**Introduction**

Epithelioid sarcoma (International Classification of Diseases for Oncology code number 8804/3) is a rare mesenchymal neoplasm that displays variable epithelioid morphology, presents a high rate of recurrences and metastases, and frequently poses diagnostic dilemmas (1). It usually affects young adults and arises in the distal extremities (classic-type epithelioid sarcoma) or, more rarely, in proximal sites of the trunk (proximal-type epithelioid sarcoma). Proximal-type epithelioid sarcoma is more frequently associated with epithelioid or rhabdoid morphology and higher mitotic activity (2).

Epithelioid sarcoma presents a high rate of recurrences and metastases and frequently poses diagnostic dilemmas. We previously reported loss of tumor suppressor SMARCB1 protein expression and SMARCB1 gene deletion in the majority of epithelioid sarcoma cases. Unfortunately, no appropriate preclinical models of such genetic alteration in epithelioid sarcoma are available. In the present report, we identified lack of SMARCB1 protein due to a homozygous deletion of exon 1 and upstream regulatory region in epithelioid sarcoma cell line VAESBJ. Restoration of SMARCB1 expression significantly affected VAESBJ cell proliferation, anchorage-independent growth, and cell migration properties, thus supporting the causative role of SMARCB1 loss in epithelioid sarcoma pathogenesis. We investigated the translational relevance of this genetic background in epithelioid sarcoma and showed that SMARCB1 ectopic expression significantly augmented VAESBJ sensitivity to gamma irradiation and acted synergistically with flavopiridol treatment. In VAESBJ, both activated ERBB1/EGFR and HGFR/MET impinged on AKT and ERK phosphorylation. We showed a synergistic effect of combined inhibition of these 2 receptor tyrosine kinases using selective small-molecule inhibitors on cell proliferation. These observations provide definitive support to the role of SMARCB1 inactivation in the pathogenesis of epithelioid sarcoma and disclose novel clues to therapeutic approaches tailored to SMARCB1-negative epithelioid sarcoma. Mol Cancer Ther; 12(6); 1060–72. ©2013 AACR.

The SMARCB1 gene located at 22q11 chromosomal region encodes for an invariant subunit of SWI/SNF chromatin remodeling complex and has been reported to act as a tumor suppressor gene in infantile malignant rhabdoid tumor (MRT; refs. 3, 4), a highly aggressive neoplasm affecting renal or extra-renal soft tissue, and cerebral tissue in pediatric patients. Previously published studies indicate that SMARCB1/INI1 gene is involved in the control of genomic stability and in the regulation of cell-cycle progression (5). SMARCB1 stimulates the p16/Rb tumor suppressor pathway by activation of CDKN2A and inhibition of Cdk/cyclinD (6). As a result, in MRT cell lines, it has been shown that SMARCB1 loss is associated with responsiveness to Cdk/cyclin inhibitors, such as 4-HPR (7) and flavopiridol (8). In addition, the in vivo spontaneous tumorigenesis in SMARCB1−/− knockout mice is increased by TP53-null genetic background (9) and is prevented by CCND1 ablation (10), further supporting the interaction with crucial molecules controlling cell-cycle progression.

We first reported evidence of SMARCB1 inactivation in epithelioid sarcoma (11), an event frequently associated with homozygous gene deletions (12). The relevance of SMARCB1 in epithelioid sarcoma pathogenesis was then supported by other reports of subtle intragenic mutations, including small deletions and point mutations (13, 14).
Subsequent studies reported the occurrence of SMARCB1 protein and/or genetic alterations in pithelioid sarcoma ranging from 60% to 93% (12–16). Although these differences may be attributable to the different molecular approaches undertaken to assess SMARCB1 inactivation, the lack of appropriate preclinical models for this enigmatic sarcoma subtype has so far prevented the assessment of the causal role in epithelioid sarcoma pathogenesis.

Here, we provide evidence that the epithelioid sarcoma cell line VAESBJ carry a homozygous SMARCB1 deletion and that in this cell line SMARCB1 actually acts as a tumor suppressor. Moreover, we provide evidence that SMARCB1 inactivation results in hyperactivation of ERBB1/EGFR and HGFR/MET pathways, thus disclosing novel avenues for the treatment of patients with epithelioid sarcoma.

Materials and Methods

Cancer cell lines

VAESBJ was purchased from Interlab Cell Line Collection and the other cell lines were from American Type Culture Collection (ATCC). Sarcoma subtypes represented were osteosarcoma (CRL1543/HOS; MG63), rhabdomyosarcoma (CRL7262/H1229.T; CRL7226/T174, CRL7763/TE381.T), epithelioid sarcoma (VAESBJ, ATCC number CRL-2138), fibrosarcoma (HTB152/Hs913.T; MES-SA), leiomyosarcoma (HTB88/SK-LMS-1), renal rhabdoid sarcoma (G401), and renal leiomysoblastoma (G402). Hek293 and IMR90 are immortalized noncancer cell lines originally derived from human embryonic kidney and fibroblasts, respectively. Cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, in a humidified incubator at 37°C and 5% CO2. Cell lines were authenticated regularly by microsatellite DNA fingerprinting. To establish the G402 and VAESBJ xenograft, 5 × 106 cells were injected subcutaneously in the flank of nude mice. After 3 weeks, the tumor was removed, formalin-fixed, and processed as reported for immunohistochemistry analysis. For infection experiments, viral supernatants were injected subcutaneously in the flank of nude mice.

Western blot analysis and quantitative PCR analysis. In cell line experiments, 10⁶ cells from pBabe-SMARCB1 and pBabe-control infected VAESBJ cells from 3 independent infection experiments were subcutaneously injected in the flank of nude mice. Tumor growth was monitored over time and calculated using the formula 1/2(r²). Once mice were sacrificed, tumors were explanted, weighted, and photographed. Animal experimentation was approved by Institutional Review Board and conducted according to National laws.

Protein expression

For Western blot analysis, protein lysates were prepared in radioimmunoprecipitation assay buffer (Sigma), 40 micrograms of cell lysate were loaded on 4%–15% gradient PAGE gels (Bio-Rad) and electrophoresed onto polyvinylidene difluoride membranes (Amersham Biosciences). Subsequently, membranes were incubated 1 hour at room temperature in a solution of TBST [10 mmol/L Tris-HCl (pH 8.0), 0.15 mol/L NaCl, and 0.05% Tween 20] supplemented with 5% nonfat dry milk. For immunodetection, the anti-BAF47/SNF5 antibody (BD Transduction Laboratories) was used diluted 1:250. After overnight incubation at 4°C with the primary antibody, membranes were washed in TBST, followed by Alexa Fluor 680- or IRDye 800CW-conjugated goat-anti-mouse or goat-antirabbit antibodies (from Invitrogen and Li-Cor, respectively). Odyssey infrared imaging system (Li-Cor) was used for detection. Protein loading equivalence was assessed using an anti-Gapdh antibody (Sigma). Additional antibodies used were: TP53 (DO-1 1:1,000; Santa Cruz), CCND1 (DCS-6 1:500; Santa Cruz), CDKN2A/p16 (C-20 1:1,000; Santa Cruz), CDKN1A/p21 (H-164 1:500, Santa Cruz), PARP (1:1,000; Cell Signaling), CASP3 (1:1,000; Cell Signaling), CASP7 (1:1,000; Cell Signaling), P(Tyr1068)-EGFR (1:1,000; Cell Signaling), EGFR (1:1,000, Cell Signaling), P(Ser473)-AKT (1:1,000; Cell Signaling), AKT (1:1,000; Cell Signaling), P-ERK1/2 (1:1,000; Cell Signaling), ERK1/2 (1:1,000; Cell Signaling), P(Tyr1003)-MET (1:1,000; Cell Signaling), MET (1:1,000; Santa Cruz), and Vinculin (1:10,000; Santa Cruz). Immunophenotyping with CD34, cytokeratins, epithelial membrane antigen, and CD31 antibodies was conducted for uniform pathologic reexamination of xenograft tumors. Protein expression of SMARCB1 was investigated by immunohistochemistry using anti-BAF47/SNF5 antibody 1:100 (BD Transduction Laboratories). Endogenous peroxidise was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes. For antigen retrieval, the slides were immersed in citrate buffer solution 5 mmol/L pH 6 and heated in autoclave at 95°C for 15 minutes. Immunohistochemistry analysis was done using Ultra vision detection system (LabVision). Expression of phosphorylated receptor kinases was detected using the RTK Proteome Profiler Array kit (R&D Systems). The procedures were carried out according to the manufacturer’s protocol using 300 μg of protein lysate per array and signals generated by horseradish peroxidase-
conjunctive secondary antibody were visualized and quantified with Chemidoc imaging system (Bio-Rad).

**Real-time PCR**

*SMARCB1* mRNA expression was analyzed by quantitative real-time PCR. Total RNA was extracted using TRIzol reagent (Ambion) and 1 μg RNA was retrotranscribed using Superscript II reverse transcriptase (Gibco) with random primers. Ten nanograms of cDNA were used as template in 20 μL PCR reactions with 1 × TaqMan Universal PCR master mix (Applied Biosystems) as endogenous reference control and 293 cell line as calibrator. *SMARCB1* assay used was Hs00268260_m1, encompassing exons 4–5.

**Mutational analysis**

Mutational analysis was conducted by exon amplification and sequencing was conducted as previously described (11). For multiplex ligation-dependent probe amplification (MLPA), tumor DNA (100 ng) was subjected to DNA copy number analysis using MLPA kits P258-B1, P294-A1, and P171 (MRC-Holland), following manufacturer instructions and together with normal DNA samples and cancer cell lines with known *SMARCB1* gene copy number alterations as controls. Fragment separation was conducted on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) by comparative Ct method, using the hypoxanthine phosphoribosyltransferase (*HPRT*) gene (HPRT PDAR, 4326321E) as endogenous reference control and 293 cell line as calibrator. *SMARCB1* assay used was HS00268260_M1, encompassing exons 4–5.

**Cell proliferation assay**

Cells were plated in multiwell plates and cell proliferation was assessed at different time points by either Trypan blue cell counting or with sulphorhabdamine B (SRB) staining (18). For SRB staining, cells (5 × 10^4) were grown in 96-well plates for 72 hours, fixed using cold 50% trichloroacetic acid for 1 hour at 4°C, then were stained using Sulphorhabdamine B (Sigma) 0.4% in 1% acetic acid. Tris-Base 10 mmol/L was used for solubilization. The absorbance of protein biomass, which is proportional to the cell number, was read at 550 nm. Time zero plates were fixed after overnight cell adhesion and served for background subtraction and calculation of relative absorbance values as described (18). Drugs used were flavopiridol (Sigma), ferretinide (Sigma), PHA665752 HGFR/MET inhibitor (Santa Cruz), and 324674 EGFR inhibitor (Calbiochem). Drugs were resuspended in dimethyl sulfoxide (DMSO) or ethanol (in case of ferretinide) and added 24 hours postplating at the indicated concentrations. Cells were fixed 72 hours later. No treatment, DMSO or ethanol vector control served as calibrator sample. Combination index of drug combinations was calculated with CompuSyn software (CompuSyn Inc.).

**Anchorage-independent growth**

Cells (10^5) were resuspended in 0.35% agar complete medium and seeded on 0.5% bottom agar medium in 6 cm petri dishes. After 2 weeks, plates were stained with iodonitrotetrazolium violet (1 mg/mL, Sigma) and clones were counted at the microscope.

**β-Galactosidase assay**

The β-galactosidase (β-Gal) staining was used as a surrogate marker of senescence. Cells were washed once in PBS (pH 7.2), fixed with 0.5% glutaraldehyde for 15 minutes, and washed in PBS (pH 7.2) supplemented with 1 mmol/L MgCl2. Cells were stained with senescence-associated β-Gal (SA-β-Gal) stain solution (11) supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 0.12 mmol/L K3Fe(CN)6, 0.12 mmol/L K4Fe(CN)6, 1 mmol/L MgCl2 in PBS (pH 6) and incubated overnight at 37°C and 5% CO2. The β-Gal-positive cells in 20 microscope fields were counted under bright field on a Olympus IX70 microscope and representative photographs were taken.

**Migration assay**

Transwell permeable supports, 6.5 mm diameter inserts, 8.0 μm pore size, polycarbonate membrane (Corning Inc.) were used to conduct migration assay. Cells (10^5) in 1% FBS containing Dulbecco’s Modified Eagle Medium were seeded in the top chamber. The bottom chamber of the Transwell was filled with 600 μL of culture medium containing 10% FBS. Cells were incubated at 37°C for 16 hours. The Transwells were then removed from the 24-well plates and stained with 0.1% crystal violet in 25% methanol. Nonmigrated cells were scraped off the top of the Transwell with a cotton swab. Migrated cells were quantified by eluting crystal violet with 1% SDS and reading the absorbance at 550 nm. In parallel, equal amounts of cells were plated in a 96-well plate, incubated at 37°C for 16 hours, stained with crystal violet as described, and the absorbance values obtained from Transwell elution were normalized over absorbance values of the 96-well plate to obtain the percentage of migrated cells in relation to the different proliferation capacity between *SMARCB1* expressing and control cells.

**Cell-cycle analysis**

Guava cell-cycle instrument (Millipore) was used to carry out cell-cycle analysis. *SMARCB1* reexpressing and control cells were plated in p60 tissue culture dish. At different time points after plating, cells were harvested and fixed in ethanol 70%. Ethanol was eliminated by centrifugation, cells were washed in PBS 1×, centrifuged, and stained with Guava cell-cycle reagent, incubated for 40 minutes, shielded from light, and the data were acquired on the Guava instrument.
Bromodeoxyuridine assay
BrdUrd Flow Kit (BD Pharmingen) was used to quantify cells that were actively synthesizing DNA. Cells were pulsed with 2 hours of bromodeoxyuridine (BrdUrd) incorporation washed and released for additional 2 hours in complete medium. Cells were permeabilized and processed according to the manufacturer’s instructions. Stained cells were analyzed with flow cytometer (Beckman Coulter).

Caspase-3/7 assay
Caspase-3/7 activity was assessed using Caspase-Glo 3/7 assay (Promega). Cells were irradiated in suspension using a Gammacell 1000 Elite biologic irradiator (Nordion Inc.), plated in 96 white-walled plates and analyzed after 48 hours. Caspase 3/7 GLO Reagent was added to each well and gently mixed for 1 minute. The luminescence of each sample was measured with a plate-reading luminometer 30 minutes after staining. As a control, all samples analyzed were plated also in a 96-multivell and stained with SRB, as indicated above. To take into account the different proliferation between SMARCB1-expressing and control cells, the measured Caspase-Glo 3/7 assay luminescence was normalized over SRB absorbance values.

Clonogenic assay
Cells (10^3) were plated in 6 cm culture dishes and incubated for 10 days in a humidified incubator at 37℃ and 5% CO2. Plates were stained with 0.1% crystal violet in 25% methanol, cells were photographed and then eluted immediately fixed and corresponding absorbance readings were used to normalize for variations in cell seeding among experimental conditions.

Cell death evaluation
Cells (2 × 10^3) were irradiated in suspension and plated in 6-well plates. After 48 hours of treatment, cells were harvested, washed in PBS 1× and stained with Annexin V-FITC (Clontech; 20μg/mL) and 7 amino Actinomycin D (BD Pharmingen). Following incubation at room temperature for 15 minutes in the dark, cells were analyzed with FACScan flow cytometer.

Statistical analysis
Comparisons between 2 classes have been carried out by 2-sample unpaired Student t test. Comparisons between 3 or more classes have been carried out by repeated-measures one-way ANOVA. Two classes’ comparisons with multiple measurement points have been carried out by 2-way ANOVA. The GI50 values were calculated by nonlinear regression curve fit of dose versus response based on Hill equation. All statistical analyses were carried out using Prism 5 software (GraphPad Inc.). At least 3 independent biologic replicate experiments were carried out and average ± SD of these replicates were considered. P values less than 0.05 were considered statistically significant.

References

Results
SMARCB1 protein expression in sarcoma cell lines
Western blot analysis of a panel of commercially available sarcoma cell lines revealed that, in addition to previously reported rhabdoid sarcoma cell lines, the VAESBJ and G402 cell lines displayed a dramatic reduction of SMARCB1 protein expression (Fig. 1A and Supplementary Fig. S1A). To ascertain the histology of these cell lines, subcutaneous xenograft tumors were established in nude mice. Morphology (Fig. 1B) and immunophenotype (CD34 and EMA positive, SMARCB1 negative, Fig. 1C–E) of VAESBJ xenografts were coherent with an origin of this cell line from an epithelioid sarcoma, as originally described (19). On the contrary, morphology, immunophenotype analysis of the G402 xenografts (CD34 and SMARCB1 negative, SMA, and EMA positive, Supplementary Fig. S1B–S1E) as well as available clinical data (a kidney cancer in an infant 6-month-old patient), suggest that G402 originated from a misclassified renal malignant rhabdoid tumor, rather than from a leiomyoblastoma as listed in ATCC.

SMARCB1 is homozygously deleted in VAESBJ and G402 sarcoma cell lines
Exon amplification and MLPA analysis indicated that VAESBJ and G402 lost the chromosome region encompassing SMARCB1 exon 1 and exon 3, respectively (Fig. 2A and Supplementary Fig. S1F and S1G). In particular, PCR-based chromosomal walking allowed the identification of an 8.4 kb homozygous deletion encompassing the genomic region from MMP11 exon 3 to SMARCB1 exon 1 in VAESBJ (Supplementary Fig. S2A and S2B). Primer pairs flanking the region of deletion allowed cloning and sequencing of the breakpoint in VAESBJ (Fig. 2B). Interestingly, high-resolution single-nucleotide polymorphism array-based copy number analysis of this cell line carried out by the Sanger Centre (Hinxton, Cambridge; http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghview/CghHome.cgi) failed to identify the presence of such a homozygous deletion (Supplementary Fig. S2C and S2D), thus supporting the subtle nature of such genetic alteration. Moreover, real-time PCR analysis using primers encompassing exons 4-5 (Fig. 2C) evidenced that the mRNA expression of SMARCB1 in VAESBJ was significantly impaired compared with control 293T cells, consistent with lack of exon 1/upstream regulatory regions and indicating that no aberrant MMP11-SMARCB1 fusion transcript was significantly expressed as a result of this deletion. Real-time PCR analysis revealed a highly reduced expression also in G402, consistent with nonsense-mediated mRNA decay, as predicted by the formation of a premature stop codon due to exon 3 deletion. Additional MLPA analysis of VAESBJ cells revealed concurrent homozygous deletion of CDKN2A and CDKN2B loci encoding p16, p14, and p15 proteins (Fig. 2D).
SMARCB1 loss sustains VAESBJ tumorigenic properties

To establish the relevance of SMARCB1 inactivation in VAESBJ cell line, we reintroduced SMARCB1 via retroviral infection (Fig. 3A). After virus infection, cells were selected and the bulk cell population was analyzed for cell proliferation, cell-cycle distribution, senescence, anchorage-independent growth, and cell migration. For comparison, selected experiments were conducted in parallel in G401 malignant rhabdoid tumor cell line (Supplementary Fig. S3), which was previously reported to be affected by SMARCB1 restoration (7, 8, 20).

Ectopic SMARCB1 expression significantly reduced cell proliferation without affecting the extent of spontaneous cell death, as assessed by Trypan blue staining ($P = 0.019, 0.014, \text{and } 0.004$ at 24, 48, and 72 hours, respectively, 2-way ANOVA $P < 0.001$ for global trend, Fig. 3B). The observed reduction in cell proliferation correlated with a reduction of cells in S and G2–M phases and an increase in the G0–G1 phase, as assessed by flow cytometry (Fig. 3C). Concordantly, BrdUrd incorporation and clonogenic potential were reduced in SMARCB1-engineered cells ($P = 0.025$, Fig. 3D and $P = 0.001$, Fig. 3E respectively). Also anchorage-independent growth was reduced in SMARCB1 reexpressing VAESBJ cells ($P = 0.019$, Fig. 3F), in part likely as a result of impaired proliferation. Different from the rhabdoid cell line G401, the ectopic expression of SMARCB1 in VAESBJ failed to result in the induction of premature senescence, as determined by SA-β-Gal staining (Supplementary Fig. S3). Moreover, similar to what was described for rhabdoid cell lines (21), SMARCB1 reexpression significantly reduced VAESBJ cell migration, as assayed by Transwell chamber assay ($P < 0.001$, Fig. 3G and Supplementary Fig. S3). Concordant with the observed reduction of anchorage-independent growth,
also in vivo tumorigenesis was significantly reduced and tumors grown were significantly smaller in SMARCB1-reexpressing VAESBJ cells ($P < 0.0001$ and $P = 0.036$ respectively, Fig. 3H).

**SMARCB1 restoration sensitizes VAESBJ cell line to irradiation**

The SWI/SNF chromatin-remodeling complex and SMARCB1 itself have been reported to influence the
response to DNA damage (22). We here show that SMARCB1 reexpression increased sensitivity to gamma irradiation in VAESBJ cell line. Reduced cell viability ($P = 0.039$, $0.045$, $0.02$ at 200, 400, and 800 rad, respectively, 2-way ANOVA $P = 0.085$, Fig. 4A) and increased cell death ($P = 0.001$, $0.013$, $0.002$, $0.02$ at 100, 200, 400, and 800 rad, respectively, 2-way ANOVA $P = 0.008$, Fig. 4B) were associated with increased of caspase-3/7 activity ($P = 0.014$ at 1200 rad, Fig. 4C) and caspase-mediated protein cleavage (Fig. 4D). The enhanced SMARCB1-mediated sensitization to irradiation was also confirmed by Annexin V/7-AAD staining ($P = 0.022$ and $P = 0.002$, untreated vs. 800 rad and 1,200 rad–treated cells, respectively, Fig. 4E).

VAESBJ cell line is susceptible to flavopiridol inhibition and to concurrent inhibition of activated ERBB1/EGFR and HGFR/MET oncogenic pathways

We exploited VAESBJ epithelioid sarcoma cell model to investigate the response to pharmacologic treatments that proved promising in preclinical models of MRT. Fenretinide (7) and flavopiridol (8) are reported to act as effective CDKs/cyclins inhibitors in the cellular context of rhabdoid tumor cell lines. We compared the cytotoxicity
compared with G401 (GI50 450 nmol/L; VAESBJ displayed a lower sensitivity to fenretinide (EGFR and/or ERBB1) inhibition plays a minor role in fenretinide sensitivity. Furthermore, flavopiridol efficiently downmodulated CCND1 and upregulated both p53 and p21 (Fig. 5B, left), consistent with a significant reduction of cell proliferation and increased cell death (one-way ANOVA P < 0.001 and P = 0.008, respectively; Fig. 5B, right). Indeed, the siRNA-mediated silencing of CCND1 (Fig. 5C and D) potently inhibited cell proliferation (Fig. 5E, P = 0.006), as did flavopiridol. Finally, flavopiridol effect was significantly potentiated upon SMARCB1 restoration (P < 0.001 and P = 0.012 at 100 nmol/L and 300 nmol/L, respectively; Fig. 5F), suggesting the presence of a synergistic effect.

To gain insights on the oncogenic pathways underlying VAESBJ tumorigenic phenotype that can be exploited for targeted therapeutic interventions, we conducted phospho–protein array analyses. These assays identified significant expression of activated/phosphorylated ERBB1/EGFR and HGFR/MET (Fig. 6A), that in turn impinge on AKT and ERK phosphorylation (Fig. 6B). Pharmacologic inhibition of HGFR/MET with PHA665752 resulted in impaired cell motility and correlated with reduced FAK kinase phosphorylation/activation (Fig. 6E), whereas the combined inhibition of both ERBB1/EGFR and HGFR/MET using selective small-molecule inhibitors Calbiochem 324674 and PHA665752, respectively, displayed a synergistic effect on proliferation reduction compared with single-agent treatment (Fig. 6C and D). Notably, Western blot analysis and immunohistochemistry analysis of epithelioid sarcoma tumor samples showed that multiple cases presented ERBB1/EGFR and/or HGFR/MET overexpression and activation (Fig. 6F).

**Discussion**

We here show that SMARCB1 loss plays a crucial role in the tumorigenic phenotype of VAESBJ cell line, thus providing definitive evidence of the causative role of SMARCB1 loss in epithelioid sarcoma pathogenesis. SMARCB1 is a core subunit of the mammalian SWI/SNF complex.

**Figure 4.** Gamma irradiation treatment in VAESBJ cell line after SMARCB1 restoration. Cell proliferation reduction (2-way ANOVA P = 0.008, A) and increased cell death (2-way ANOVA P = 0.008, B) in SMARCB1 reexpressing cells as assessed by SRB assay and Trypan blue count, respectively, following 48 hours γ-irradiation treatment. SMARCB1 restoration increased caspase 3/7 and PARP activity in VAESBJ cell line treated with high dose of γ radiation, as assessed by caspase assay (C) and Western blot analysis (D). E, increased apoptosis is evident by AnnexinV/7AAD staining and subsequent fluorescence-activated cell sorting (FACS) analysis. Graphs display average ±SD of one representative out of 3 independent experiments. *, P < 0.05 by 2-tailed unpaired Student t test.
ATP-dependent chromatin remodeling multisubunit complexes. By epigenetically affecting histone-DNA contacts and nucleosome remodeling, the SWI/SNF complexes regulate a broad range of cellular pathways (23, 24). Loss of SMARCB1 has been shown to occur at high frequency in MRT and epithelioid sarcoma. Accordingly, genetic inactivation of SMARCB1 tumor suppressor gene has been documented in several MRT-derived cell lines. Instead, occasional reports of SMARCB1 mutations in cell lines of other tumor types,
Figure 6. A, phospho–protein array analysis of VAESBJ cell line, showing significant expression of activated/phosphorylated ERBB1/EGFR and HGFR/MET but not other receptor kinases investigated. B, Western blot analysis of protein expression in VAESBJ cell line, showing that both activated EGFR and HGFR/MET impinge on AKT and ERK phosphorylation. C, sulforhodamine-B cell cytotoxicity assay showing dose–response plots of EGFR inhibitor 324674, HGFR/MET inhibitor PHA665752, and combined treatment with both inhibitors. D, combination index (CI) plot showing synergism (CI < 1) of EGFR inhibitor 324674 and HGFR/MET inhibitor PHA665752 at all doses of combined treatments tested. E, reduction of migration capability upon treatment with HGFR/MET inhibitor PHA665752 (*, P = 0.012 by 2-tailed unpaired Student’s t test). F, evidence of EGFR and HGFR/MET expression and activation in epithelioid sarcoma tumor samples by Western blot and immunohistochemistry.
such as rhabdomyosarcoma (e.g., A204 line; ref. 25) and Wilms’ tumor (e.g., G401 line; ref. 26), have been subsequently invalidated by reevaluation of such cell lines as misclassified malignant rhabdoid tumors (27, 28). To our opinion, this is also the case of G402 cell line, originally indicated as a leiomyoblastoma, that we show to carry an exon 3 SMARCB1 deletion. Morphologic and immunophenotypic characterization of this cell line, together with the available clinical data regarding an infant renal tumor (ATCC, www.atcc.org), allowed us to establish that G402 is also a bona fide MRT cell line. On the contrary, morphology and immunophenotype of the VAESBJ xenograft (including CD34-positive staining) as well as available clinical data on the tumor of origin, reporting a paraspinal sarcoma in a patient aged 42 years (19), support the diagnosis of epithelioid sarcoma.

We provide evidence that VAESBJ carry a homozygous deletion of SMARCB1 involving exon 1. This result indicates that VAESBJ represents a unique model to investigate the role of SMARCB1 inactivation in the context of epithelioid sarcoma. Because in MRTs cells, SMARCB1 tumor suppressor activity has been shown to impinge on cell proliferation (29, 30), apoptosis (31), senescence (20, 27), and cell motility (21), we investigated these phenotypes in SMARCB1-deleted VAESBJ epithelioid sarcoma cell line. Here, we provide evidence that SMARCB1 restoration in this cell line affects cell proliferation, enhances the sensitivity to genotoxic stress, and reduces cell migration. Notably, different from what was reported for MRT cell models, restoration of SMARCB1 in VAESBJ expression failed to result in premature senescence. In MRTs cells, such a response relied on p16 activation (20). The fact that VAESBJ cells carry homozygous deletion of CDKN2A/p16 locus may account for the attenuation of the senescent phenotype in this cell line.

We exploited VAESBJ epithelioid sarcoma cell model to gain insight into activated oncogenic pathways and response to targeted as well as conventional genotoxic treatments. We here provide evidence that VAESBJ cells display activation of both the receptor tyrosine kinase ERBB1/EGFR and HGFR/MET. Recently, a role for EGFR activation has been proposed in epithelioid sarcoma (32). Although VAESBJ does not carry EGFR mutation, it displayed sustained, EGFR-independent, phospho-AKT activation, and showed limited response to EGFR inhibition (32). We found that the HGFR/MET pathway cooperates with EGFR in sustaining AKT and ERK phosphorylation. Concordantly, combined inhibition of ERBB1/EGFR and HGFR/MET pathways resulted in enhanced and synergic growth suppression. Thus, our observations provide an explanation for the incomplete response of VAESBJ cell line to EGFR inhibition (32).

Similar to MRTs cell lines (7, 10), flavopiridol treatment in VAESBJ induced both CCND1 protein downmodulation and cell-cycle arrest. Unexpectedly, we observed a synergistic effect on cell-cycle block with combined Flavopiridol treatment and SMARCB1 ectopic reexpression, indicating that flavopiridol affects cell proliferation and viability through pathways at least in part independent of SMARCB1 tumor suppressor.

The cancer genome of pediatric MRTs is extraordinarily stable and the only recurrent alteration reported so far is SMARCB1 inactivation (33), showing that in specific cellular contexts unique genetic alterations are sufficient to sustain tumorigenesis. In contrast, epithelioid sarcoma (11, 34) and VAESBJ epithelioid sarcoma model system (ref. 35; see also http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghHome.cgi), despite the shared feature of recurrent SMARCB1 inactivation, show a much more unstable genome, thus suggesting that in the adult setting, the cancer-initiating cell of epithelioid sarcoma requires the overriding of multiple pathways, including the one involved in the control of genome integrity.

The p16/CCND1/CDK4/RB/E2F pathway inactivation has been initially reported as the prominent downstream effect of SMARCB1 inactivation in MRTs (20, 30, 36). However, more recently, engineered mouse models showed that SMARCB1 haploinsufficiency cooperates with both RB and TP53 haploinsufficiency to increase penetrance and reduce latency of tumor onset (9, 22, 37) and in vitro studies showed that SMARCB1 inactivation globally affects the epigenetic status of the cancer genome (38, 39). We observed that VAESBJ cells retain a wild-type TP53 but carry a homozygous deletion of CDKN2A locus, thus displaying impaired p16/RB and p14/TP53 responses. Overall, these data suggest that SMARCB1 collaborates with the inactivation of both TP53 and RB signals to promote tumorigenesis by, at least in part, independent pathways.

A previous report described the involvement of SMARCB1 in the modulation of DNA repair after UV irradiation (40). We here show that indeed SMARCB1 inactivation is responsible for resistance to gamma irradiation and its restoration significantly augments the apoptotic response to irradiation-induced DNA damage.

In conclusion, we provide the first characterization of a cell model of epithelioid sarcoma carrying genetic inactivation of SMARCB1 by homozygous gene deletion. Our observations provide definitive support to the role of SMARCB1 inactivation in the pathogenesis of epithelioid sarcoma and disclose novel clues to therapeutic approaches tailored to SMARCB1-negative epithelioid sarcoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Brenca, R. Maestro, P. Modena
Development of methodology: M. Brenca, E. Lorenzetto, A.P. Dei Tos, P. Modena
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Rossi, E. Piccinin, S. Piccinin, F.M. Rossi, A. Giuliani, A.P. Dei Tos, P. Modena
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**SMARCB1/INI1 Genetic Inactivation Is Responsible for Tumorigenic Properties of Epithelioid Sarcoma Cell Line VAESBJ**

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