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Loss of HSulf-1 expression enhances tumorigenicity by inhibiting Bim expression in ovarian cancer

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Abstract

The expression of human Sulfatase1 (HSulf-1) is downregulated in the majority of primary ovarian cancer tumors, but the functional consequence of this downregulation remains unclear. Using two different shRNAs (Sh1 and Sh2), HSulf-1 expression was stably downregulated in ovarian cancer OV202 cells. We found that HSulf-1-deficient OV202 Sh1 and Sh2 cells formed colonies in soft agar. In contrast, nontargeting control (NTC) shRNA-transduced OV202 cells did not form any colonies. Moreover, subcutaneous injection of OV202 HSulf-1-deficient cells resulted in tumor formation in nude mice, whereas OV202 NTC cells did not. Also, ectopic expression of HSulf-1 in ovarian cancer SKOV3 cells significantly suppressed tumor growth in nude mice. Here, we show that HSulf-1-deficient OV202 cells have markedly decreased expression of proapoptotic Bim protein, which can be rescued by restoring HSulf-1 expression in OV202 Sh1 cells. Enhanced expression of HSulf-1 in HSulf-1-deficient SKOV3 cells resulted in increased Bim expression. Decreased Bim levels after loss of HSulf-1 were due to increased p-ERK, because inhibition of ERK activity with PD98059 resulted in increased Bim expression. However, treatment with a PI3 kinase/ AKT inhibitor, LY294002, failed to show any change in Bim protein level. Importantly, rescuing Bim expression in HSulf-1 knockdown cells significantly retarded tumor growth in nude mice. Collectively, these results suggest that loss of HSulf-1 expression promotes tumorigenicity in ovarian cancer through regulating Bim expression.

Keywords

tumorigenicity; HSulf-1; Bim; ovarian cancer

Human Sulfatase1 (HSulf-1) encodes an endosulfatase that regulates the sulfation of heparan sulfate proteoglycans (HSPGs).[1,2] Sulfated HSPGs on the cell surface serve as coreceptors for many growth factors and cytokines to enhance their downstream signaling. HSulf-1 inhibits the function of HSPGs by desulfation and thus suppresses multiple receptor tyrosine

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kinase signaling pathways, particularly by the heparin-binding growth factors, bFGF, VEGF, HGF, PDGF and HB-EGF.[1–3] Loss of HSulf-1 expression has been found to augment both autocrine and paracrine proliferation signaling through various heparin-binding growth factors.[1–3] The tumor suppressor effect of HSulf-1 has been suggested in various tumors including ovarian cancer.[3–7]

Our previous work identified *HSulf-1* as a downregulated gene in ovarian cancer through the mechanism of epigenetic silencing and loss of heterozygosity (LOH). It is undetectable in the majority of ovarian carcinoma cell lines and markedly diminished or undetectable in ~75% of ovarian cancer specimens. These results suggest that HSulf-1-mediated pathway may play an important role in tumorigenicity. However, owing to the lack of HSulf-1 expression in the majority of ovarian carcinoma cell lines, we have not successfully established a HSulf-1 knockdown ovarian cancer cell line until recently. Our previous limited understanding of the role of HSulf-1 was mostly based on the work using overexpression of this gene. No direct evidence has been reported to support the critical role of HSulf-1 in the development of ovarian cancer and the detailed downstream signaling mechanism of HSulf-1 remains unknown. Clarifying the role and mechanism of loss of HSulf-1 in the process of ovarian cancer development and progression may provide new treatment targets.

In our study, we generated *HSulf-1* knockdown clones in the OV202 ovarian cancer cell line using shRNA. We found that loss of HSulf-1 resulted in a significant increase in the ability of OV202 cells to form anchorage-independent colonies *in vitro* and enhanced tumorigenicity in nude mice *in vivo*. On the contrary, ectopic expression of HSulf-1 in SKOV3 cells resulted in the inhibition of tumor growth in nude mice. Downregulated Bim expression, as a result of increased activity of ERK pathway after loss of HSulf-1, was proved to be a critical downstream factor contributing to the enhanced tumorigenicity in ovarian cancer cells.

Material and Methods

Cell culture and drug treatment

The human ovarian cancer cell line SKOV3 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured according to the provider's recommendation. Ovarian cancer OV202 cell line was established and cultured as described previously.[6] PD98059 and LY294002 were purchased from Calbiochem (San Diego, CA). QVD (Q-VD-Oph), a pancaspase inhibitor, was purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids

pcDNA-HSulf-1 plasmid was cloned as described previously.[7] Cells were transfected with plasmids using Lipofectamine Plus (Invitrogen, Grand Island, NY) according to the manufacturer's recommendation. Transfected cells were selected with G418 at the concentration of 600 mg/ml 48 hr post-transfection.

ShRNA

HSulf-1-targeting ShRNA1 (Sh1), which targets the 3′-untranslated region (UTR), was cloned into lentiviral vector pLKO.1-puro as described previously.[1] HSulf-1-targeting ShRNA2 (Sh2) and nontargeting control ShRNA (NTC shRNA) cloned into the lentivirus vector pLKO.1-puro were chosen from the human library (MISSION TRC-Hs 1.0) and purchased as glycerol stock from Sigma-Aldrich (St. Louis, MO). Transfection with Fugene (Roche, South San Francisco, CA) was performed according to the manufacturer's instructions. Transfected cells were selected with 1 lg/ml puromycin. Two populations of transduced cells (OV202 Sh1 and Sh2) were generated instead of clonal lines generated from individual colonies.

Western blot analysis

Western blot analysis was performed as described previously.[8] Whole-cell lysates were analyzed with the following antibodies: anti-HSulf-1, Bim, p-AKT (Ser 473), AKT, p-42/44 ERK (Thr202/ Tyr204), ERK and β-actin. Anti-HSulf-1 antibody was rabbit polyclonal antibody purchased from Abcam (Cambridge, MA). Monoclonal antibody against β-actin was purchased from SigmaAldrich (St. Louis, MO). All other antibodies were obtained from Cell Signalling Technology (Danvers, MA). Densitometric analysis was performed for immunoblots using Image J software (NIH).

Soft agar assay

A soft agar assay was performed as described previously.[8] Cells were seeded at 2,000 cells per well and incubated for 1–2 weeks. The resulting colonies were stained with 0.5 mg/ ml *p*-iodonitrotetrazolium violet in phosphate-buffered saline (PBS). Colonies larger than 50 mm in diameter were counted and photographed.

Quantitative real-time reverse-transcription polymerase chain reaction

Reverse-transcription polymerase chain reaction (RT-PCR) was used to determine the influence of loss of HSulf-1 on Bim mRNA levels. For cell lines, RNA was isolated and purified using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and further purified by DNAse treatment with Ambion Turbo DNAse. After extraction and purification, RNA was reverse transcribed and amplified using superscript first-strand cDNA synthesis (Invitrogen, Grand Island, NY) as per the manufacturer's instructions. Real-time PCR primers for Bim were obtained from Applied Biosystems (Foster City, CA). The gene-expression level was normalized using GAPDH mRNA as the endogenous control.

In vivo animal studies

All mice were handled according to the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Institutional Animal Care and Use Committee at the Mayo Clinic College of Medicine. Female nu/nu mice (National Cancer Institute-Frederick Cancer Research and Development Center), aged 4–6 weeks, were used for this study with five mice in each group. Tumors were generated in mice by subcutaneous injection of 5×10^6 OV202 clonal cells or SKOV3 clonal cells (see Ref. 7) into the right flanks of mice. Tumor size was measured every 3–4 days for 28 days. The perpendicular diameters of the

tumors were measured using a caliper and the tumor volumes were calculated using the formula: tumor volume (V)=π/6 × larger diameter × smaller diameter^2.

Statistical analysis

Analysis of variance (ANOVA) followed by Newman–Keuls test were performed using Prism 3.0 (GraphPad Software, La Jolla, CA); $p<0.05$ was considered statistically significant.

Results

Downregulation of HSulf-1 increased the anchorage-independent growth *in vitro* and tumor growth *in vivo*

To examine the role of loss of HSulf-1 in ovarian cancer tumorigenesis, HSulf-1 expression was stably downregulated in a population of cells with two different ShRNAs, one targeting the 3′-UTR (Sh1) and the other targeting the open reading frame (ORF; Sh2) in OV202 cells. Nontargeting ShRNA (NTC) served as controls. Immunoblot analysis of HSulf-1 in these cells indicated stable downregulation of HSulf-1 in both ShRNA clones with almost 100% downregulation in the Sh1 clone (Fig. 1a). Soft agar growth assay showed that OV202 HSulf-1-deficient cells formed colonies on soft agar within 1 week with colony number of 4806 ± 12 (mean±SD) and 44±69 (mean ± SD) in Sh1 and Sh2 knockdown cells, respectively (Fig. 1b). In contrast, OV202 NTC cells did not form any colony on soft agar. These results indicated that downregulation of HSulf-1 resulted in increase in the ability to form anchorage-independent colonies of OV202 cells.

To further determine if the *in vitro*-enhanced ability of colony formation of OV202 HSulf-1-deficient cells can be reflected *in vivo*, we injected 5×10^6 OV202 isogenic (NTC, Sh1 and Sh2) cells subcutaneously into nude mice. At the end of 2 weeks, 80% (four of five) mice in Sh1 group and 40% (two of five) in Sh2 group formed tumors; at the end of 4 weeks postinjection, all five mice in OV202 Sh1 group and 60% (three of five) mice in Sh2 group formed tumors (Fig. 1c). Consistent with the *in vitro* data, the mice injected with NTC cells did not form any xenograft at the end of the experiments. The average xenograft weights in Sh1 and Sh2 groups 4 weeks postinjection reached 3.71 and 1.42 g, respectively (Fig. 1d). Conversely, cells with enhanced expression of HSulf-1 in SKOV3 cells also demonstrated significant inhibition of tumor growth in nude mice starting from 14 days postinoculation ($p<0.05$) (see Supporting Information Fig. 1).

Knockdown of HSulf-1 is associated with downregulated Bim expression

Defects in apoptosis provide cancer cells with an intrinsic survival advantage. BCL-2 family proteins are central players in the regulation of apoptosis and modulate death signaling through the intrinsic or mitochondrial pathway.[9] Therefore, to understand the mechanism by which loss of HSulf-1 is associated with increased tumorigenicity, we examined the expression profile of Bcl-2 family members in OV202 isogenic cells with or without HSulf-1 expression. As shown in Figure 2a, knockdown of HSulf-1 led to a notable reduction in the levels of the proapoptotic Bim protein in OV202 Sh1 and Sh2 cells. Densitometric analysis of Bim levels was performed and expression values were normalized

with β-actin expression. The results showed that Bim level in OV202 NTC cells was 10.2- and 2.4-fold higher than in OV202 Sh1 and Sh2 cells, respectively. No significant changes were observed in other proapoptotic proteins including puma, Bax and Bak, or the prosurvival proteins Bcl-2. Bcl-xL was also notably downregulated. However, based on its prosurvival function,[9,10] decreased Bcl-xL is less likely to contribute to enhanced tumor growth observed in OV202 Sh1 and Sh2 cells. The prosurvival protein A1 was below the level of detection (data not shown).

As HSulf-1ShRNA1 targets 3prime; -UTR of HSulf-1, HSulf-1 expression in Sh1 cells can be rescued by exogenous expression of HSulf-1 using the construct containing HSulf-1 ORF. To determine whether HSulf-1 regulates the expression of Bim, we rescued HSulf-1 expression following transient transfection of HSulf-1 expression construct in OV202 Sh1 cells (Fig. 2b). As shown in Figure 2b, re-expression of HSulf-1 resulted in an increased Bim expression. Alteration in Bim levels regulated by HSulf-1 was not unique to OV202 cell line because stable enhanced expression of HSulf-1 in SKOV3 cells also resulted in upregulation of Bim (Fig. 2c).

Involvement of ERK pathway in HSulf-1 regulated Bim expression

Bim expression and function is regulated at both the transcriptional and post-translational levels through AKT pathway and p44/p42 ERK, respectively.[11,12] Therefore, Western blot analysis was performed to determine the activation status of AKT and ERK in OV202 isogenic cell lines. As shown in Figure 3a, there was an increase in both p-ERK and p-AKT at the basal level in HSulf-1-deficient OV202 cells when compared to NTC cells. Rescue of HSulf-1 expression after transient transfection of HSulf-1 construct in OV202 Sh1 cells resulted in a decreased level in both p-ERK and p-AKT (Fig. 3b), clearly demonstrating the inhibitory role of HSulf-1 in both pathways. To determine the contribution of these two pathways involved in regulating Bim expression, OV202 Sh1 cells were treated with ERK inhibitor PD98059 and PI3K inhibitor LY294002 separately. As shown in Figure 3c, although the treatment with LY294002 did result in significant change in AKT phosphorylation level, no significant change was observed in Bim level. However, pharmacological inhibition of ERK phosphorylation with PD98059 was associated with decreased p-ERK as well as increased Bim level, suggesting a regulatory role of ERK pathway on Bim expression. As Bim can be regulated at the transcriptional level through AKT,[11,12] real-time RT-PCR was performed to determine the mRNA level of Bim in OV202 clonal cell lines. Bim protein is expressed in OV202NTC and to a lesser extent in Sh2 cells (Fig. 2a). As shown in Figure 3d, an increase of Bim mRNA was observed in OV202 Sh1 cells. These results suggest that Bim is not transcriptionally regulated by increased AKT phosphorylation in Sh1 cells. Meanwhile, no significant difference of Bim mRNA was observed in OV202 Sh2 cells compared to NTC cells (Fig. 3d). The absence of increased Bim mRNA in Sh2 cells despite increased AKT phosphorylation compared to NTC cells also indicates that Bim is not transcriptionally regulated by AKT in Sh2 cells. Taken together, these results do not support the regulation of Bim expression at transcriptional level by AKT pathway in this system.

Reduced Bim is critical for loss of HSulf-1-induced tumorigenesis *in vivo*

To verify if Bim downregulation in OV202 Sh1 and Sh2 cells plays an important role in promoting tumorigenesis, we generated ectopic Bim-expressing clones in OV202Sh1 cells (OV202 Sh1 Bim C2 and C16) using CMV-driven Bim expression constructs. Immunoblot analysis confirmed enhanced expression of Bim in these clonal lines (Fig. 4a). We quantified Bim expression in these clonal cells and the results showed that the rescued Bim levels in Sh Bim C2 and C16 were 2.4- and 3.0-fold higher than in OV202 Sh1 V cells, respectively, suggesting that Bim expression was restored to a level less than the value before knockdown of HSulf-1 expression in OV202 cells. As shown in Figure 4, when these cells were injected subcutaneously into nude mice at a cell number of 5×10^6 per mouse, enhanced Bim expression significantly arrested tumor growth, as defined by tumor volume shrinkage of at least 80% compared to the control group (Fig. 4b). At the end of the experiments, the average tumor weights were 0.12 and 0.93 g in mice injected with Bim C2 and C16 cells, respectively, compared to 4.81 g in the control groups ($p < 0.01$) (Fig. 4c).

Discussion

In our study, we observed that OV202 ovarian cancer cells, after knockdown of HSulf-1 by shRNA, acquired the ability to establish anchorage-independent colonies on soft agar (Fig. 1b) and formed xenografts in nude mice (Fig. 1c). These results are particularly interesting because this model shows that knockdown of only one gene, *HSulf-1*, in this cancer cell line was sufficient to alter the tumorigenicity both *in vitro* and *in vivo*. Therefore, our data provided the direct evidence to link the loss of HSulf-1 expression and tumorigenesis in ovarian cancer. Using this model, we also demonstrated that Bim, as a downstream signaling molecule of HSulf-1, plays an important role in ovarian tumor growth.

Bim belongs to the Bcl-2 protein family that contains three subgroups consisting of essential regulators of apoptosis.[12,13] The first subgroup includes the antiapoptotic members such as Bcl-2, Bcl-XL and Bcl-W, sharing three to four homology domains called the BH region. The other two groups include the proapoptotic members, one with two to three BH domains such as Bax and Bak, and the other with a unique BH3 domain and comprising Bim, Noxa and Puma. In response to diverse intracellular damage signals, the cell's decision to undergo apoptosis is determined by interactions between these three fractions of the Bcl-2 protein family.[9,10] In fact, the damage signals are transduced by the diverse “BH3-only” proteins. Apoptotic stimuli trigger activation of specific BH3-only proteins, which then engage the prosurvival Bcl-2 family members and liberate the downstream effectors, Bax and Bak, to elicit the caspase cascade and culminate in cell demolition.[9,10] Bim is a critical BH3-only protein that can engage all the prosurvival proteins. Interference with the innate apoptotic activity is a hallmark of neoplastic transformation and tumor formation.[14] Our Western blot results showed that Bim expression was hardly detected after knocking down of HSulf-1 in OV202 cells (Fig. 2a), suggesting that it may contribute to the overgrowth of OV202 cells after loss of HSulf-1.

Indeed, the important role of Bim in maintaining homeostasis has been reported to be associated with tumorigenesis in multiple tumors.[11,15] Transgenic expression of microRNAs (miR) of the miR-17B92 cluster that destabilizes Bim mRNA in mice led to

lymphoproliferative disease with autoimmune pathology and premature death of these animals.[16] Consistently, deletion of the miR-17B92 cluster led to increased Bim expression and defects in early B-cell development.[17] In support of a tumor suppressor role for Bim, Bim was found to be frequently silenced in human Burkitt's lymphoma. The biallelic deletion of Bim was observed in 42% of mantle cell lymphoma cell lines.[18,19] The direct evidence of tumor suppressor function of Bim was first reported under conditions of oncogenic expression of c-Myc.[20] In Em-myc mice, the loss of even one Bim allele accelerated tumorigenesis. Importantly, a significant number of tumors arising from the sole deregulation of c-Myc required additional defects along the p53 axis, while loss of Bim relieves the selection pressure for the inactivation of p53 pathway, clearly demonstrating that Bim acts as a suppressor in Myc-induced tumorigenesis. Recently, Bim has been proved to be a tumor suppressor in epithelial solid tumors.[15] In line with these observations, OV202 HSulf-1-deficient cells that can generate xenografts *in vivo* demonstrated dramatically reduced expression of Bim. Ectopic expression of Bim in these transfected OV202 Sh1 cells significantly retarded xenograft growth by at least 80% in volume in nude mice, providing strong evidence of the tumor suppressor role of Bim in ovarian cancer cells.

Our data also demonstrated that Bim is an effector molecule for HSulf-1. Bim is regulated at both the transcriptional and post-translational levels.[11,12,21] Phosphorylation of AKT and its downstream transcriptions factor FoxO3a have been reported to repress transcription of Bim, while activation of the ERK pathway is associated with the phosphorylation of Bim leading to its proteasomal degradation.[11,15] Although the activity of AKT pathway was increased in OV202 Sh1 and Sh2 cells, the minimal change of Bim level following the treatment with AKT inhibitor argues against the possible role of AKT on Bim expression in this system. The involvement of AKT pathway was not supported by the results from quantitative RT-PCR either because an increased rather than decreased mRNA level of Bim was observed in Sh1 cells. We speculated that loss of feedback inhibition due to dramatically decreased Bim at the protein level in Sh1 cells might account for the increased mRNA level of Bim. Instead, the dramatic increase in Bim level following the treatment with ERK inhibitor strongly supports the notion that ERK pathway is responsible for the decreased Bim level in OV202 Sh cells, thus providing evidence for the cross-talk between survival and apoptotic machineries in tumor cell (Fig. 5).

Despite the initially high response rate to standard frontline debulking surgery followed by platinum-based chemo-therapy, the relapse rate in ovarian cancer is high and many patients will recur within 6 months of completing platinum-based treatment.[22] Understanding the molecular mechanisms that control tumor growth has the potential to contribute to the development of improved chemotherapeutic treatment options. Our study demonstrated that blocking Bim by loss of HSulf-1 expression may represent a new mechanism used by some ovarian cancer cells to acquire higher tumorigenic ability. This finding suggests that the modified Bim protein may be a potential candidate as an antitumor agent, giving strong supporting evidence for the combination of cisplatin and a BH-3 mimetic.[14,23] This combination could represent a powerful new approach in the clinical setting for treating patients harboring loss of HSulf-1 in their tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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What's new?

The authors of this study previously identified the endosulfatase gene HSulf-1 as a down-regulated gene in ovarian cancer. In this study, and for the first time, they provide direct evidence of the critical role of HSulf-1 loss in promoting the tumorigenesis of ovarian cancer in a nude mice model. The *in vitro* and *in vivo* data also reveal a novel link between the expression of HSulf-1 and the important pro-apoptotic factor Bim. The study thus demonstrated that blocking Bim by loss of HSulf-1 expression may represent a new mechanism employed by some ovarian cancer cells to acquire higher tumorigenic ability.

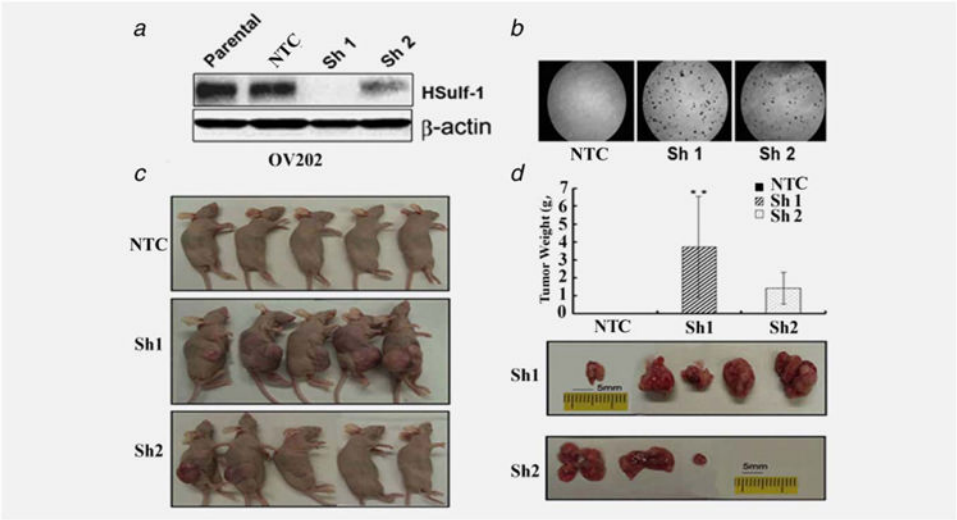

Figure 1.

Loss of HSulf-1 results in enhanced tumorigenicity of OV202 cells *in vitro* and *in vivo*. (*a*) Immunoblot analysis shows knockdown of HSulf-1 in OV202 cells with HSulf-1 shRNAs (sh1 and sh2). (*b*) Downregulated HSulf-1 results in colony formation in soft agar. OV202 stable clones expressing HSulf-1 shRNAs (sh1 or sh2), and NTC shRNA were seeded at 2,000 cells per well in six-well plates and incubated for 10 days. The resulting colonies were stained with 0.5 mg/ml p-iodonitrotetrazolium violet in PBS and photographed. Data are representative of two independent experiments. (*c*, *d*) HSulf-1-deficient OV202 cells formed subcutaneous xenografts in nude mice. Mice were s.c. inoculated with OV202 NT, Sh1 and Sh2 cells and tumor growth was monitored over time. All five mice in Sh1 group and three mice in Sh2 group formed tumors at the end of experiment (*c*). The xenografts removed from mice in Sh1 and Sh2 groups are shown and the average weights of the xenografts in each group are graphically represented (*d*). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

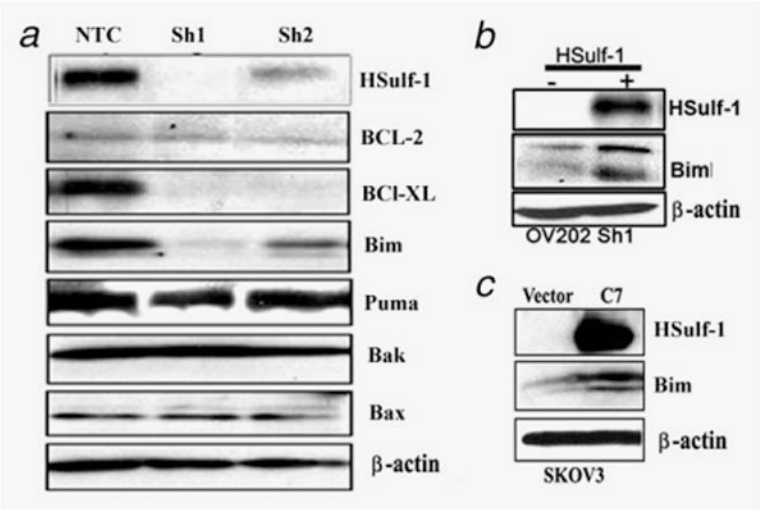

Figure 2.

Bim is regulated by HSulf-1 expression. (*a*) Bim was downregulated after loss of HSulf-1. OV202 clones were assessed by Western blotting for the indicated apoptosis-related proteins and reduced Bim levels were observed in OV202 Sh1 and Sh2 cells. (*b*) Rescue of HSulf-1 in OV202 Sh1 cells resulted in increased Bim expression. (*c*) Upregulation of Bim was observed in SKOV3 cells with ectopic expression of HSulf-1. Data are representative of two independent experiments.

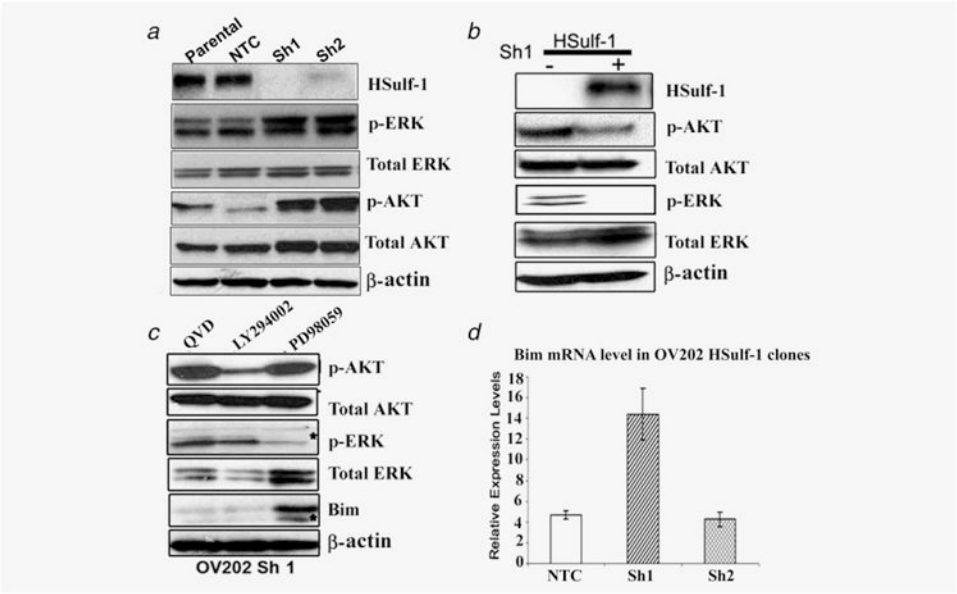

Figure 3.

HSulf-1 regulates Bim expression through ERK pathway. (*a*) Loss of HSulf-1 caused an increased phosphorylation of ERK and AKT. (*b*) Rescue of HSulf-1 resulted in decreased phosphorylation of ERK and AKT. (*c*) Pharmacological inhibition of ERK phosphorylation with PD98059 but not inhibition of AKT phosphorylation with LY294002 increased Bim protein levels. OV202 Sh1 cells seeded at 5×10^5 per well in six-well plates were treated with 10 μM LY294002 and 10 mM PG98059 separately. QVD, a pancaspase inhibitor, was added to both the untreated and treated cells to reduce apoptosis. Cell lysates were collected and subjected to Western blot analysis. (*d*) Bim mRNA levels in HSulf-1-deficient and -proficient cells as measured by real-time RT-PCR. Data are representative of two independent experiments.

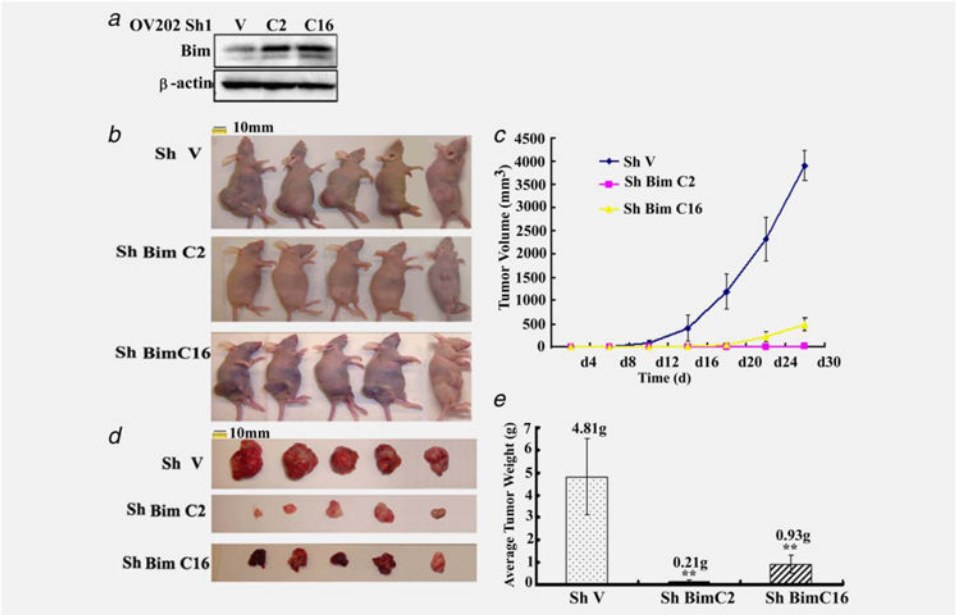

Figure 4.

Bim significantly inhibits tumor growth *in vivo*. (*a*) Ectopic expression of Bim in OV202 HSulf-1-deficient Sh1 cells. Data are representative of two independent experiments. (*b–e*) Ectopic Bim expression retarded tumor growth in nude mice. A total of 5×10^6 Bim re-expressing OV202 Sh cells (C2 and C16) were injected subcutaneously into nude mice. OV202 Sh1 cells transfected with empty vector served as the control. Ectopic Bim expression retarded tumor growth in nude mice (*b*). The average tumor volumes were monitored for 28 days and graphically represented in the right panel (*c*). Xenografts removed from mice were shown (*d*) and the average xenograft weights at the end of experiments were graphically represented (*e*). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

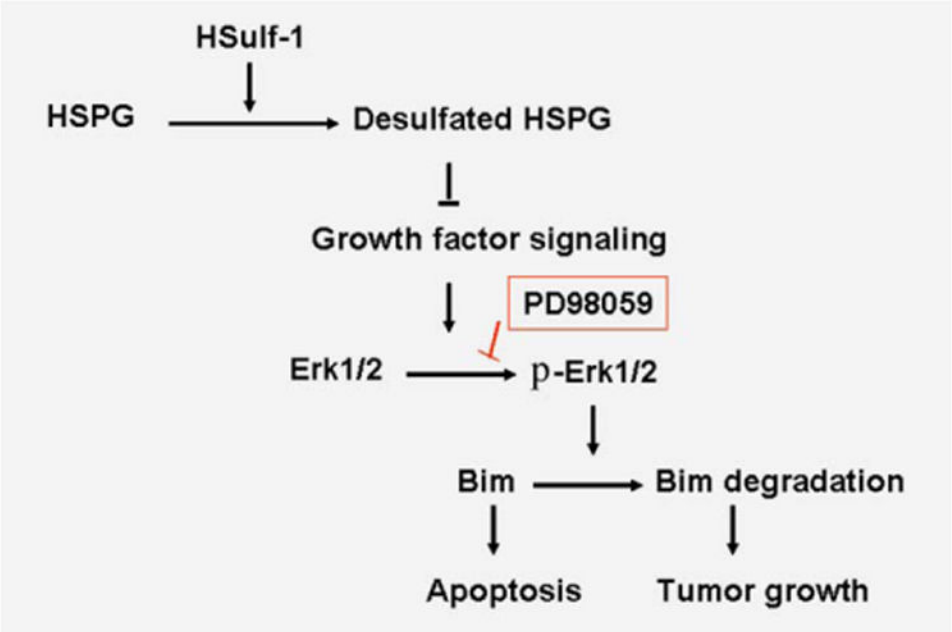

Figure 5.
Working model for the effect of HSulf-1 on Bim expression. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]