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HSulf-1 inhibits cell proliferation and invasion in human gastric cancer

Jie Li,[1] Min-Li Mo,[1] Zhao Chen,[1] Jie Yang,[1] Qiu-Shi Li,[1] Dian-Jun Wang,[2] Hui Zhang,[3] Ying-Jiang Ye,[3] Jun-Pu Xu,[4] Hai-Long Li,[5] Fang Zhang[5] and Hai-Meng Zhou[1,5,6]

[1]School of Life Sciences, Tsinghua University, Beijing; [2]Department of Pathology, The Chinese PLA General Hospital, Beijing; [3]Surgical Oncology Laboratory and Department of Gastroenterological Surgery, Peking University People's Hospital, Beijing; [4]Beijing ACCB Biotech Ltd, Beijing; [5]Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region Institute of Tsinghua University, Jiaxing, Zhejiang, China

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The *HSulf-1* gene encodes an extracellular 6-*O*-endosulfatase and regulates the sulfation status of heparan sulfate proteoglycans (HSPG). We have demonstrated that promoter hypermethylation is correlated with the *HSulf-1* silencing in gastric cancer. To investigate the functional importance of *HSulf-1* silencing in gastric cancer, we restored *HSulf-1* expression in the gastric cancer cell line MKN28, which lacks endogenous *HSulf-1*. Following restoration of expression, *HSulf-1* inhibited cell proliferation, motility, and invasion *in vitro*, as well as significantly suppressing the MKN28 xenograft model ($P < 0.05$). No noticeable changes in proliferation and motility were observed following restoration of *HSulf-1* in another gastric cancer cell line, namely AGS cells. Interestingly, in MKN28 cells, which have been reported to be dependent on extracellular Wnt signaling, we found that *HSulf-1* inhibited the transcriptional activity of the Wnt/β-catenin pathway and downregulated its targeted genes. Conversely, in AGS cells, in the constitutive Wnt/β-catenin pathway is active, *HSulf-1* had no effect on the activity of the Wnt/β-catenin pathway. Furthermore, transfection of *Wnt3a* cDNA or β-catenin shRNA resulted in rescue or enhancement, respectively, of the effects of *HSulf-1* in MKN28 cells. Furthermore, HSPG epitope analysis confirmed that HSulf-1 affected the structure of heparan sulfate on the cell surface. Together, the results of the present study suggest that extracellular *HSulf-1* may function as a negative regulator of proliferation and invasion in gastric cancer by suppressing Wnt/β-catenin signaling at the cell surface. (*Cancer Sci* 2011; 102: 1815–1821)

Gastric cancer is the second most common cause of cancer-related deaths worldwide.[1,2] Understanding the mechanisms involved in gastric tumorigenesis and metastasis is important for the development of new effective therapeutic agents.

The *HSulf-1* gene, characterized as Human ortholog of *Qsulf-1*, can hydrolyze the sulfate ester bonds of heparan sulfate proteoglycans (HSPG), leading to removal of the sulfate at the 6-*O* position of glucosamine.[3,4] It is believed that changes in the sulfation status of HSPG can affect their interactions with signaling molecules and therefore modulate signal transduction.[3,5–8] Recent evidence indicates that *HSulf-1* is downregulated in several types of cancer, including hepatocellular carcinoma (HCC), ovarian and breast cancer.[5,7] When *HSulf-1*-transfected myeloma cells are implanted into SCID mice, the rate of tumor growth is significantly reduced.[9] In addition, *HSulf-1* has been shown to enhance the suppression of tumorigenesis by histone deacetylase inhibitors in HCC.[10] It has also been reported that *HSulf-1* suppresses carcinogenesis and angiogenesis by inhibiting the activation of the heparin-binding growth factor pathway, including fibroblast growth factor (FGF)-2, hepatocyte growth factor (HGF), epidermal growth factor (EGF),[5–7,9,11] and vascular endothelial growth factor (VEGF) signaling.[8] It has also been documented that the HSPG, which are coreceptors for cytokines,[12,13] are required for Wnt-dependent regulation in *Drosophila*, *Xenopus laevis*, and mammals.[14–16] However, a role has been reported for *HSulf-1* as a positive regulator of the Wnt signaling pathway, promoting tumor growth, in pancreatic cancer.[17] Therefore, the possible relationship between HSulf-1 and Wnt signaling requires further exploration.

The Wnt signaling pathway plays a critical role in cell fate determination, tissue development, and cancer.[18–20] Secreted Wnt proteins bind to Frizzled and lipoprotein receptor 5/6 (LPR 5/6), resulting in the stabilization of β-catenin via inhibition of phosphorylation-dependent degradation.[19,20] The stabilized β-catenin accumulates in the cytoplasm and further translocates to the nucleus, interacting with transcription factors in the TCF/LEF family to activate target genes.[21] Accumulating evidence indicates a close correlation between the Wnt signaling pathway and the initiation and progression of gastric cancer.[22,23]

We have recently demonstrated downregulation of *HSulf-1* in human gastric cancer.[24] To further evaluate the role of *HSulf-1* in the tumorigenesis and metastasis of gastric cancer, in the present study we restored *HSulf-1* expression in gastric cancer cell lines that lack endogenous *HSulf-1* expression and investigated its effects on the growth rate and invasiveness of the cells *in vitro* and *in vivo*. The results suggest that *HSulf-1* may inhibit tumor growth and invasion by suppressing canonical Wnt signaling in gastric cancer.

Materials and Methods

Constructs. The *HSulf-1* expression plasmid (Plasmid 13003) was purchased from Addgene (Cambridge, MA, USA). The TOPflash and FOPflash reporter plasmids containing wild-type and mutant TCF/LEF binding sites, respectively, were kindly provided by Professor Yeguang Chen (Tsinghua University, Beijing, China). The *CTNNB1* shRNA plasmid was purchased from Origene (Rockville, MD, USA). The targeted *CTNNB1* gene sequence used in the present study was 5′-GGTCCTC-TGTGAACTTGCTCAGGACAAGG-3′. The scrambled or target hairpin oligonucleotides were subcloned into pSliencer, provided by Professor Peng Li (Tsinghua University). The *DKK1* and *mWnt3a* genes were subcloned into pCDNA3.1/myc-his vectors (Invitrogen, Carlsbad, CA, USA). Transfections were performed using Lipofectamine 2000 (Invitrogen).

Cell culture. The human gastric cancer cell lines used in the present study (AGS and MKN28) were obtained from the China Center for Type Culture Collection (Wuhan, China). The MKN28 and AGS cells stably expressing HSulf-1 or empty vector were selected with 0.5 and 1 mg/mL G418 (Invitrogen),

[6]To whom correspondence should be addressed.
E-mail: zhm-dbs@tsinghua.edu.cn

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respectively. Stable, pooled populations of individual clones were verified by immunoblotting analysis for HSulf-1. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

RNA extraction and semiquantitative RT-PCR. RNA extraction and semiquantitative RT-PCR were performed as described previously.[24] The sequences of the primer pairs used in the present study are listed in Table 1.

Immunoblotting. The stable cell lines were cultured to confluence. Cells were then trypsinized, pelleted by centrifugation at 500*g* for 5 min. To obtain total cell lysates, cell pellets were resuspended in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) and then vortexed for 5 s. After incubation on ice for 15 min, the suspension was centrifuged at 20,000*g* at 4°C for 5 min and the supernatant was collected for immunoblotting.

The cytosolic protein fraction was obtained as follows. Cell pellets were suspended in hypotonic buffer (20 mM Tris-HCl, pH 7.5, 25 mM NaF, 1 mM EDTA) and put on ice for 30 min. The cell suspension was centrifuged in an ultracentrifuge (Optima MAX; Beckman Coulter, Fullerton, CA, USA) at 124,500*g* for 30 min at 4°C. The supernatant was collected as the cytosolic fraction.

Both total protein (20 μg) and cytosolic protein extracts (40 μg) were subjected to immunoblotting. The primary antibodies used were anti-β-actin (1:5000; Sigma-Aldrich, St Louis, MO, USA), anti-β-catenin (1:2000; Sigma-Aldrich), anti-cyclin D1 (1:500; BD Biosciences, San Jose, CA, USA), anti-c-Myc (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Dvl-3 (1:200; Santa Cruz Biotechnology), and anti-HSulf-1 (1:250; Abnova, Taipei, Taiwan).

Cell proliferation assay. Cell growth rates were determined using the CellTiter 96 Aqueous Proliferation Assay Kit (also called MTS proliferation assay [Promega, Madison, WI, USA]). Stable cell lines were plated into 96-well culture plates at a density of 5×10^2 cells/well. After the cells had been cultured for 0, 2, 3, and 4 days, the MTS solution was added to the culture medium and cells were incubated for a further 1.5 h. Absorbance was measured at 490 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA).

Colony formation and soft agar assays. Stable cells (1×10^3) were seeded in triplicate into 100-mm dishes. The culture medium, containing G418, was replaced every 3–4 days. After cells had been cultured for 2–3 weeks, they were fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 20 min, and then photographed.

The soft agar assay was performed in six-well plates in triplicate using cells plated at a density of 1×10^4 cells/well. Cells were resuspended in medium with 0.35% agarose (Sigma-Aldrich) and seeded onto plates precoated with 0.5% base agarose. Cells were then cultured for 2–3 weeks. The colonies were counted manually after cells had been stained with 0.005% crystal violet.

Analysis of HSPG epitopes. Stable cells (1×10^5) were harvested, washed twice with PBS, and then incubated at 4°C for 45 min with mouse monoclonal anti-heparan sulfate (HS) antibody 10E4 (Seikagaku America, Falmouth, MA, USA), or IgM (Sigma-Aldrich) as a control, at a dilution of 1:50. After cells had been washed twice with PBS, they were incubated in PBS containing FITC-conjugated rabbit anti-mouse Ig (DakoCytomation, Carpinteria, CA, USA) at a dilution of 1:50 for 30 min. Cells were the washed twice with PBS, fixed in 2% paraformaldehyde, and then analyzed by flow cytometry (FACScan; BD Biosciences).

Luciferase assay. Cells were seeded into 24-well plates at a density of 5×10^3 cells/well prior to transfection. Cells were then transfected with the TOPflash or FOPflash plasmid in addition to the PRL-TK plasmid (as an internal control; Promega). After a further 18 h culture, luciferase activity in the cells was measured using the Dual-Glo Luciferase Assay (Promega).

Wound healing assay. Stable cell lines were seeded in six-well plates and cultured until they reached confluence. Wounds were scratched on the monolayer of cells using 200-μL pipette tips. The images of the wounds were photographed using an Olympus IX71 inverted microscope (Olympus Japan, Tokyo, Japan) after the cells had been cultured for 0, 24, and 48 h and wound size was measured.

Transwell migration and invasion assay. For the migration assay, 5×10^4 cells were suspended in serum-free DMEM and plated on chambers (8-μm pore size; Corning Costar, Cambridge, MA, USA) that were not coated with matrigel. For the invasion assay, the upper chamber was precoated with 5 mg/mL matrigel (Sigma-Aldrich) before 5×10^5 cells in serum-free DMEM were added to the chamber. For both assays, medium containing 10% FBS was added to the lower chamber as a

Table 1. Nucleotide sequences of the primers used in RT-PCR

Gene	Primers	
	Forward	Reverse
18S rRNA	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGTGTG
Wnt3a	AAGCAGGCTCTGGGCAGCTA	GACGGTGGTGCAGTTCCA
Dvl1	CATCCTTCCACCCTAATGTGTCC	TAAAGCCCGGGTCCTGGTAGGC
Dvl2	CATCCTTCCACCCTAATGTGTCC	GTCCCCCAGGCTGGTACTCT
Dvl3	CACGTGGTTGCTTCACATTGC	GACAAGTGGAAGTCGTCTAGG
Fzd2	GCACTACACGCCGCGCATGTC	CCCACCCCGGGCGGAGGAAAG
Fzd3	GTGAGTGTTCGAAGCTCATGG	ATCACGCACATGCAGAAAAG
Fzd5	GACTGTCTGCTCTTCTCGGC	GACGCACACAGGCAGAGGAA
Fzd6	ACTCTTGCCACTGTGCCTTTG	GTCGAGCTTTTGCTTTTGCCT
Fzd7	GCCTCGACGCTCTTTACC	GAGGCCAACGTAGCACACC
HSulf-1	ACTGTACCAATCGGCCAGAG	CCTCCTTGAATGGGTGAAGA
Axin-2	GCAGACGACGAAGCATGTC	GCCTTTCCCATTGCGTTTGG
c-Myc	TTCGGGTAGTGGAAAACCAG	CAGCAGCTCGAATTTCTTCC
CCND1	AGCTCCTGTGCTGCGAAGTGGAAAC	AGTGTTCAATGAAATCGTGCGGGGT
DKK4	AGCTCTGGTCCTGGACTTCA	CAACCCACGACATGTAGCAC
MMP-2	ATCATGATCAACTTTGGCCGCT	CAGCTGTTGTACTCCTTGCCAT
S100A4	AGCTGATGAGCAACTTGGACAG	CATCAAGCACGTGTCTGAAGGA
S100P	CAGGAGGAAGGTGGGTCTGAA	TGTGACAGGCAGACGTGATTG

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chemoattractant. After 24 h culture, cells in the upper chamber were removed using a cotton swab and stained with 0.5% crystal violet. The motility and invasiveness of the cells were determined by dissolving the stained cells in 10% acetic acid and measuring absorbance at 560 nm.

Xenograft model. Experiments in mice were conducted in the Animal Facility of Tsinghua University and were approved by the Institutional Animal Care and Use Committee of Tsinghua University. Gastric cancer xenografts were established in 6-week-old female BALB/c nude mice. Briefly, MKN28 cells stably expressing empty vector or HSulf-1 were trypsinized and resuspended in PBS (pH 7.4) and then mixed 1:1 (v/v) with matrigel (Vigorous, Beijing, China) at 4°C prior to injection into one mouse in a total volume of 100 μL. The mixture, containing 5×10^6 cells, was injected s.c. into the right flank of eight female mice (four in each group). Tumor size was determined using the formula ($0.5 \times \text{width}^2 \times \text{length}$) at various times over the 5-week period.

Five weeks after injection, tumors were collected and treated for RNA extraction, semiquantitative RT-PCR, and western blot analysis, as described above.

Statistical analysis. Data were analyzed using GraphPad Prism 5.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± SD of three independent experiments performed in triplicate. The significance of differences between groups was determined by Student's *t*-test. Two-tailed $P < 0.05$ was considered significant.

Results

***HSulf-1* inhibits the proliferation of gastric cancer cells.** First we examined the effect of *HSulf-1* on the growth of gastric cancer cells. The growth rate of MKN28 cells stably transfected with *HSulf-1* was significantly slower than that of cells stably transfected with empty vector ($P < 0.001$; Fig. 1a). However, stable transfection of *HSulf-1* did not affect the growth rate of AGS cells ($P > 0.05$; Fig. 1b). We also confirmed these observations in the colony formation (Fig. 1c,d) and soft agar (Fig. 1e,f) assays. Colony numbers were significantly less in MKN28 cells stably transfected with *HSulf-1* than in control cells ($P < 0.01$; Fig. 1c–f). To determine the effect of Hsulf-1 expression on the sulfation status of HSPG in gastric cancer cells, we used the anti-HS antibody 10E4, which has been reported to recognize HS 6-*O*-sulfated glucosamine residues as well as HS the N-sulfated glucosamine moiety.[3,25–27] Using flow cytometry, we found that the expression of the 10E4 epitope on the cell surface was significantly higher in MKN28 cells than in AGS cells (Fig. 1g,h), indicating possible differences in the HS structures between these two cell lines. Stable transfection of *HSulf-1* in MKN28 cells significantly decreased the 10E4 epitope on the cell surface compared with control cells (Fig. 1g). However, the same treatment of AGS cells had a relatively small effect on the cell surface 10E4 epitope (Fig. 1h).

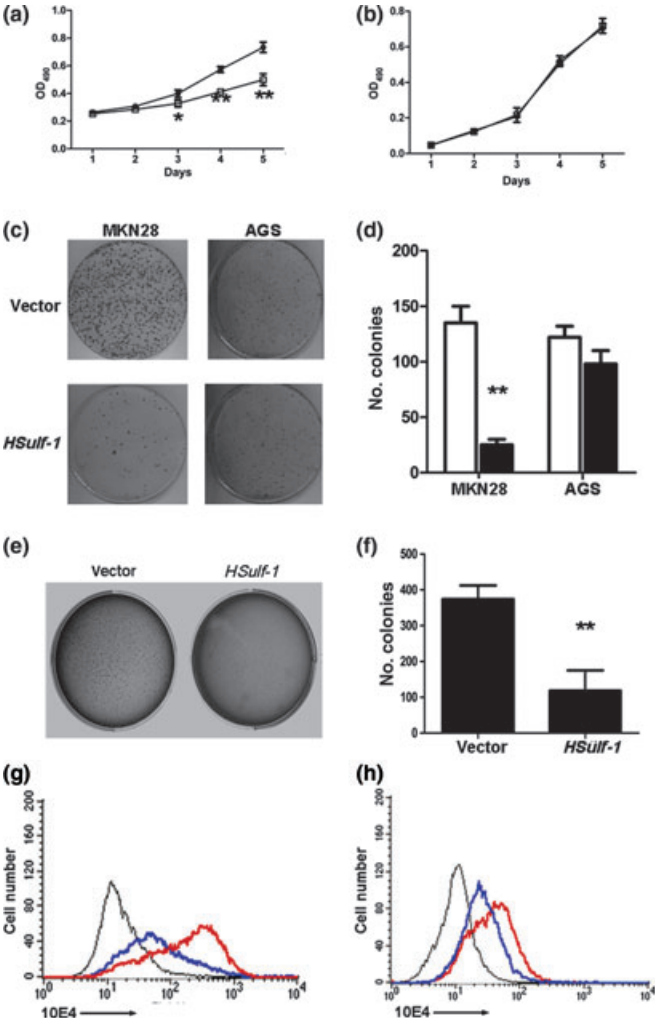

Fig. 1. Effect of *HSulf-1* on cell proliferation in gastric cancer cell lines. A and B: Growth curves for (a) MKN28 and (b) AGS cells, as determined by the MTS assay, following transfection of cells with either empty vector (–●–) or *HSulf-1* (–□–). (c) Representative images and (d) quantification of the results of the colony formation assay in MKN28 and AGS cells stably transfected with either empty vector (□) or *HSulf-1* (■). (e) Representative images and (f) quantification of the results of the soft agar assay in MKN28 cells stably transfected with *HSulf-1*. (g,h) Flow cytometry analysis of the effects of *HSulf-1* expression on the cell surface expression of the 10E4 epitope in MKN28 (g) and AGS (h) cells. Cells stably expressing empty vector (red line) or *HSulf-1* (blue line) were stained with anti-HS antibody or with an irrelevant antibody as a negative control (gray line). Data are the mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with empty vector.

***HSulf-1* the inhibits migration and invasion of gastric cancer cells.** In the wound-healing assay, we observed that 48 h after the wound had been made, the migration of MKN28 cells stably transfected with *HSulf-1* was markedly inhibited (~40% reduction) compared with control cells (Fig. 2a). In contrast, no noticeable change in the migration of AGS cells was observed (Fig. 2a). We next performed the transwell assay to examine the mobility and invasion of those stable cells (Fig. 2b,c). In MKN28 cells stably transfected with *HSulf-1*, an approximate 50% reduction in both mobility and invasion was seen compared with control cells ($P < 0.001$). However, there was no significant difference in the mobility of AGS cells stably transfected with *HSulf-1* and control cells. In accordance with these observations, we found that mRNA levels of metastasis-related genes, such as *DKK1*, *MMP-2*, *S100A4*, and *S100P*,[22,28–30] were downregulated in *HSulf-1* transfected MKN28 cells, but that there were no noticeable change in the expression of these genes in AGS cells (Fig. 2d).

***HSulf-1* regulates Wnt signaling in gastric cancer cells.** *HSulf-1* can regulate signal transduction pathways by affecting interactions between HSPG and extracellular signals. In addition, it has been reported that *HSulf-1* is involved in the regulation of Wnt signaling in pancreatic cancer.[17] Dysregulation of the Wnt signaling pathway has been implicated in gastric carcinogenesis and metastasis.[22,23,31–33]

Therefore, in the present study, we first assessed the expression profile of Wnt pathway genes in AGS and MKN28 cells. As shown in Figure 3(a), the expression of several Wnt ligands was

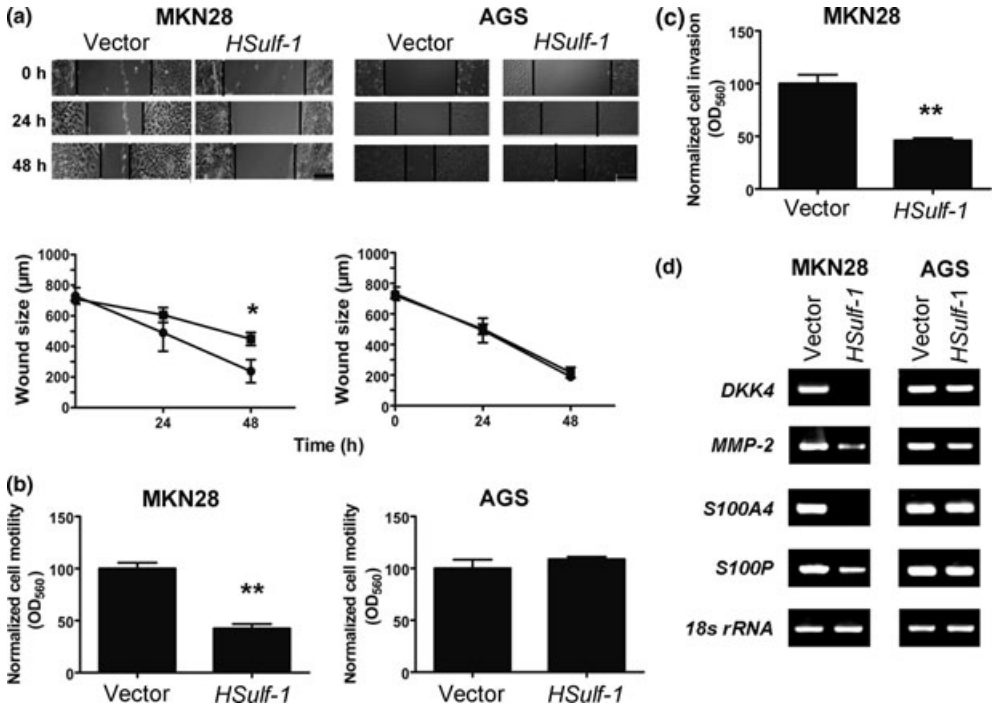

Fig. 2. Effect of *HSulf-1* on the migration and invasion of cells from the tow gastric cancer cell lines. (a) Wound healing assay. Wound size was monitored (top) and measured (bottom) at the time points indicated in MKN28 and AGS cells stably transfected with either empty vector (–●–) or *HSulf-1* (–■–). Scale bar, 200 μm. (b) Transwell migration assay of MKN28 and AGS cells stably transfected with empty vector or *HSulf-1*. OD_{560}, optical density at 560 nm. (c) The invasion assay for MKN28 cells stably transfected with empty vector or *HSulf-1*. Data are the mean ± SEM percentage absorbance at 560 nm compared with control. $*P < 0.05$, $**P < 0.01$ compared with empty vector. (d) Semiquantitative RT-PCR analysis of the mRNA expression of metastasis-related genes in MKN28 and AGS cells stably transfected with empty vector or *HSulf-1*.

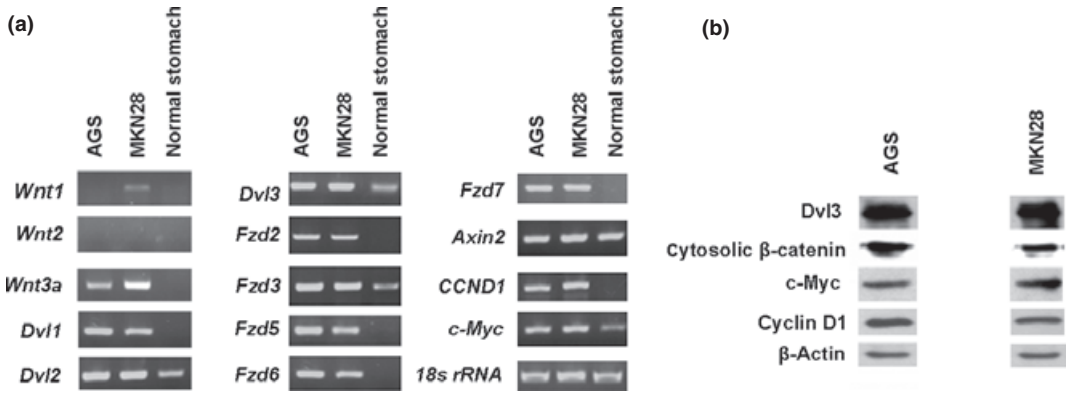

Fig. 3. Analyses of gene expression profiles in the two gastric cancer cell lines. (a) Semiquantitative RT-PCR analysis of the mRNA expression of Wnt signaling genes in MKN28 and AGS cells. Normal stomach tissue was used as a control, with *18s rRNA* used as loading control. (b) Western blot analysis of β-catenin in the cytosolic fraction and the Dvl3 and Wnt targets c-Myc and Cyclin D1 in whole cell lysates. β-Actin was used as a loading control.

elevated in both cell lines (i.e. *Wnt3a* in AGS cells; *Wnt1* and *Wnt3a* in MKN28 cells). The expression of these Wnt ligands was not detectable in normal stomach tissue, used as a control. The expression of Frizzled (Fzd) family members (*Fzd2, Fzd3, Fzd5 Fzd6*, and *Fzd7*), receptors for Wnt ligands, was also increased in both AGS and MKN28 cells compared with expression in normal stomach tissue. In addition, the expression of a key effector of the Wnt pathway, namely Dishevelled (*Dvl*), and the downstream target genes *CCND1* and *c-Myc* was upregulated in the AGS and MKN28 cells (Fig. 3a). In accordance with these observations, the expression levels of the proteins of these Wnt pathway effectors (Dvl and cytosolic β-catenin) and downstream target genes (cyclin D1 and c-myc) were increased in AGS and MKN28 cells (Fig. 3b). Furthermore, transcription factor TCF/LEF reporter activity, another hallmark of Wnt signaling activation, was high in AGS and MKN28 cells (Fig. 4). These results confirm the aberrant activation of Wnt signaling in AGS and MKN28 cells.

To investigate the function of *HSulf-1* in Wnt activation in gastric cancer, we examined the mRNA expression of

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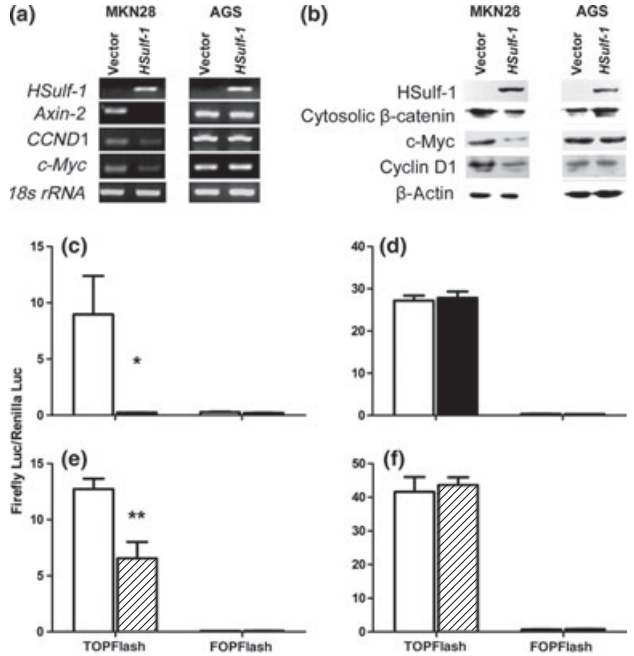

Fig. 4. Effect of *Hsulf-1* on Wnt signaling pathway in the two gastric cancer cell lines. (a) Semiquantitative RT-PCR analysis of the mRNA expression of *HSulf-1* and Wnt/β-catenin downstream genes and (b) western blot analysis of protein levels of HSulf-1, cytosolic β-catenin, and Wnt targets in MKN28 and AGS cells stably transfected with empty vector or *HSulf-1*. (c,d) TCF/LEF reporter activity and (e,f) TCF/LEF luciferase reporter activity in MKN28 (c,e) and AGS (d,f) cells stably transfected with empty vector (□), *HSulf-1* (■), or *DKK1* (▨). Data are the mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with empty vector.

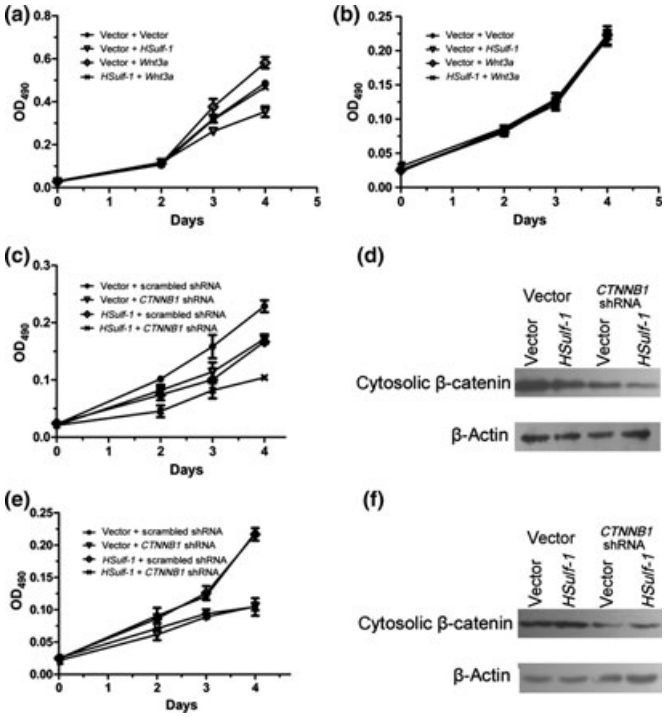

Fig. 5. Effects of *HSulf-1* and the Wnt pathway on cell proliferation in two gastric cancer cell lines. (a,b,c,e) Results of the MTS assay after stable MKN28 (a,c) and AGS (b,e) cells were transfected with *Wnt3a* (a,b) or *CTNNB1* shRNA (c,e). (d,f) Western blots of cytosolic β-catenin in MKN28 (d) and AGS (f) cells transiently transfected with *CTNNB1* shRNA. Data are the mean ± SEM.

Wnt target genes in AGS and MKN28 cells stably transfected with *HSulf-1*. In MKN28 cells stably transfected with *HSulf-1*, the expression of all target genes examined was downregulated. In contrast, there were no obvious changes in the expression of these genes in AGS cells (Fig. 4a). We also determined the protein levels of cytosolic β-catenin, a key component of the Wnt pathway, and cyclin D1 and c-myc as downstream targets of Wnt.[19] Consistent with our RT-PCR results, cytosolic accumulation of β-catenin and levels of cyclin D1 and c-myc were markedly downregulated in MKN28 cells stably transfected with *HSulf-1*, but not in AGS cells (Fig. 4b). Using the TOPflash/FOPflash reporter system to evaluate downstream transcription activity of the Wnt signaling pathway, we found that TCF/LEF reporter activity was significantly inhibited in MKN28 cells stably transfected with *HSulf-1* ($P < 0.05$; Fig. 4c). In contrast, *HSulf-1* transfection had no effect on TCF/LEF reporter activity in AGS cells (Fig. 4d). As a control, we transfected cells with *DKK1*, an extracellular antagonist for Wnt signaling,[34] into MKN28 and AGS cells to confirm the regulation of upstream Wnt signaling on its downstream transcription activity. We found that normalized TOPflash reporter activity was significantly decreased in MKN28 cells ($P = 0.006$), but unaltered in AGS cells ($P > 0.05$), after *DKK1* transfection (Fig. 4e,f). These results indicate that *HSulf-1* may be an extracellular suppressor inhibiting upstream Wnt signaling in gastric cancer cells.

To further test this hypothesis, we transfected *Wnt3a* into AGS and MKN28 cells stably transfected with *HSulf-1* and examined cell growth rates. Following *Wnt3a* transfection, the inhibitory effect of *HSulf-1* on MKN28 cell proliferation could be totally rescued (Fig. 5a). Conversely, neither *HSulf-1* nor *Wnt3a* had any effect on the proliferation of AGS cells (Fig. 5b). We also transfected *CTNNB1* shRNA to knockdown β-catenin in both cell lines (two different shRNA targeting *CTNNB1* tested in the present study showed consistent results; one set of data are shown in Fig. 5). The efficiency of β-catenin knockdown was confirmed by immunoblotting (Fig. 5d,f). We found that *CTNNB1* shRNA alone inhibited cell proliferation in both MKN28 and AGS cells. In MKN28 cells, knocking down β-catenin potentiated the inhibitory effects of *HSulf-1* on cell proliferation (Fig. 5c). However, in AGS cells, transfection of the combination of *HSulf-1* and *CTNNB1* shRNA resulted in the same inhibitory effect on cell proliferation as that seen with transfection of *CTNNB1* shRNA alone (Fig. 5e). Together, the results suggest that *HSulf-1* may inhibit MKN28 cell growth by suppressing upstream Wnt signaling.

***HSulf-1* inhibits growth of gastric cancer *in vivo*.** Finally, MKN28 xenografts were established to evaluate the effect of *HSulf-1* on the growth of gastric cancer *in vivo*. Three weeks after inoculation in mice, we found that the tumor volume of tumors resulting from the injection of MKN28 cells stably transfected with *HSulf-1* was significantly less than that of the control ($P < 0.05$; Fig. 6a). In addition, at the completion of this experiment (i.e. 5 weeks after inoculation), the weight of the tumors arising after injection of MKN28 cells stably transfected with *HSulf-1* was significantly less than that of the control ($P < 0.05$; Fig. 6b,c). Furthermore, the expression of cytosolic β-catenin and Wnt downstream target genes was downregulated in the tumors arising from injection of MKN28 cells stably transfected with *HSulf-1* (Fig. 6d), a finding that is consistent with the *in vitro* results. These data indicate that *HSulf-1* may inhibit the tumor growth of MKN28 cells *in vivo* by suppressing the Wnt signaling pathway.

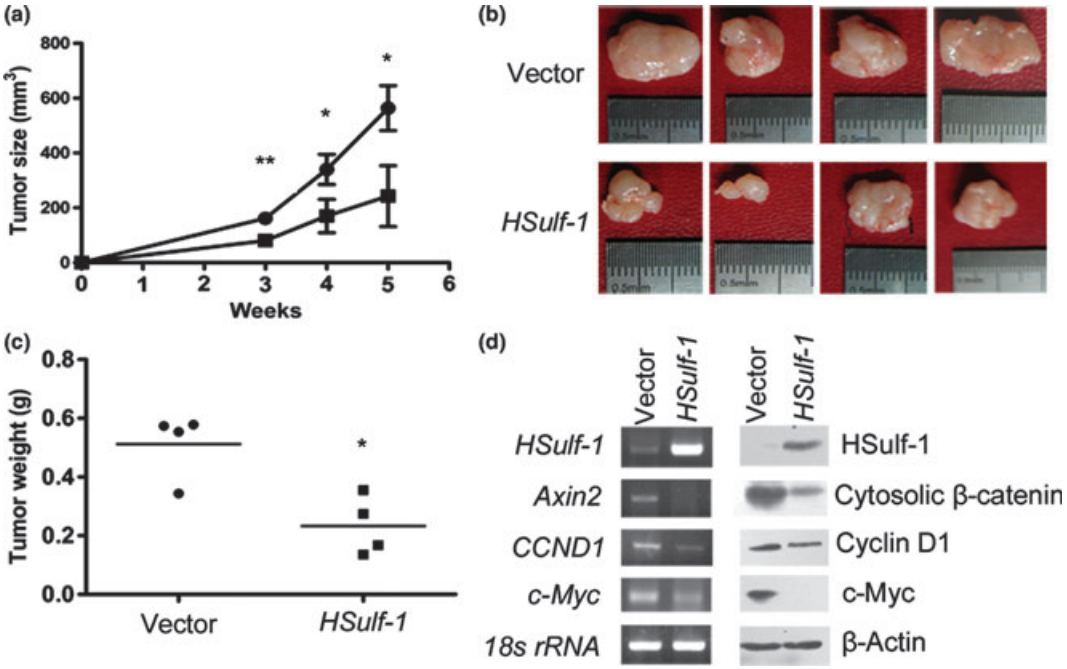

Fig. 6. Effect of *HSulf-1* on the tumorigenicity of MKN28 cells *in vivo*. (a) Tumor volumes measured at different time points in MKN28 cells transfected with either empty vector (–●–) or *HSulf-1* (–■–). Data are the mean ± SEM. **P* < 0.05, ***P* < 0.01 compared with empty vector. (b) Tumors harvested from mice 5 weeks after injection of MKN28 cells transfected with either empty vector or *HSulf-1*. (c) Individual tumor weights for each mouse at the end of the experiment. (d) Semiquantitative RT-PCR and western blot analyses of the Wnt signaling pathway in tumors arising in mice following the transfection of MKN28 cells transfected with either empty vector or *HSulf-1*.

Discussion

Although it has been shown that *HSulf-1* has a role in tumorigenesis and metastasis in several types of cancer,[5–10] its role in gastric cancer had not been investigated. In the present study, we found that *HSulf-1* suppressed cell proliferation, tumor growth, and activity of the Wnt signaling pathway in MKN28 cells, but not in AGS cells. These results suggest a possible function for *HSulf-1* in the suppression of upstream Wnt signaling to inhibit gastric cancer cell growth. The mechanisms underlying aberrant Wnt activation in these two cell lines appear to be different. It has been reported previously that AGS cells have the G34E mutant allele in *CTNNB1*.[35] We also confirmed this mutation in AGS cells by direct sequencing (data not shown). As a result, in AGS cells the Wnt pathway is constitutively active and independent of upstream Wnt signals. Conversely, MKN28 cells are dependent on upstream Wnt signaling. Consistent with these findings, we observed that low 10E4 epitope expression on the cell surface of AGS cells and a relatively small reduction in 10E4 epitope expression in response to HSulf-1 in AGS cells compared with MKN28 cells. To confirm this differential cell surface regulation of Wnt signaling in MKN28 and AGS cells, we transfected *DKK1*, an extracellular antagonist of Wnt signaling,[36] into AGS cells and demonstrated no noticeable reduction in the transcriptional activity of the downstream Wnt pathway. Conversely, *DKK1* transfection did affect MKN28 cells. We also confirmed aberrant activation of Wnt pathway in the two cell lines. To further test our hypothesis, we performed gain-of-function and loss-of-function experiments (Fig. 5). We observed that *Wnt3a* transfection rescued the *HSulf-1* inhibition of MKN28 cell proliferation, but that it had no effect in AGS cells. In the loss-of-function experiment, although the proliferation of both AGS and MKN28 cells was inhibited by *CTNNB1* shRNA knockdown alone, knocking down *CTNNB1* only potentiated the inhibitory effects of *HSulf-1* on the proliferation of MKN28 cells, and not AGS cells. Together, these results indicate that *HSulf-1* may function as a negative extracellular regulator of upstream Wnt signaling important for cell growth in gastric cancer through its enzymatic effects on HSPG, consistent with a recent report that *HSulf-1* mediates the affinity of HSPG for extracellular ligands.[36]

In addition, we found that *HSulf-1* inhibited the migration and invasiveness of MKN28 cells, as well as the expression of several well-known metastasis-related genes (Fig. 2). Of these genes, *DKK4*, *S100A4*, and *S100P* have been identified as Wnt signaling downstream target genes.[30] Although *DKK4* is a Wnt antagonist, it is associated with the malignant properties of cancer cells.[29] However, another metastasis-related gene, namely *MMP-2*, was not a target of Wnt signaling, indicating possible regulation of multiple signaling pathways by *HSulf-1* in the invasion of gastric cancer cells.

In summary, the results of the present study demonstrate that *HSulf-1* functions as a negative regulator of gastric carcinoma and metastasis, at least in part by regulating the sulfation status of HSPG and further suppression of upstream Wnt signaling.

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Disclosure Statement

The authors have no conflict of interest.

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