Regulation of integrin αV subunit expression by sulfatide in hepatocellular carcinoma cells

Wei Wu[†#], Yi Wei Dong[‡#], Peng Cheng Shi[†], Mei Yu[†], Da Fu[†], Chun Yi Zhang[†], Qian Qian Chai[†], Qian Lei Zhao[‡], Ming Peng[†], Li Hui Wu[‡], Xing Zhong Wu[†]*

[†] Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, Key Lab of Glycoconjugate Research, Ministry of Public Health, Shanghai 200032, P.R. China.

[‡] Yuying Children's Hospital, Wenzhou Medical College, Zhejiang 325003, P.R. China

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*To whom correspondence should be addressed.

Xing Zhong Wu, PhD, MD, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, P.R. China，Tel & Fax: 8621 54237697, E-mail: xz_wu@shmu.edu.cn

# Both authors contributed equally to this work

The abbreviations used: SM3, lactosyl sulfatide, sulfated lactocerebroside, Sulfo-Lacto-Cer; SM4, galactosyl sulfatide, sulfated galactocerebroside; Lacto-Cer, lactocerebroside; Gal-Cer, galactocerebroside; *CST*, Cerebroside sulfotransferase; ECM, extracellular matrix； ManN-pro, ManN propanyl perac; Cyclo-ManN-pro, cyclo-ManN propanyl perac; HUEVC, human umbilical vein endothelial cells; EMSA, Electrophoretic Mobility Shift Assay; ChIP, Chromatin Immunoprecipitation

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ABSTRACT

Integrin is important in migration and metastasis of tumour cells. Changes of integrin expression and distribution will cause an alteration of cellular adhesion and migration behaviours. In this study, we investigated sulfatide regulation of the integrin αV subunit expression in hepatoma cells and observed that either exogenous or endogenous sulfatide, elicited a robust up-regulation of integrin αV subunit mRNA and protein expression in hepatoma cells. This regulatory effect occurred with a corresponding phosphorylation (T739) of the transcription factor Sp1. Based on the EMSA (electrophoretic mobility shift assay), sulfatide enhanced the integrin αV promoter activity and strengthened the Sp1 complex super-shift. The results of ChIP analysis also indicated that sulfatide enhanced Sp1 binding to the integrin αV promoter *in vivo*. Silence of Sp1 diminished the stimulation of integrin αV expression by sulfatide. In the early stage by sulfatide stimulation, phosphorylation of Erk as well as c-Src was noted, and inhibition of Erk activation with either U0126 or PD98059 significantly suppressed Sp1 phosphorylation and integrin αV expression. Herein, we demonstrated that sulfatide regulated integrin αV expression and cell adhesion, which was associated with Erk activation.

Keywords: Cell adhesion, Integrin **αV**, Sulfatide, Cerebroside

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INTRODUCTION

Integrins have been implicated as very important adhesion molecules that are involved in multiple physiological processes, such as cell adhesion, proliferation, and survival (1-3). Each integrin generally consists of a noncovalently linked α- and β-subunit, with each subunit having a large extracellular domain, a single membrane-spanning domain, and a short, non-catalytic cytoplasmic tail. Integrins seem to be the major receptors by which cells attach to components of the ECM (extracellular matrix), such as vitronectin, etc. (4) and involve in the metastasis signalling of hepatocellular carcinoma (HCC) (5).

The integrin αV subunit associates with one of five integrin β subunits, β1, β3, β5, β6 or β8, to form five distinct αVβ heterodimers (6). The integrin αVβ heterodimers on the cell surface interact with cell adhesive proteins, such as collagen, fibrinogen, fibronectin, and vitronectin. These interactions play an important role in cell adhesion or migration, especially in tumour metastasis. Integrins increase in invasive tumours and distant metastases, characterize the metastatic phenotype and play a key role in tumour metastasis (7, 8). Many studies have documented marked differences in the surface expression and distribution of integrins between malignant tumours and pre-neoplastic tissues. For example, the integrin αVβ3 complex is strongly expressed in the invasive front cells of malignant melanoma and angiogenic blood vessels, but is weakly expressed on pre-neoplastic melanomas and quiescent blood vessels (9). Also, it has been demonstrated that αVβ3 integrin is specifically required to sustain neovascularization induced in vivo by fibroblast growth factor-2(10). Integrin αVβ3 physically associates with phosphorylated and activated insulin-like growth factor receptor, and may be involved in the HCC cell migration and progression(11). Furthermore, inducing the expression of the integrin αV (7) or β3 (12) subunit in melanoma cells increases their metastatic potential. A change of the expression and distribution of integrins on the cell surface can modulate the strength of cell adhesion and migration. The regulation of cell adhesion and migration involves coordinated events of tumour metastasis.

Many primary and metastatic cancer cells display altered integrin expression levels and/or activation status, leading to abnormal cell adhesion, growth, and survival, which are pathological hallmarks of cancer. Although the influence of integrin αV and β3 on metastasis has been documented

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in several studies (13, 14), there is little information about cerebroside sulfation signalling, and the molecular regulation mechanisms underlying integrin expression have not been elucidated. Sulfatide, the product of galactose-3-O-sulfotransferase 1 *(Gal3ST-1,* also called cerebroside sulfotransferase, *CST)*, is highly expressed in high metastasis potential HCC cells (MHCC97H) compared to low metastasis potential HCC cells (MHCC97L) (15). After retinoic acid treatment and inhibition of cell migration, the cellular sulfatide production is decreased(15), but the precursor cerebroside is not affected, suggesting the inhibition of *CST* activity. Hep3B cells overexpressing *CST* that produces sulfatide significantly promotes the metastasis behaviours in nude mice. Apart from *CST (Gal3ST-1), Gal3ST-2* is also observed to be involved in tumour metastasis (16). Both genes encode galactose-3-O-sulfotransferases, which catalyse and transfer sulfate to the 3' hydroxyl group of the galactosyl residue in the glycol chain. This sulfation makes the substrate molecule negatively charged, changes its affinity with binding molecules and involves HCC metastasis or progression (17). The HCC Hep3B cells transfected by *CST* expressed an elevated level of integrin αV and intensively adhered to vitronectin, the ligand of integrin αVβ3 (15, 18). However, the mechanism by which the *CST* is involved in regulation of integrin αV and cell adhesion is currently not fully understood. The relationship between the promotion mechanism of cancer cells and elevated expression of sulfatide remains unknown(19). A recent study showed that sulfatide can serve as a laminin-binding glycolipid and can anchor laminin-1 and -2 to the Schwann cell surface, form a laminin-associated complex and enable basement membrane assembly to initiate c-Src activation(9). Sulfatide was also identified as an interacting partner of P-selectin and promoted a P-selectin-mediated metastasis process in colon cancer cells(20). Sulfatide and P-selectin interactions led to subsequent platelet aggregation (21) and played an important role in the formation of cancer embolus. Our previous study (15, 18) revealed that hepatoma cells expressed sulfatide after *CST* transfection. The enzyme *CST* in HCC can only catalyse the production of sulfatide which acts as the endogenous sulfated cerebroside. We thus hypothesize that the enzyme product sulfatide is responsible for the regulation of integrin αV subunit and involves the metastasis process. To test this, we investigated, in this study, the regulatory effect of both exogenous and endogenous sulfatide, the product of *CST,* and demonstrated that sulfatide, but not cerebroside, played an important role in the regulation of the expression of the integrin αV subunit which can form the molecule αVβ3 in hepatocellular carcinoma cells.

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MATERIALS AND METHODS

Materials

Lactocerebroside (Lacto-Cer), galactocerebroside (Gal-Cer) and sulfatide from bovine brain were obtained from Sigma (Sigma–Aldrich, St.Louis, MO, USA). ManN-pro (ManN propanyl perac) and Cyclo-ManN-pro (cyclo-ManN propanyl perac) were kindly provided by Professor Wermer Ruetter (22, 23). Peptides GRGDSP and GRGESP with 99% purity were provided by ChinaPeptides Co., Ltd (Shanghai, China). Polyclonal rabbit, monoclonal mouse anti-human integrin αV antibodies and polyclonal rabbit anti-human Sp1 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse O4 antibody (anti-galactosyl sufatide and lactosyl sulfatide) was obtained from Chemicon (Temecula, CA, USA). Monoclonal mouse anti-lactosyl sulfatide antibody was obtained from Seikagaku (Tokyo, Japan). FITC-anti-human integrin αV was obtained from BioLegend (San Diego, CA). Antibodies against Erk, p38, Rac, mTOR, JNK and phospho-Raf (S338) were obtained from Cell Signalling Technology Inc (3 Trask Lane, Danvers, MA 01923, USA). Phospho-Raf (Y341) was from Beijing Boisynthesis Biotechnology Co. LTD (Bioss, Beijing, China). Akt antibody was from Bioworld (Atlanta, Georgia 30305, USA). Anti-phosphoserine antibody was from Millipore (Billerica, MA 01821,USA). Fibrinogen was from Sigma-Aldrich (St. Louis, MO), collagen type I was from Roche (Indianapolis, IN, USA), and fibronectin and vitronectin were from Calbiochem (San Diego, CA).

Cell culture

Hepatoma cells (SMMC-7721, BEL-7404), HUVECs (human umbilical vein endothelial cells), HeLa and HEK-293T cells were obtained from the Institute of Cell and Biochemistry Research of the Chinese Academy of Science. *CST*-overexpressing cells (*CST*-1, *CST*-8), *CST*-knockdown cells (Chp2,

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Chp5) and their corresponding Mock cells were established by our laboratory previously (18). *CST* over-expressed HCC cells mainly produced lactosyl sulfatide. The cells were maintained in RPMI 1640 medium supplemented with 10% newborn bovine serum (PAA, Austria) at 37°C under a 5% CO_2 atmosphere. For the treatment, cells were cultured in RPMI 1640 medium containing 2 μM sulfatide, lactocerebroside, or galactocerebroside added from stock solution in DMSO. An equal amount of DMSO(0.1% v/v) was added to control group.

Plasmid Construction

The short hairpin sequences including (1) 5`-AGGAGUUGGUGGCAAUAAU-3` and (2) 5`-UAUUAGGCAUCACUCCAGG-3`, which specifically interfered and targeted Sp1 mRNA, were designed according to the protocol from Ambion (24). The synthesised 55 bp forward and reverse oligonucleotides containing the siRNA sequence were annealed and ligated to the pSilencer 4.1 vector. The pcDNA3.0-Sp1 expression plasmid was kindly provided by Dr. Jian-Hai Jiang. A human *CST* cDNA expression plasmid was previously constructed (15, 18). The integrin αV promoter fragments from -1295 to +207 bp, -795 to +207 bp, -309 to +207 bp, and -16 to +207 bp were obtained by PCR from the genomic DNA of SMMC-7721 cells. The following primers were used: integrin αV/Kpn I -1295: 5'- CCCGGTACGGTCCACACAATGCACTTAAA-3', integrin αV/Kpn I -795: 5'-AAAGGTACGCAAGAGGCTATGCTGGC-3', integrin αV/Kpn I -309: 5'-AAAGGTACGCCTCCTTCCAGGTCTCC-3', integrin αV/Kpn I -16: 5'-AAAGGTACGTGGGGCGGGGGGAGGT-3', integrin αV/Xho I +207: 5'-CCCGTCGAGAGAAATCCACGGCGAA-3'. The PCR products were inserted into the Xho I/Kpn I sites of the pGL3-basic vector (Promega, Madison, MI) and designated as pGL3- integrin αV. All of the constructs were verified by sequencing.

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RT-PCR and Real-time PCR

Total RNA was extracted from cells with the Trizol reagent according to the manufacturer's instructions and was used as the template for cDNA synthesis. Reverse transcription was then carried out by M-MLV. The following primer sets were used for RT-PCR and Real-time PCR: integrin αV subunit (sense 5`-GACAGTCCTGCCGAGTA-3`, anti-sense 5`-CTGGGTGGTGTTTGC-3`), Sp1 (sense 5`-TCACAAGCCAGTTCCAGCTCC-3`, anti-sense 5`-GGGTGCACCTGGATTCCTGAA-3`), Sp3v1 (sense 5`-GAAATGGCTGCCTTGGACG-3`, anti-sense 5`-AGCGGTGACGGCTGAGTGT-3`), Sp3v2 (sense 5`-ACCCCCTCCCCCTGTCTCCCTC-3`, anti-sense 5`-CTCCCATCGGTTTGGTGCTCCT-3`), ETS (sense 5`-TCACAAGCCAGTTCCAGCTCC-3`, anti-sense 5`-GGGTGCACCTGGATTCCTGAA3`), AP2 (sense 5`-GCTGGGCACTGTAGGTC-3`, anti-sense 5`-ACTTGGACAGGGACACG-3`), EGR1 (sense 5`-CAGCAGCCTTCGCTAACC-3`, anti-sense 5`-CATCGCTCCTGGCAAACT-3`), EGR2 (sense 5`-CAGCCTCATCCAGCGTCAC-3`, anti-sense 5`-TCGTCAGAGCGGGAGAACC-3`), β-actin (sense 5`-CTCCATCCTGGCCTCGCTGT-3`, anti-sense 5`-GCTGTCACCTTCACCGTTCC-3`), CST (sense 5`-CAAGTTCGCCTTCCCTAA-3`, anti-sense 5`-CACAGCAGGTCCTTCAG-3`). The PCR amplifications were performed, and the products were analysed by 2% agarose gel electrophoresis. β-actin was used as an internal control. Real-time PCRs were performed using a BIO-RAD IQ5 Real-time PCR System (Bio-Rad).

Transfection

For transient transfection, SMMC-7721 cells were seeded in six-well plates at 5×10^5 cells/well. The cells were then transfected with Sp1 expression vectors for 24 h and then treated with Lacto-Cer and sulfatide for 24 h respectively. The empty pcDNA3.0 vector was used as a Mock control. The

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pSilencer 4.1-CMV puro vector (Ambion Inc, USA) encoding Sp1 siRNA (si-Sp1) was transfected to 80% confluent SMMC-7721 cells. The pSilencer 4.1-CMV puro vector containing scramble oligonucleotides was used as the control. The transient transfection of SMMC-7721 cells was performed using the transfection reagent (SunBio, Shanghai, China) according to the manufacturer's instructions.

Cell Adhesion Assay

The adhesion assay was performed as previously reported (16). Briefly, 96-well plates were coated at 100 µl/well with collagen type I (40 µg/ml), fibrinogen (20 µg/ml), fibronectin (10 µg/ml), and vitronectin (2 µg/ml) at 4°C overnight. The negative control groups were coated with 1% BSA, and the positive groups were coated with poly-lysine. After blocking with 1% BSA for 1 h at room temperature and washing twice with PBS, 200 µl of the cell suspension (4×10^4/ml) was added to each well. After incubation at 37°C for 1 h, all wells were washed twice with PBS, stained with MTT {3-(4,5)-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide}, and the absorbance value was measured at 570 nm with a reference wavelength of 630 nm. The adhesion rate was calculated by the formula (A570 value in test group－A570 in negative group) / (A570 in positive group－A570 in negative group) × 100%.

Cell ELISA

The method was based on our previous report (25). Briefly, *CST*-1, *CST*-8 and Mock cells (1×10^4) were incubated in a 96-well plate. After 24 h of culture, the cells were washed and fixed with 4% paraformaldehyde in PBS. Endogenous alkaline phosphatase was saturated and consumed with the substrate. The plates were blocked with 1% blocking reagent in PBS overnight at 4°C, incubated with O4 antibody (anti-Sulfo-Lacto-Cer and Sulfo-Gal-Cer) and mouse IgG as control for 2 h at room

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temperature, washed three times, and incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins. The absorbance was measured at 450 nm after incubation with the alkaline phosphatase substrate pNPP(*p*-nitrophenyl phosphate).

Immunofluorescence

The immunofluorescence method was based on the previous report (26). The cells of *CST*-1 and *CST*-8 were fixed with 2% paraformaldehydrate and not permeabilized, but exogenous sulfatide-treated cells were permeabilized. After blocking and staining with a mouse anti-sulfatide antibody for 2 h, and subsequently with FITC-conjugated secondary antibody for 1 h, the coverslips containing the cells were mounted on glass slides with Vectashield, and the cells were viewed under a fluorescence microscope.

Cell Adhesion to HUVEC

HUVECs in 150 μl of medium were seeded onto 96-well plates at 1×10^4 cells per well and incubated overnight at 37°C to confluence. Next, the HUVEC monolayer was stimulated with 10 ng/ml TNF-α for 4 h prior to the addition of the variously treated SMMC-7721 (1×10^5/ml) cells in 200 μl RPMI 1640 medium with 1% newborn bovine serum. After incubation at 37°C for 1 h, unattached cells were vigorously washed off with PBS, and the attached cells were fixed with 4% paraformaldehyde for 10 min. Subsequently, adherent cells were counted under a phase contrast microscope. The numbers of adherent cells in all groups were analyzed and compared to show the cell adhesion to HUVEC as described earlier (16). Data were reported as mean ± SD for at least three wells, and the experiment was repeated three times independently.

Flow Cytometry Analysis

Based on our previous report (25), sub-confluent SMMC-7721 cells were detached with 0.02%

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EDTA, and the single-cell suspensions were washed and maintained in suspension for 1 h in RPMI 1640 medium with 10% newborn bovine serum. Cells were not permeabilized and then incubated with 2 μg of FITC-labelled primary anti-human CD51 antibody per million cells for 2 h at 4°C with gentle shaking. After washing, the cells were resuspended in 1% paraformaldehyde, and cell surface immunofluorescence was analysed by a flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Luciferase Assay

SMMC-7721 cells were seeded into 96-well plates and the next day transiently transfected with 0.2 μg of specific expression vectors. The cells were incubated for 24 h at 37°C, washed once with PBS, and then lysed in 20 μl of Lysis Buffer (Promega, Inc., Madison, WI) to measure the luciferase reporter gene expression by the luciferase reporter assay system (Promega, USA). The intensity of luminescence was measured by a luminometer (Lumat LB 9507, Berthold, Germany). The data of relative luciferase activities were normalised to the control. The results are presented as the mean of the experiments performed in triplicate. Experiments were repeated at least three times.

Western blotting

Western blot analysis was carried out essentially as described previously (17). Briefly, 30 μg of protein was resolved on 10% SDS-polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride membrane. The membrane was then incubated with a series of antibodies against GAPDH, integrin αV, Sp1 and histone 4. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody, and protein bands were visualised using an enhanced chemiluminescence detection kit.

Nuclear extracts

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Nuclear extracts were prepared with the nuclear extraction kit (CHEMICON, Cat .No.2900). The concentration of protein was determined by the Lowry method.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed with a modified method according to a previous protocol (27). The double-stranded probes were labelled with fluorescence by a 5'oligolabelling kit (RPN 5755, Amersham Pharmacia Biotech) according to the manufacturer's instruction. EMSA reaction mixtures were incubated in 1× binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl pH 7.5), 4% glycerol and nuclear extracts at room temperature for 20 min with or without unlabelled competitor, and fluorescence-labelled oligonucleotides for 30 min at room temperature. For the super-shift assays, anti-Sp1 antibody was added to the EMSA reaction mixture prior to the addition of the labelled oligonucleotide probes for 30 min at room temperature. For the competitive binding assay, the non-labelled probe was added to the binding reaction at 100-fold excess over the labelled probe. The samples were electrophoresed on a native 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 80 V for 1 h at 4°C and then at 120 V for 60 min. Finally, the binding reactions were visualised and analysed by a multi-function imaging scanner (Typhoon Tyio).

Chromatin Immunoprecipitation Assays (ChIP)

Chromatin isolated from SMMC-7721 cells was used for the ChIP assays according to the manufacturer's instructions (EZ-ChIP, Millipore Corporation, Temecula, CA, USA). Briefly, SMMC-7721 cells, treated as indicated, were washed and chemically cross-linked using 1% formaldehyde in PBS for 10 min at room temperature. After quenching by the addition of 2 ml of 10× glycine solution (2.5 mol/L), cells were washed twice with ice-cold PBS and collected in 2 ml of PBS containing protease inhibitor cocktail II and centrifuged at 700 x g at 4°C for 5 minutes. Next, the cell

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pellets were resuspended in 1 ml of SDS lysis buffer containing 1× Protease Inhibitor Cocktail II and sonicated on wet ice by a Bioruptor sonicator (Ningbo, China) to disrupt genomic DNA to an average size of 200 bp – 750 bp. After preclearing with protein A-agarose for 1 h at 4°C, the chromatin was incubated at 4°C overnight with a specific antibody against Sp1, with anti-RNA polymerase II as the positive control and anti-IgG as the negative control, followed by incubation with protein A-agarose for 1 h. The precipitates were washed, and the chromatin complexes were eluted. After reversal of the cross-linking at 65°C for 4 h, the DNA was purified and used as the template for PCR. Primers P1/P2 and P3/P4 were used for detection of the integrin αV promoter containing the putative Sp1 binding sites. P1 : 5′-CAACAGTCGCACGGAAGT-3′, P2: 5′-AGCTCCTGAGCCTGGGT-3′; P3: 5′-TCCTCCTTCCAGGTCTCC-3′, P4: 5′-AGGGCTGAGCTTCGGA-3′. PCR amplification was carried out for 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 20 s, and a final extension at 72°C for 10 min. PCR products were analysed by electrophoresis on a 2% agarose gel.

Statistic analysis Mean values ± SD given were obtained from at least 3 culture wells. All experiments presented were repeated at least 3 times and representative experiments are shown. For single comparisons of the average differences between 2 samples, statistical significance was determined by the two-tailed Student's t test.

RESULTS

Exogenous sulfatide stimulates integrin αV subunit expression

SMMC-7721 cells were treated with exogenous sulfatide, Gal-Cer or Lacto-Cer for 12 h, 24 h, and 36 h (Fig. 1A), and the mRNA level of the integrin αV subunit in the cells was analysed by RT-PCR. As shown in Fig. 1A, treatment with sulfatide stimulated the mRNA expression of the integrin αV

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subunit gene in a time-dependent manner, and the up-regulation of the integrin αV subunit was the most obvious at 24-36 h after the treatment(Fig. 1A). After 48 h the up-regulation effect attenuated (data not shown). The stimulation by sulfatide was also observed in a dose-dependent manner, and a concentration of 2 μM was necessary to stimulate the expression of the integrin αV subunit (Fig. 1B). Furthermore, the protein expression level of the integrin αV subunit was also promoted by sulfatide, but not by galactocerebroside (Gal-Cer) or lactocerebroside (Lacto-Cer) (Fig. 1C). Lacto-Cer was then used as the control for sulfatide in the following experiments since both Lacto-Cer and Gal-Cer did not affect integrin αV expression significantly. To determine the distribution on the cell membrane, flow cytometry analysis of non-permeabilized cells with integrin αV specific antibody revealed that up-regulation of the surface integrin αV subunit was observed after sulfatide treatment (Figs. 1D & 1E). The positive rate was elevated from 37.8±0.8 % to 54.5±0.95 %. No significant increase of the integrin αV subunit expression was observed on the cells induced by exogenous Lacto-Cer. Other subunits such as β3, β5, and α5 did not change significantly after the treatment (Fig. 1F) and after sulfatide overexpression as in our previous reports (15, 16). After sulfatide treatment of the cells, high positive staining with O4 antibody which recognizes sulfatide was seen in the treated cells (Fig. 1G), while not in the control (Lacto-Cer treatment), confirming that glycosphingolipids could incorporate into cells.

Sulfatide is the sulfation product of cerebroside. The addition of a sulfate moiety gives the sulfatide molecule a negative charge. To test whether the sulfatide or just the negative charge was the cause of up-regulation of the integrin αV subunit gene, SMMC-7721 and BEL-7404 cells were incubated with 2 μM ManN propanyl perac and cyclo-ManN propanyl perac, which were reported to incorporate into a cellular sugar chain with a sialyl group (22, 23, 28).. RT-PCR (Fig. 1H) and real-time PCR (Fig. 1I and 1J) showed that in both the SMMC-7721 and the BEL-7404 cells, only sulfatide enhanced the

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expression of the integrin αV subunit, while the negatively charged ManN propanyl perac and cyclo-ManN propanyl perac did not. The increased αV subunit was mainly jointed with β3 subunit to form αVβ3 as observed in our previous studies (15-17). Current results suggest that exogenous sulfatide up-regulated the expression of the integrin αV subunit.

Endogenous sulfatide also promotes the expression of integrin αV subunit

To further confirm the regulation of the integrin αV subunit expression by sulfatide, we established stable transfection cell lines with a *CST* plasmid in SMMC-7721 cells to produce endogenous sulfatide. We obtained *CST*-1, *CST*-8 and the corresponding Mock cells, which over-express *CST* and sulfatide (18). A cell ELISA assay (Fig. 2A) and immunofluorescence staining (Fig. 2B) demonstrated that there was over-expression of *CST* and its product sulfatide. Next, we established *CST* knock-down cells by stable transfection with specific siRNA plasmids in SMMC-7721 cells and established cell lines Chp2, Chp5 that reduce *CST* expression. The RT-PCR results (Fig. 2C) confirmed successful *CST* interference in the cells. The mRNA and protein levels of the integrin αV subunit gene were significantly increased in SMMC-7721 cells that were transfected with *CST* plasmid and not in the Mock cells (Fig. 2D). In addition, the expression of the integrin αV subunit was decreased significantly in *CST* siRNA cells, especially in the Chp2 cells (Fig. 2E). Therefore, either exogenous or endogenous sulfatide could stimulate the expression of the integrin αV subunit gene.

Sulfatide enhances adhesion of hepatoma cells

High expression of the integrin αV subunit can influence tumour progression through regulation of cell migration and adhesion (29). The results above showed that sulfatide could stimulate the expression of the integrin αV subunit gene. We then examined the influence of sulfatide on cell adhesion. As shown in Fig. 3A, the adhesion to HUVECs, stimulated by TNF-α, was significantly

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increased in cells treated with sulfatide as compared to the control, Lacto-Cer, ManN-pro, and Cyclo-ManN-pro groups (Fig. 3A). The adhesion of the treated SMMC-7721 cells to vitronectin, collagen type I, fibrinogen, and fibronectin, is shown in Fig. 3B-E. With the exogenous sulfatide, the cells were more adhesive to vitronectin, collagen type I, fibrinogen, and fibronectin than to the control. The adhesion rate increased to 22.7±4.04 % from 1.763±0.234 % for vitronectin, and to 63.75±1.43 % from 6.05±1.472 % for collagen type I, respectively. The adhesion to vitronectin was significantly inhibited by adding the antibody against integrin αV (64.4%)(Fig. 3B). Adhesion to collagen and was diminished by integrin αV antibody, while adhesion to fibrinogen not (Fig. 3C,D). Adhesion to fibronectin was also significantly inhibited by the antibody (Fig. 3E). GRGDSP peptide suppressed the adhesion to vitronectin and fibronectin, as well as collagen. The heparin seemed mainly to reduce the adhesion to vitronectin and fibronectin in these cells. The adhesion of ManN-pro and Cyclo-ManN-pro groups was similar to the Lacto-Cer group (data not shown). Thus, sulfatide stimulated the expression of the integrin αV subunit and enhanced the adhesion of SMMC-7721 cells to extracellular matrix proteins that were the ligands of the integrin αV subunit.

Sp1 is involved in the integrin αV subunit gene regulation induced by sulfatide.

According to bioinformatic promoter analysis and previous reports (29, 30)，transcription factors, including Sp1, Ets1, Ap2, Egr1, Egr2 and Sp3, may be important for the expression regulation of the integrin αV subunit gene. To test and screen these transcription factors which might be involved, we measured all these transcription factors. Among them, Sp1 expression was elevated (Figs. 4A & 4B) and its phosphorylation on threonine 739 (T739) was significantly enhanced after sulfatide treatment (Figs. 4C & 4D). However, the tyrosine phosphorylation was not obvious on Sp1(data not shown) by the detection with phospho-tyrosine antibody after Sp1 immunoprecipitation. The serine

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phosphorylation on Sp1 was slightly enhanced in the blot with anti-phospho-serine antibody after precipitation with Sp1 antibody (Fig. 4C & 4D). To investigate the role of Sp1 in the sulfatide-induced regulation of the integrin αV subunit further, we constructed an Sp1 interference plasmid and confirmed its activity by RT-PCR (Fig. 4E). We transfected Sp1 siRNA plasmid (si-Sp1) into SMMC-7721 cells after treatments with exogenous sulfatide (Fig. 4F) and into *CST* transfectants (Fig. 4G). Results showed that Sp1 siRNA could disturb the expression of the integrin αV subunit gene that is induced by both exogenous and endogenous sulfatide. The Sp1 plasmid (Sp1) was transfected into si-*CST* transfectants (Chp2 and Chp5), in which *CST* was knocked down by siRNA. Sp1 up-regulated the expression of the integrin αV subunit gene, and the expression of Sp1 directly correlated with both the mRNA and the protein expression levels of the integrin αV subunit gene (Figs. 4F, G & 4H). Additional Sp1 over-expression increased the integrin αV subunit distribution on the surface of SMMC-7721 cells (Figs. 4I & 4J). These observations are consistent with the results above, indicating that sulfatide regulated Sp1 transcription factor and enhanced the gene expression of the integrin αV subunit.

To investigate the interaction partner of Sp1 after its phosphorylation, Sp1 protein complex was precipitated by Sp1 specific antibody for the detection of Western blot. In the complex immunoprecipitated by Sp1 antibody, Stat3 was observed (Figs. 4K & 4L). Furthermore in the sulfatide group much more Stat3 was noted than in control. Interestingly, Stat3 was highly phosphorylated after sulfatide stimulation in both SMMC-7721 and BEL-7404 cells (Figs. 4K & 4M). Therefore Sp1 was complexed with Stat3 after phosphorylation induced by sulfatide.

Analysis of the human integrin αV subunit gene promoter

An analysis of the human integrin αV subunit gene promoter (−1295/+207) (Gene Bank Accession

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Number: 23999) based on Transfac website predicted Sp1 binding sites, which had 4 Sp1 binding enrichment regions. The first site includes 1 Sp1 binding consensus site; the second site includes 4 Sp1 binding consensus sites; the third site includes 3 Sp1 binding consensus sites; and the forth includes 1 Sp1 binding consensus site (Fig. 5). To define the integrin αV subunit gene promoter, we cloned the Sp1 transcription factor binding sites in the promoter. These promoter fragments (Fig. 6) were inserted into the promoter-less pGL3-basic vector. Figure 6A shows the integrin αV subunit gene promoter fragments, including different Sp1 binding sites. The basal and proximal regulatory regions of the integrin αV subunit promoter were characterised by constructing a series of luciferase reporter plasmids and transfected into HeLa and HEK293T cells. Deletion of the sequence between −1295 and −794 increased the transcriptional activity (Fig. 6B). To further define the roles of the Sp1 binding sites of the integrin αV subunit gene promoter in response to sulfatide regulation, HeLa and HEK293T cells were transfected with pGL3 (-795/+207) along with treatments of 2 μM sulfatide and controls (Fig. 6C). Sulfatide significantly increased the activity of the integrin αV subunit gene promoter. In order to test whether sulfatide increased the activity of the Sp1 transcription factor regulation of the integrin αV subunit gene promoter, HeLa and HEK293T cells were co-transfected with pGL3 (-795/+207) and pcDNA3.0-Sp1 or si-Sp1 along with treatments of 2 μM sulfatide and Lacto-Cer. A significant increase in the reporter activity by the treatment with sulfatide (Figs. 6D & 6E) supported the Sp1 transcription factor being involved in transcriptional regulation of the integrin αV subunit gene and Sp1 regulation activity being enhanced by sulfatide.

Sulfatide enhances the binding of Sp1 to the integrin αV subunit gene promoter.

Electrophoresis mobility shift assays (EMSA) were performed to test whether sulfatide enhanced the binding of the Sp1 transcriptional factor to the integrin αV subunit gene promoter. As shown in Fig.

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5 and Fig. 6, there are four binding sites of Sp1 in the integrin αV subunit gene promoter, but the forth Sp1 binding site did not affect the activity of the integrin αV subunit gene promoter. Therefore, we designed three pairs of probes for the first three binding sites, which encode the Sp1 consensus sequences and the corresponding mutant probes (Fig. 7A). According to the luciferase analysis (Fig. 6B), the second Sp1 binding site had the strongest promoter activity. The nuclear extracts from the untreated SMMC-7721 cells were incubated with the fluorescence-labelled DNA probes for EMSA. The super-shift band of the protein-DNA complex was indeed detected in the presence of the Sp1 antibody (Fig. 7B, lane 5) and the probe designed for the second Sp1 binding site had the highest binding capability among the three pairs of probes (Fig. 7C). This band was eliminated in the presence of unlabelled double-stranded oligonucleotide competitor (Fig. 7B, lane 3), but it was not affected by mutated oligonucleotides (Fig. 7B, lane 4). Histone 4 was the internal loading control of the nuclear extracts of the variously treated cells (Fig. 7D). The level of the super-shift bands was enhanced in cells transfected with pcDNA3.0-Sp1 (Fig. 7E) and also in the cells treated with sulfatide (Fig. 7F). This suggested the impact of sulfatide on Sp1 binding to the integrin αV subunit gene promoter.

Sulfatide regulates Sp1 binding to the integrin αV subunit promoter *in vivo*.

The status of the Sp1 on the human integrin αV subunit gene promoter was determined using chromatin immunoprecipitation (ChIP). The presence of the integrin αV subunit gene promoter in the chromatin immunoprecipitates was analysed by semi-quantitative PCR using specific primer pairs that spanned the integrin αV subunit gene promoter region from -632 to -452. To test whether Sp1 directly binds the regulatory regions of the integrin αV subunit gene promoter, we performed ChIP assays with untreated SMMC-7721 cells. As shown in Fig. 8A, chromatin immunoprecipitation with an antibody against Sp1 indicated that Sp1 could directly bind the regulatory regions of the integrin αV subunit

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gene promoter. The anti-RNA Polymerase II was used as a positive control, which could bind to the integrin αV subunit gene promoter, and normal mouse IgG was used as a negative control. We next probed whether sulfatide or Sp1 over-expression affected the recruitment of Sp1 to the integrin αV subunit gene promoter. As shown in Fig. 8B and 8C, chromatin immunoprecipitation with the specific antibody against Sp1 indicated that treatments with sulfatide or pcDNA3.0-Sp1 transfection induced a significant increase in Sp1 binding to the integrin αV subunit gene promoter as compared to Lacto-Cer or pcDNA3.0. Input DNA from each sample was used as the control. These data suggested that sulfatide enhanced the Sp1 occupancy of the integrin αV subunit gene promoter.

Sulfatide regulation is associated with Erk signalling

Sp1 transcription factor can change its regulation activity by phosphorylation. To further study the reason that sulfatide enhanced Sp1 phosphorylation and integrin αV subunit gene promoter, the gene expression profiles stimulated by sulfatide were investigated. Among the genes regulated, mitogen-activated protein kinase (MAPK) signalling pathway triggered in these cells was noted for its most enrichment (Fig. 8D). After 24 h of treatment with sulfatide, the Erk1/2 (extracellular signal regulated kinases) pathway activation was found in hepatocellular carcinoma cells (BEL-7404) and the levels of active phosphorylated Erk1/2 increased significantly (Fig. 8E & 8F), while Akt phosphorylation was not affected. In addition, the cells treated with Lacto-Cer or Gal-Cer had comparable level of the phosphorylated Erk1/2 as compared to the control. Apart from BEL-7404, a similar result could also be seen in SMMC-7721 cells (Fig. 8E). Further detection showed that Erk1/2 was phosphorylated 2 h after sulfatide treatment, suggesting that sulfatide stimulates Erk1/2 activation. Since Erk1/2 can be activated by Raf and Src signalling, we further measured the Src activation in the treated cells and observed that phosphorylation of Src (on tyrosine 416) was significantly higher in

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sulfatide group than that in Lacto-Cer (Fig. 8E & 8F). C-Raf especially on tyrosine 341 was found highly phosphorylated in the sulfatide group (Fig. 8G, H). The phosphorylation on the serine 338 of c-Raf was also enhanced (Fig. 8I. Although not as strong as the Erk1/2 phosphorylation, a significant activation of p38 was also observed in the sulfatide group (Fig. 8G, J). Additionally, JNK phosphorylation was found increase in the sulfatide group as well (Fig. 8G, K), while RAC was less phosphorylated in the sulfatide group (Fig. G, L). We thus reasoned that the activation of Raf/MEK/Erk pathway might be responsible to the regulation. To test this, an inhibitor of MEK1 was used in the next experiment. In results, the active phosphorylation of Erk1/2 was inhibited by the pre-treatment with 50 μM PD98059, an inhibitor of MEK1/2, even under sulfatide stimulation. Interestingly, the phosphorylation of Sp1 induced by sulfatide was also suppressed (Fig. 9A). Another inhibitor with different structure, U0126, also showed a significant inhibition of Erk1/2, Sp1 phosphorylation (Fig. 9B), and integrin αV expression, consistent with the effect of PD98059. This suggested that Erk pathway was associated with the Sp1 phosphorylation in sulfatide-induced regulation of integrin αV subunit expression.

DISCUSSION

Integrins are very important in both adhesion and metastasis of tumour cells. Integrin αV expression is required for the acquisition of a cancer metastatic stem or progenitor cell phenotype(31). Transcriptional silencing of αV and β3 integrin subunits suppresses cancer metastasis(32) . In hepatocellular carcinoma(HCC), the expression of integrins such as α5β1 and αVβ3 is greatly altered, which is directly involved in the angiogenesis and metastasis of HCC(5). Hepatocellular carcinoma expresses integrin αVβ3 which leads to extravasation of HCC cells in the liver through a process of

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metastasis possibly mediated by vitronectin(33). However, less is known about the regulation mechanism of integrin αVβ3 expression in HCC, especially by sulphated cerebroside (sulfatide). Sulfatide is the product of *CST.* In HCC cells of both Hep3B and SMMC-7721, *CST* could be over expressed by transfection to produce sulfatide in our previous study(15, 18). The enzyme *CST* overexpression was found to be involved in metastasis of HCC in nude mice and adhesion to vitronectin (15, 18), which were ascribed to the production of sulfatide. We then postulate that sulfatide leads to the expression of the integrin αV subunit that is involved in the metastasis of HCC. However the pathway and mechanism about sulfatide regulation of integrin αV subunit expression have not been known. A recent report indicated that sulfatide-laminin binding can initiate basement membrane assembly and signalling (9). Sulfatide expressed on cell surface exerts biological functions through mediating interactions with various proteins, such as laminin, hepatocyte growth factor and Disabled-2(34). Furthermore, the expression and distribution of sulfatide are often altered during development and oncogenic transformation (35). The elucidation of the biological significance of sulfatide in the regulation of integrin is one of the most important issues.

In this study, we did observe that either exogenous or endogenous sulfatide could increase the expression of the integrin αV subunit, while cerebroside not. Furthermore, this effect was rendered at transcriptional level in the regulation of the integrin αV subunit expression. The transcription expression of integrin αV subunit gene is commonly controlled by transcriptional factors among which Sp1 has the binding sites in integrin αV subunit gene promoter and the phosphorylation on threonine 739 was enhanced after sulfatide stimulation. Sp1 mediates its target genes and can change its activity by phosphorylation on threonine 739(36). Regarding the integrin αV subunit, Sp1 promoted its expression by enhanced binding activity to the promoter since Sp1 protein was phosphorylated and its expression was

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elevated. Our data confirm that sulfatide stimulated the phosphorylation and expression of Sp1 which subsequently binds to and enhances integrin αV subunit gene promoter. The issue that sulfatide stimulated Sp1 expression will be addressed in another report. More importantly, the molecule sulfatide could send a regulatory signal that stimulates Sp1 phosphorylation and promotes integrin αV subunit expression. However, the signalling pathway is currently unknown. Through large scale screening via gene expression analysis, effecters associated with MAPK pathway were noted for the most enrichment. Therefore, we investigated the MAPK-associated signal pathway triggered by sulfatide. As compared to a control, Gal-Cer and Lacto-Cer, sulfatide significantly stimulated and enhanced Erk1/2 phosphorylation. Erk1/2 is an important signalling molecule that can further activate Sp1 transcriptional factor via phosphorylation on T739 (37, 38). Pre-treatment with both PD98059 and U0126, inhibitors of MEK1/2, significantly diminished and prevented Erk1/2 and Sp1 phosphorylation that sulfatide stimulated. This demonstrated that Erk1/2 signalling activation is important in Sp1 phosphorylation and integrin αV subunit expression regulation by sulfatide. Activated p38 can also phosphorylate Sp1 on threonine 739(36). Thus activation of p38 contributes to the phosphorylation and activation of Sp1 as well, although the phosphorylation of p38 (T180/Y182) was not as robust as Erk1/2.

In current study we noted that exogenous sulfatide could incorporate into the cells treated, which was consistent with the previous observation(39). After incorporation, the glycosphingolipids can cluster in the membrane microdomain where they interact with caveolae(40) and are internalized. Then how is sulfatide recognized by the upstream components of the MAPK pathway? In this study we noted the phosphorylation and activation of c-Src and c-Raf, the upstream regulators of Erk1/2, in the cells stimulated with sulfatide within a short period (2h), but not in Gal-Cer or Lacto-Cer-treated cells. Sulfatide can bind to laminin after intercalation into plasma membrane and initiate basement membrane assembly which enables activation of

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c-Src by tyrosine phosphorylation(9, 12). Treatment with arylsulfatase abrogates the activation of c-Src(9), suggesting that the molecule without sulfate group is unable to activate c-Src. Activated c-Src can phosphorylate c-Raf on the site of tyrosine 341, which is the upstream kinase of MEK1/2.and can further activate Erk1/2 cascade. On the other hand, we also noted that c-Raf was phosphorylated on the site of serine 338 which is activated by PAK (p21-activated protein kinase) (41), a downstream effecter of RAC1(42, 43) that can be activated by Src signalling (44). RAC, a small G protein, can also be activated by integrin-related focal adhesion kinase (FAK). Activated RAC1 binds to PAK protein kinase. Activated PAK in turn phosphorylates and activates c-Raf and MEK1. However, the phosphorylation on serine 71 of RAC1 was inhibitory for its GTP binding(45). In the present study RAC1 phosphorylation on serine 71 was attenuated in sulfatide group although the Akt phosphorylation was not significantly affected. This may synergize the Src signalling to activate Erk1/2. Binding its ligands, sulfatide forms a complex with laminin and acts as a scaffold for cell signalling to activate c-Src, subsequent c-Raf/MEK/Erk which in return phosphorylates Sp1. Phosphorylated Sp1 recruits Stat3, binds to, and enhances the promoter of integrin αV subunit gene. This constitutes at least one major part of the integrin αV subunit gene regulation pathways that are caused by sulfatide in HCC cells. This study demonstrates that sulfatide is apt and able to regulate integrin αV subunit expression in the hepatocullular carcinoma cells. The increased αV subunit mainly forms the molecule αVβ3(15-17) and involves the process of metastasis of HCC. HCC cells with increased integrin αV expression showed significantly higher adhesion to vitronectin, which could significantly be inhibited by specific integrin αV antibody. Vitronectin binds to a cysteine loop region of β3 subunit and this interaction is required for the positive effects of αVβ3 ligand occupancy on IGF-I signalling(46).

Interestingly, cerebroside failed to regulate both Sp1 and the integrin αV subunit, which suggested that the sulfation of cerebroside was important in triggering the regulatory signal. However, according to

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Xianqiong Zou's report, the non-glycosylated C6-ceramide significantly increased GLTP expression by altering the binding affinity of the Sp1 and Sp3 co-effectors to the GLTP promoter(47). This hints that ceramide is also able to send signals to regulate the expression and binding promoter activity of Sp1 although the fatty acid chain in the molecule is short. Glycosylation of ceramide might hinder the signalling of the molecule. The sulfation of cerebroside may relieve such hindrance and trigger the regulatory signalling for Erk activation, Sp1 phosphorylation and integrin αV expression; Moreover, we previously observed that sulfation of a type I or type II sugar chain could also promote the expression of the integrin αV subunit in hepatoma cells (16, 17), suggesting that the sulfo group in the sulfatide molecule may directly trigger the regulatory signal for integrin αV subunit expression.

In conclusion, the present study demonstrated that sulfatide increased the integrin αV subunit expression level and adhesion to vitronectin in human hepatocellular carcinoma cells, which was associated with Erk signalling and Sp1 regulation.

CONFLICT OF INTEREST

The authors have no competing financial interests in relation to the work described.

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FIGURE LEGENDS

Fig. 1. Expression of integrin αV subunit was regulated by exogenous sulfatide. (A) SMMC-7721 cells were treated with 2 μM sulfatide, Gal-Cer, and Lacto-Cer for 12, 24 and 36 h. The cells were then used for analysis of integrin αV mRNA by RT-PCR. (B) Integrin αV mRNA was detected in SMMC-7721 cells treated with the indicated concentrations of sulfatide for 24 h. (C) SMMC-7721 cells were treated with 2 μM sulfatide, Gal-Cer, and Lacto-Cer for 24 h. The cells were lysed and analysed by immunoblotting using an antibody against the integrin αV subunit(left panel). The densitomeric analysis of the blotting was summarized(right panel). (D) Flow cytometry analysis of the cell surface integrin αV expression. Mouse immunoglobulin was used for a negative control as the unrelated primary antibody. (E) The positive rate of the surface integrin αV staining by flow cytometry analysis. (F) Integrin β3,5,6,8, α5, and αV subunit mRNA was analyzed by Real-time PCR after 24 h treatment with sulfatide. (G) Sulfatide was stained with O4 antibody after SMMC-7721 cells were treated with sulfatide and Lacto-Cer for 24 h, fixed, washed, and permeabilized. (H) SMMC-7721 cells were treated with 2 μM sulfatide, Lacto-Cer, ManN propanyl perac and cyclo-ManN propanyl perac for 24 h respectively. The expression of the integrin αV subunit mRNA and protein were analysed by both RT-PCR and Western blotting. (I & J) In SMMC-7721(I) and BEL-7404(J) cells as treated in Fig. 1H, the expression of the integrin αV subunit mRNA was analysed by Real-time PCR. All figures were the representative study and at least two experiments yielded similar results. * $P < 0.05$, ** $P < 0.01$

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Fig. 2. Regulation of the integrin αV subunit gene expression by endogenous sulfatide. (A& B) In *CST* transfectants *CST*-1, *CST*-8 and Mocks, sulfatide was analysed by a cell ELISA assay (A) and immunofluorescence detection with O4 antibody under a confocal microscope. The cells were not permeabilized. (B). (C) *CST* knockdown was achieved in the *CST* RNAi transfectants Chp2 and Chp5 and confirmed by RT-PCR. Scr: scrambles. (D) Expression of the integrin αV in *CST*-transfected SMMC-7721 cells (*CST*-1 and *CST*-8) was analysed by RT-PCR and Western blotting. Mock3 and Mock4 were transfectants of the control vector. (E) The expression of integrin αV in si-*CST* SMMC-7721 cells by *CST* RNAi (Chp2 and Chp5) was analysed by RT-PCR and Western blotting. Scr1 and Scr2 were transfected with the control vector. All figures were the representative study and at least three additional independent experiments were repeated. * $P < 0.05$,

Fig. 3. Sulfatide enhanced cell adhesion. SMMC-7721 cells were treated with 2 μM sulfatide, Lacto-Cer, ManN propanyl perac, or cyclo-ManN propanyl perac for 24 h. Then the cells were used for adhesion assay. (A) The adhesion of the treated cells to TNF-α induced HUVECs. The monolayer HUVECs were stimulated with 10 ng/mL TNF-α for 4 h prior to the adhesion assay. After incubation and washing, the attached cells were counted under a phase contrast microscope in five random fields in each well and the cell number was averaged among the test groups and compared to show the affinity of the cells to HUVECs. (B-E) The treated SMMC-7721 cells were examined for their adhesive capability to vitronectin (B), collagen type I (C), fibrinogen (D), and fironectin (E). For the inhibition, the cells were pre-incubated with 30 mg/ml GRGESP, GRGDESP, 60 μg/ml antibody against integrin αV, and 1 mg/ml heparin, respectively for 2 h at 4°C. The numbers of cells adhering

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were represented by A570, and the adhesive rate was calculated. Shown are the means ± SD from five independent experiments with equivalent results. Bars indicated the SD. * $P < 0.05$, ** $P < 0.01$

Fig. 4. Observation of Sp1 in the sulfatide-induced expression of integrin αV.

(A) The expression level of Sp1 was analysed with real-time PCR in both SMMC-7721 and BEL-7404 cells with various treatments. (B)After the treatment for 24 h, the expression of Sp1 mRNA and protein in these cells was analysed by RT-PCR and Western blotting. (C) After SMMC-7721 cells were treated with 2 μM sulfatide and Lacto-Cer for 24 h, Sp1 protein expression and phosphorylation were analysed by immunoblotting. Ser-P: phospho-serine antibody, Sp1-p: antibody against phospho-Sp1 on threonine 739. (D) Densitometry analysis of Sp1 phosphorylation was summarized (Sp1-p:left, Ser-p:right). (E) Sp1 mRNA analysis by RT-PCR. The cells were transfected with Sp1 RNAi plasmid and negative siRNA plasmid, and the Sp1 mRNA was analysed by RT-PCR. Neg siRNA was the negative control siRNA. (F) Influence of Sp1 silence by RNAi (si-Sp1) on the exogenous sulfatide-induced integrin αV expression in SMMC-7721 cells. Scr was the transfection with the control vector containing a scramble sequence. (G) Influence of Sp1 silence by si-Sp1 transfection on the endogenous sulfatide-induced αV expression in *CST*-1 and *CST*-8 cells. (H) Sp1 overexpression influence on sulfatide regulation in Si-*CST*-transfected cells (Chp2 and Chp5). Sp1 was overexpressed by Sp1 transfection (Sp1), which enhanced the endogenous sulfatide -induced αV expression. (I) Flow cytometry analysis of the cell surface integrin αV expression in cells transfected with Sp1 and a vehicle and in cells without transfection (control). (J) The positive rates of the cell surface integrin αV staining were averaged from the experiments of flow cytometry. (K) Stat3 protein was observed in the complex immunoprecipitated by Sp1 antibody (upper panel). Phosphorylation of Stat3 on

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tyrosine 705 was measured by Western blotting (bottom panel). (L,M) The graphs summarized the densitometry analysis of Stat3/Sp1 (L) and Stat3-p Y705/Stat3 (M). All figures are from a representative study and at least three additional experiments yielded similar results. * $P < 0.05$, ** $P < 0.01$, IP: immunoprecipitation, IB: immunoblotting

Fig. 5. Nucleotide sequence of the human integrin αV promoter. The major transcriptional start site as indicated is at position +1. The Sp1 transcriptional factor binding sites are boxed, and the promoter fragment primers are shaded.

Fig. 6. Characterisation of the human integrin αV promoter. (A) The promoter fragments were designed according to the Sp1 binding sites in the integrin αV promoter. (B) 5' deletion analysis of the integrin αV promoter was performed in HEK293T and HeLa cells. The activity of luciferase was statistically analysed between the construct and the vector pGL3-basic. (C) The luciferase activity was assayed in both HEK293T and HeLa cells transfected with the human integrin αV promoter plasmids, pGL3(-795/+207), or treated with 2 μM sulfatide for 24 h. The comparison was made between sulfatide and Lac-Cer groups. (D) HEK293T and HeLa cells were co-transfected with the human integrin αV promoter plasmids pGL3(-795/+207) and si-Sp1(pSilence-Sp1) or a control vector. After transfection for 12 h, HEK293T and HeLa cells were treated with sulfatide and Lacto-Cer for an additional 12 h. The luciferase activities were assayed and expressed as the percentage of the test groups over the control siRNA as the mean ± SD of three separate experiments. (E) HEK293T and HeLa cells were co-transfected with the human integrin αV promoter plasmids pGL3(-795/+207) and the Sp1 expression plasmids (pcDNA3.0-Sp1) or with pcDNA3.0. After transfection, HEK293T and

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HeLa cells were treated with sulfatide and Lacto-Cer for an additional 12 h. The luciferase activities were assayed and expressed as the mean ± SD of three separate experiments. * $P < 0.05$

Fig. 7. EMSA analysis of the integrin αV subunit gene promoter. (A) Three EMSA probes of the Sp1 transcriptional factor binding sites. SB: Sp1 binding site, SM: mutated Sp1 binding site. (B) EMSA was performed for the (-541/-519) binding site using the nuclear extract from SMMC-7721 cells without treatment. SB2: Sp1 binding site 2, SM2: mutated Sp1 binding site 2. (C) EMSA for the (-619/-597), (-541/-519) and (-176/-154) binding sites using the nuclear extract from SMMC-7721 cells. (D) Immunoblotting with histone 4 as the loading control was performed to adjust the amount of nuclear extracts from the various groups. (E) EMSA for the (-541/-519) binding site. The Nuclear extract was from SMMC-7721 cells transfected with pcDNA3.0 and pcDNA3.0-Sp1. The group without adding nuclear extract was the negative control for the complex and α-IgG was the negative control for Sp1 antibody. (F) EMSA for the (-541/-519) binding site. The nuclear extract was from SMMC-7721 cells with various treatments including sulfatide, Lacto-Cer and the vehicle. The group without adding nuclear extract was the negative control of the complex, α-IgG was the negative control for the super-shift.

Fig. 8. Sulfatide enhancing Sp1 binding to the promoter of integrin αV.

(A) Immunoprecipitation of the DNA and Sp1 complexes was performed using an antibody that was specific for Sp1 in ChIP assay. The DNA used as the template for PCR amplification of the integrin αV promoter fragment was from SMMC-7721 cells that were immunoprecipitated by anti-RNA-Polymerase II, control IgG, or Sp1 antibody. Integrin αV promoter was observed in the

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complex immunoprecipitated by Sp1 antibody, but not in the control IgG. α-RNA-Pol II: anti-RNA-Polymerase II. (B) The DNA from SMMC-7721 cells treated for 24 h with the vehicle, sulfatide, or Lacto-Cer was used as the template in the ChIP assay. The integrin αV promoter was enhanced in the group of sulfatide, but not in Lac-Cer group. (C) The DNA was immunoprecipitated by Sp1 antibody from SMMC-7721 cells transfected with pcDNA3.0 and pcDNA3.0-Sp1, and it was examined by PCR for amplification of the integrin αV promoter. (D) Cluster analysis of the differential gene expression profile in sulfatide and Lacto-Cer treated SMMC-7721 cells. (E) Phosphorylation of the Akt (S473), Src (Y416) and Erk(T202/Y204) kinases was detected by Western blotting in BEL-7404 cells and SMMC-7721 cells. After treatment with sulfatide, the cells were collected and lysed for the detection. Cells treated with Lacto-Cer or the vehicle were tested as a control. (F) The densitometry analysis of Erk (left) and Src (right) phosphorylation was summarized. (G) Phosphorylation of c-Raf(Y341), JNK(T178), Rac(S71), and mTOR(S2481) was measured simultaneously in both SMMC-7721 and BEL-7404 cells. (H – L) The graphs summarized the densitometry analysis of Raf-Y341(H), Raf-S338(I), p38-p(J), JNK-p(K), and RAC-p(L). All figures are from a representative study and at least three additional experiments yielded similar results. * $P <$ 0.05, ** $P <$0.01

Fig. 9. Inactivation of Erk prevented Sp1 phosphorylation induced by sulfatide.

(A) Prior to the treatment with sulfatide, BEL-7404 cells were pre-inhibited with 25 and 50 μM PD 98059 respectively, and assayed by Western blotting for the measurement of Erk1/2 (T202/Y204) and Sp1 (T739) phosphorylation. (B) The effect of MEK1/2 inhibitor on the phosphorylation of the Erk1/2 (T202/Y204) and Sp1 (T739) was assessed by Western blotting in both SMMC-7721 and

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BEL-7404 cells that were pre-treated with 10 μM U0126 before sulfatide treatment (top panel). The graph in bottom panel summarized the densitometric analysis of Erk-p(left) and Sp1-p(right). All figures are from a representative study and at least three additional experiments yielded similar results. * $P < 0.05$, ** $P < 0.01$

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A

bp Marker Control Lacto-Cer Gal-Cer Sulfatide

250- 100- Integrin α V (12h)

500- 250- β -actin

250- 100- Integrin α V (24h)

500- 250- β -actin

250- 100- Integrin α V (36h)

500- 250- β -actin

B

bp Marker sulfatide 0 0.5 1.0 2.0 10 20 μM

250- 100- Integrin α V

500- 250- β -actin

C

Control Gal-Cer Lacto-Cer Sulfatide

120kD Integrin α V

85kD

50kD

35kD

GAPDH

Relative protein level (fold)

2.0 1.5 1.0 0.5 0.0

** ** **

Control Lac-Cer Gal-Cer Sulfatide

D

Integrin α V

Counts 400 300 200 100 0

Positive Negative

10^0 10^1 10^2 10^3 10^4 FITC

Positive Sulfatide

10^0 10^1 10^2 10^3 10^4 FITC

Positive Lacto-Cer

10^0 10^1 10^2 10^3 10^4 FITC

Positive Control

10^0 10^1 10^2 10^3 10^4 FITC

E

Positive Rate (100%)

60 50 40 30 20 10 0

*

Negative Sulfatide Lacto-Cer Control

F

Relative mRNA levels (folds)

Lactol-Cer Sulfatide

4 3 2 1 0

*

β3 β5 β6 β8 α5 αV

G

O4 Ab

DAPI

Lacto-Cer Sulfatide

H

Sulfatide	+	−	−	−	−
Lacto-Cer	−	+	−	−	−
ManN-pro	−	−	+	−	−
Cyclo- ManN-pro	−	−	−	+	−

Integrin α V

β -actin

Integrin α V

GAPDH

I

SMMC-7721

Relative Integrin αV mRNA Levels vehicle=1.0

18 16 14 12 10 8 6 4 2 0

**

Sulfatide Lacto-Cer ManN-pro Cyclo-ManN-pro Control

J

BEL-7404

Relative Integrin αV mRNA Levels vehicle=1.0

16 14 12 10 8 6 4 2 0

**

Sulfatide Lacto-Cer ManN-pro Cyclo-ManN-pro Control

Figure 1

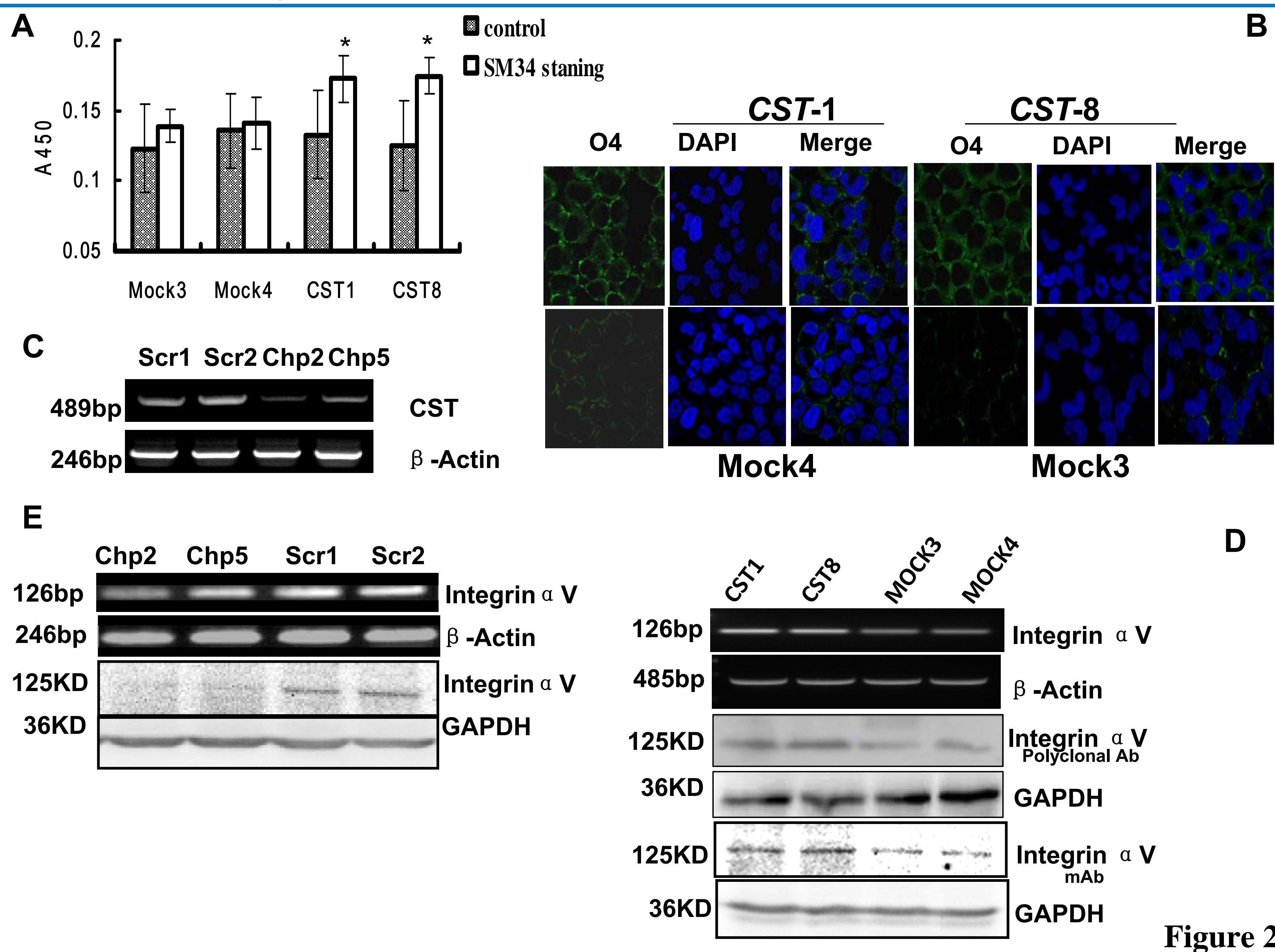
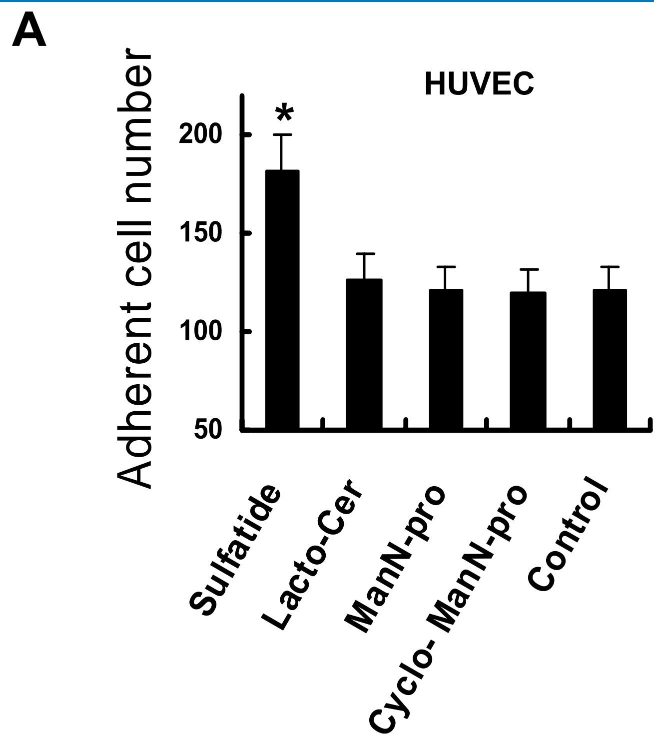
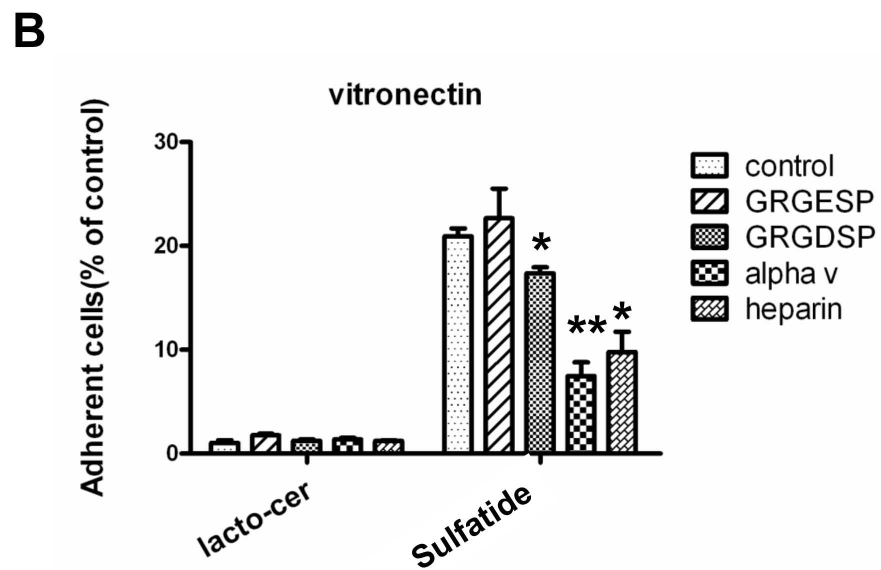
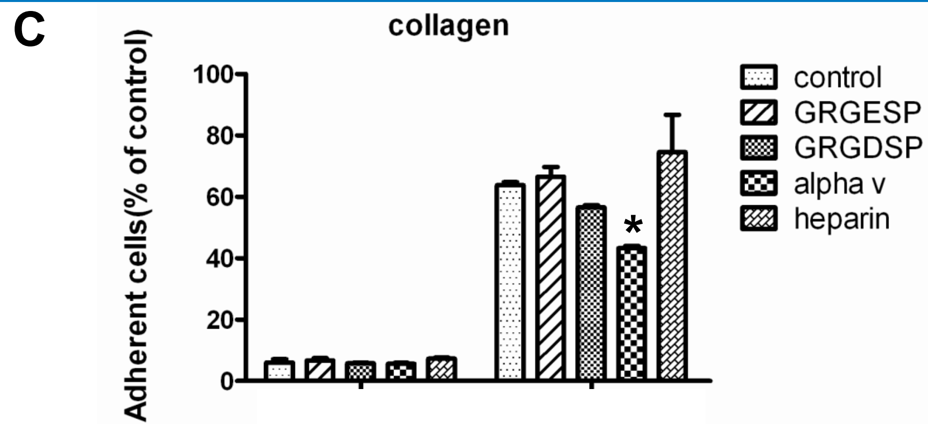
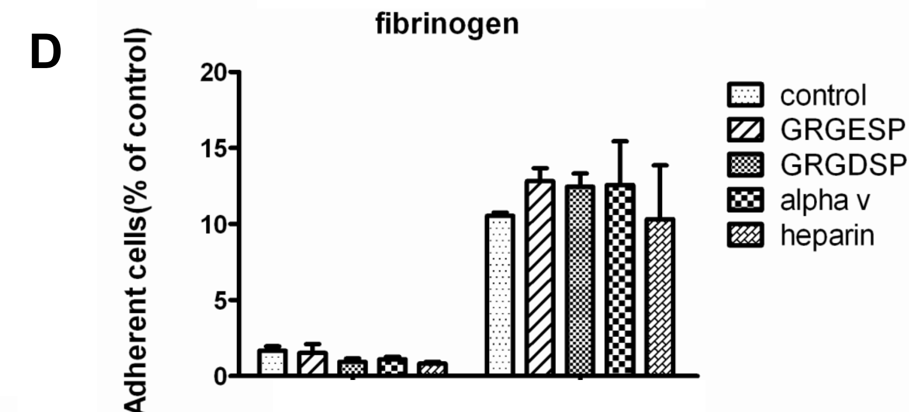


Figure 2









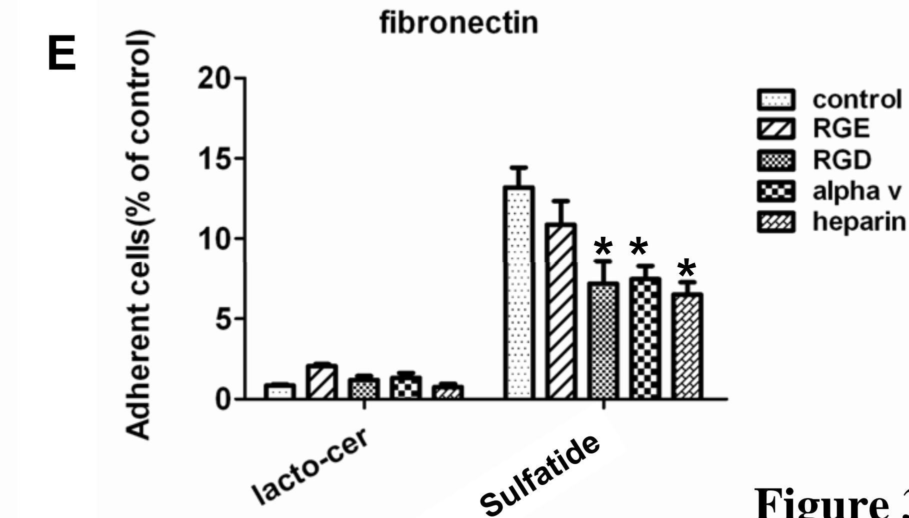


Figure 3

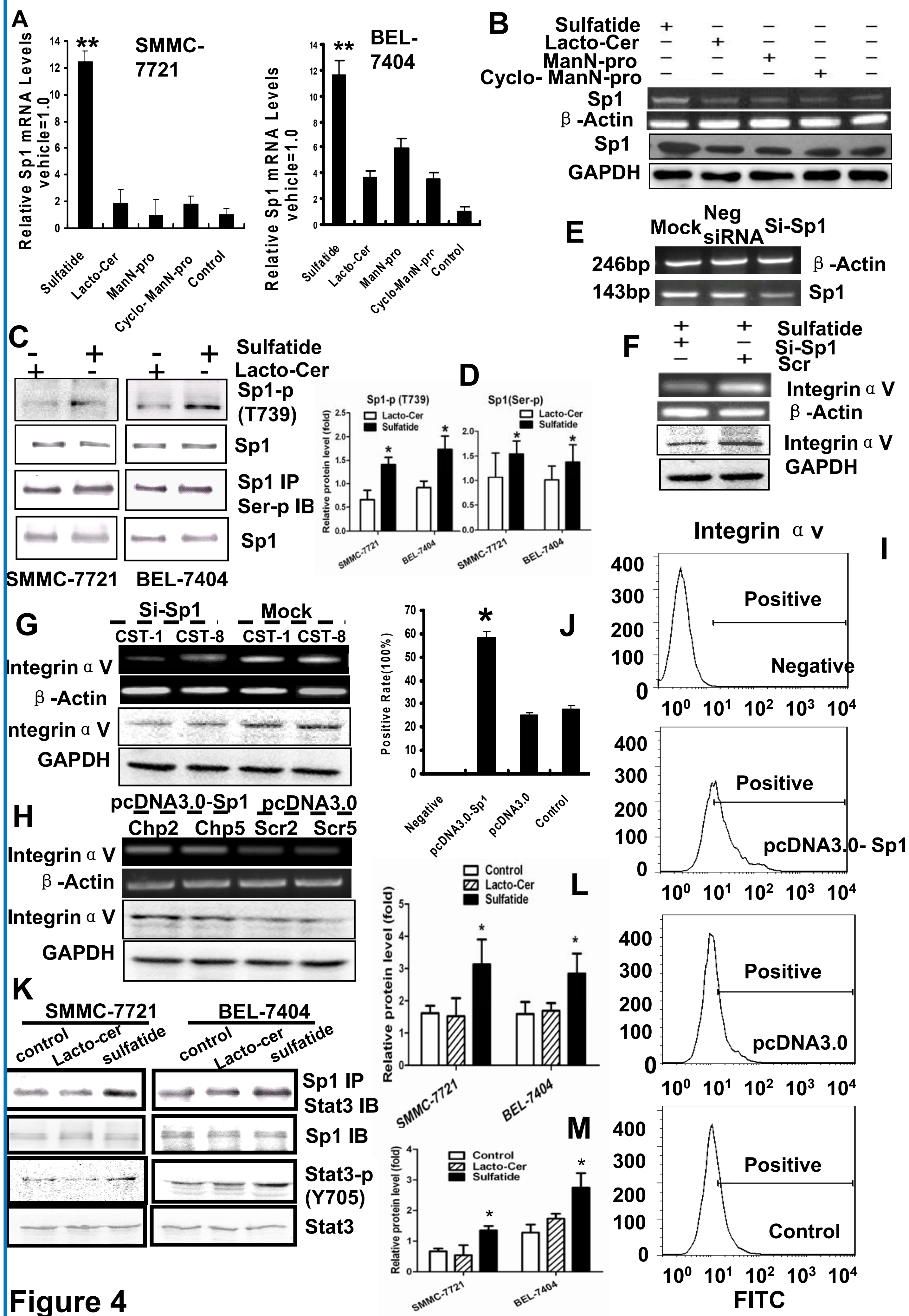


Figure 4

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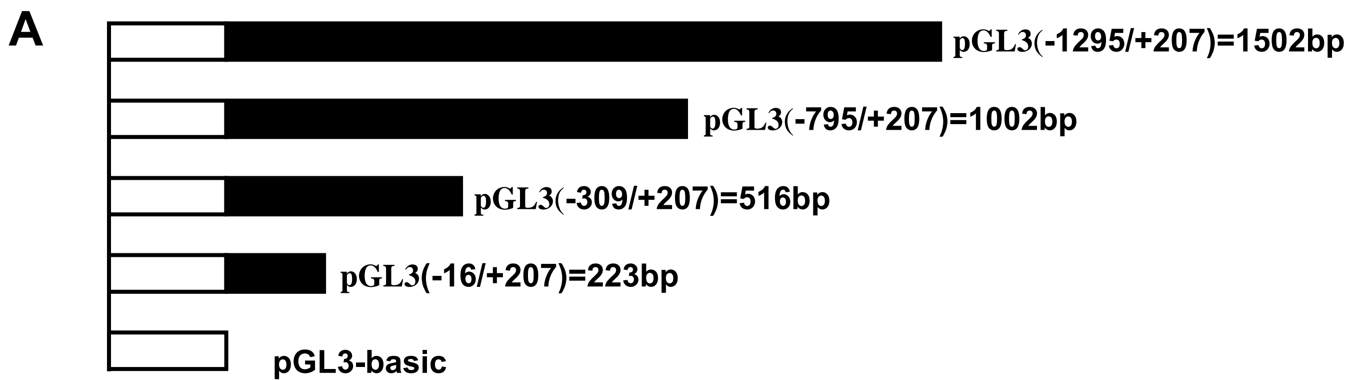
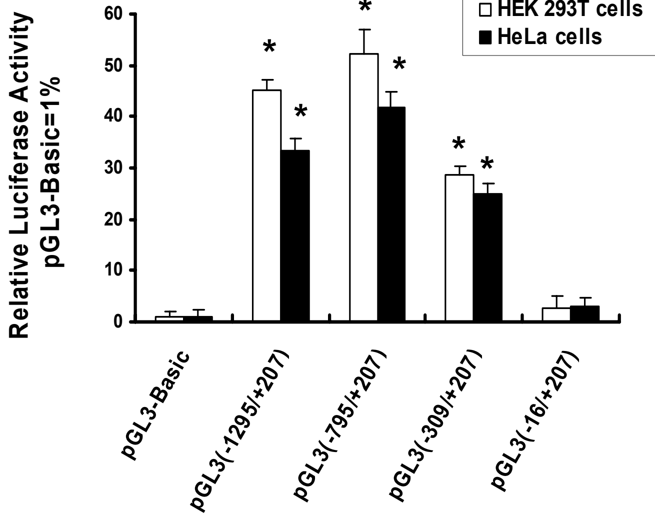
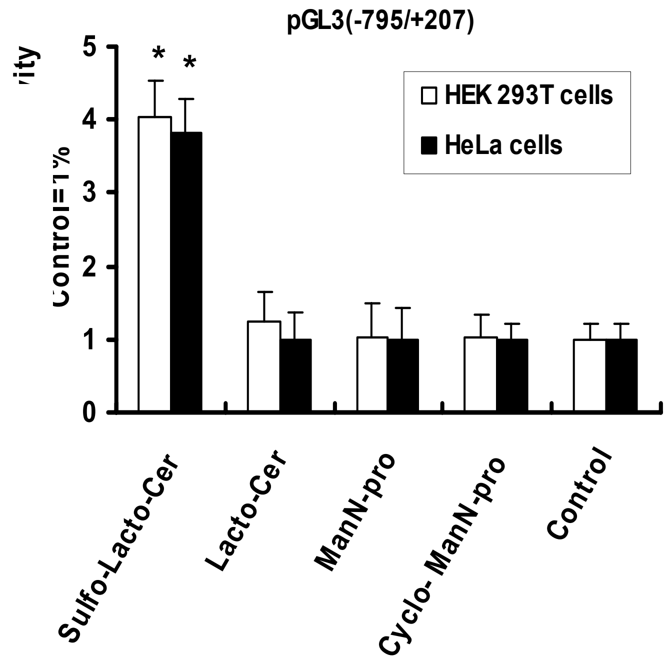
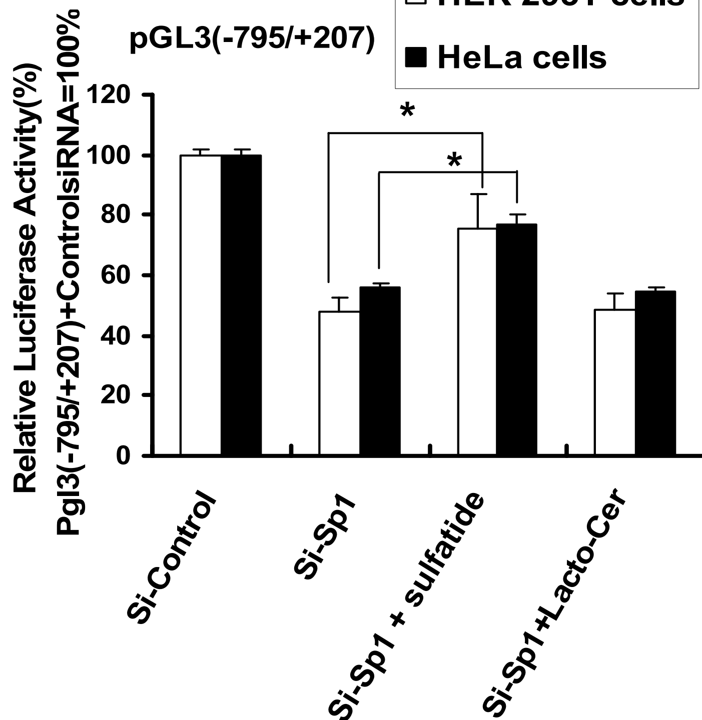
-1358                                 AGTTTTAATAGTTACTTTGGGAAAACAGAAATTACTGTTCCAGTACTTACTTGTAAGT.
-1300   TGTAAGTCCACACAATGCACTTAAACTTCACCTTGTTCAACAGATTCTGTAGCACATGAAACACTTGAAATATGGAAGAGTCACTTCTCTGGGCACTTTG.
             Primer1: sense .
-1200   CTCATTTCGTCTGCTATTCATTCACTGATTGGACATTTTATTGATCGACTACTATCTGCTTGGCAAGGTGCTAGAAATAAGAACACTAAGAAAAAGGTCA.
-1100   AGGTTCCTGTCCTCAAGGCCCTCTCTCTCAGTCCAGTGTTGTCAAGGCTTGATGAAACACACCAGTGGCAAACAAGCACGCGGGGCCTTATTTCACCGGT.
-1000   GTGCGGTCCTTCTTCCAGCTCTGAAATTATGTCTATCCAAACATCCTAAATCAACAAATCAGTGTTTCTAAATCATACAACCGCAGCTAACAAACTGGGA.
-900    ATAAGGCTTCGATTATTCTGACTTGATTTAAAACGATCACTGCCAATTCTTTGCCCTGCGAATCCTTTCTTTGACTACTAAGTTAAGCTTTACATACCTC.
-800    ACTTGCAAGAGGCTATGCTGGCTTTCTGGAAATCCGTAATTAAGTTCAGTGTTTGTGGAATGGAGTGTAATTAACGACCATTAATTAACAGGGTTTCGTT.
            Primer2: sense .
-700    CGTGAGAGCCTGCCAGGCTAAGCAGCAGAAACACGAGAAACTGATAAAAAGCTTTCCTCATTTTTAAACAACAGTCGCACGGAAGTTCCCGGCGGGACAA.
                                                                                         Sp1 site: 1 .
-600    GGGAACGTGGGTGCCCTTGCTACTCCCGTGGACGCGGGTAGATTGGACGCTGGACCGTATCTCCCCGCCCCCGCCCCACGCCTCCTCAGGTGCTCAGC.
                                                                           Sp1 site: 2 .
-500    CTGAGGCCTTCGTCCAGGAGCGCTGCCGCTGACCCAGGCTCAGGAGCTGGGGGCCCCTGCACAGACGCCCAGGTCTCGGGACAGGCGGCGACTGCACTCA.
-400    CGGAAGTACGCTGAGCTCTCCCCTGTAGAAGGGCGCCTCTCCTCCCCCACTTCCTCCTCCAGCTCCACAGCAGCCTCCCGGGCCGGCTCCTCCTCCTTCC.
                                                                                          Primer3: sense.
-300    AGGTCTCCTCCCAGTGCCGCCGCGGCTCTCAGGCCTGAGGTGCGGCGCTCACCCCGGCAGTCCCAGCCTCAGACGCTGCGTGGAGCGGCGGAGCCGGAG.
-200    GGAAGCAAAGGACCGTCTGCGCTGCTGTCCCCGCCCCGCGCGCTCTGCGCCCCTCGTCCCTGGCGGTCGCTCCGAAGCTCAGCCCTCTTGCCTGCCCCGG.
                                  Sp1 site: 3 .
-100    AGCTGTCCCGGGCTAGCCGAGAAGAGAGCGGCCGGCAAGTTTGGGCGCGCGCAGGCGGCGGGCCGCGGGCACTGGGCGCCTCGCTGGGGCGGGGGAGGT.
                                                             Sp1 site: 4                  Primer4: sense.
+1      GGCTACCGCTCCCGGCTTGGCGTCCCGCGCGCACTTCGGCGATGGCTTTTCCGCCGCGGCGACGGCTGCGCCTCGGTCCCCGCGGCCTCCCGCTTCTTCT.
101     CTCGGGACTCCTGCTACCTCTGTGCCGCGCCTTCAACCTAGACGTGGACAGTCCTGCCGAGTACTCTGGCCCCGAGGGAAGTTACTTCGGCTTCGCCGTG.
                                                                                     Primers: anti-sense.
201     GATTTCTTCGTGCCCAGCGCGTCTTC.
```

Figure 5









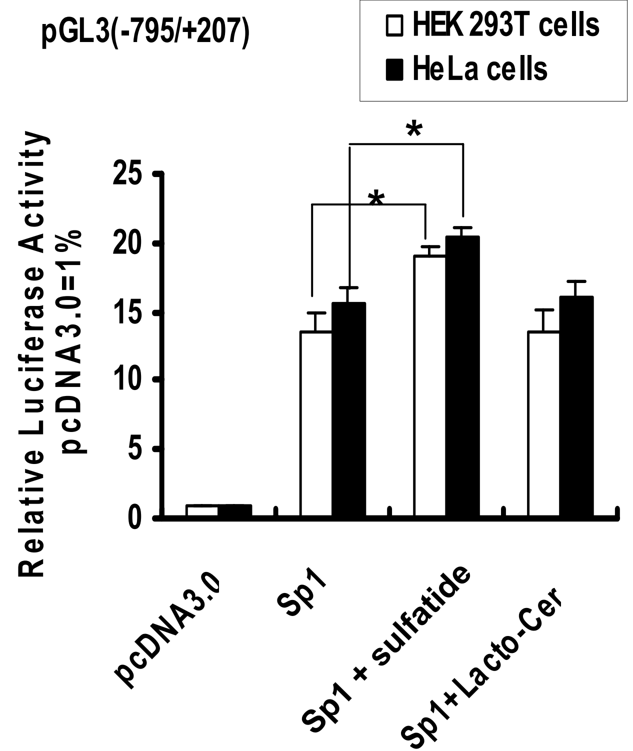
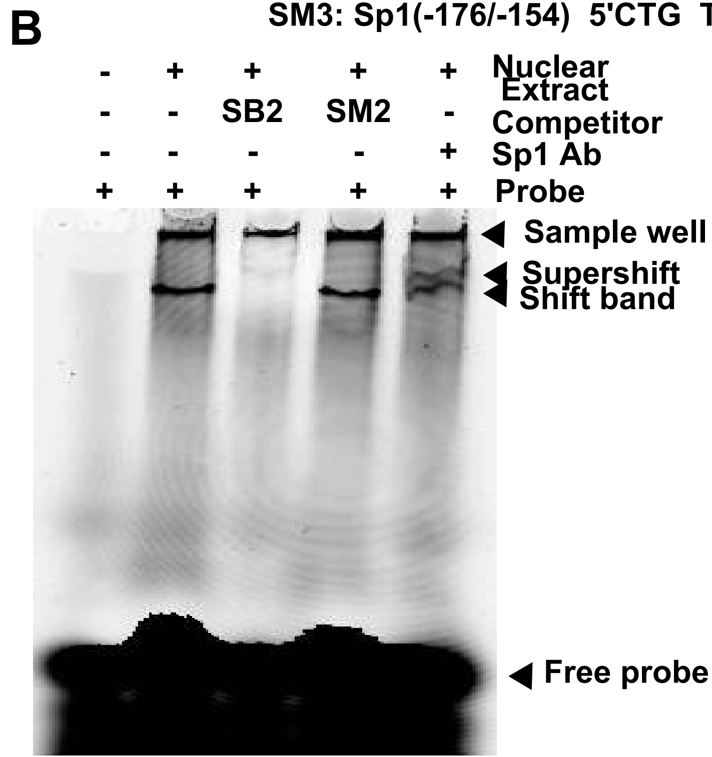
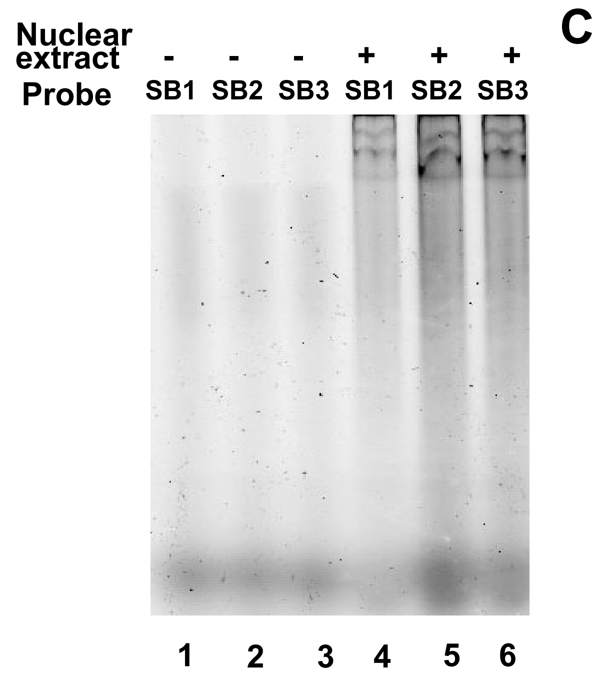


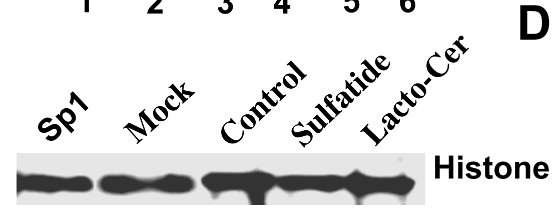
Figure 6

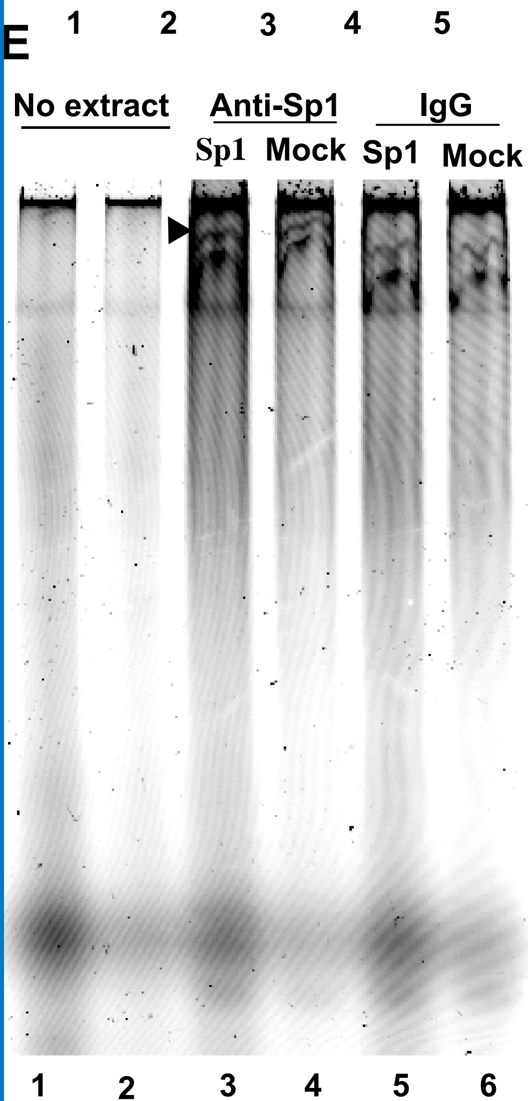
A

(1)SB1: Sp1(-619/-597) 5' GAA GTT CCC GGC GGG ACA AGG G 3'
SM1: Sp1(-619/-597) 5' GAA GTT CCC TGC GTG ACA AGG G 3'
(2)SB2: Sp1(-541/-519) 5' ATC TCC CCG CCC CCG CCC CCA C 3'
SM2: Sp1(-541/-519) 5'ATC TCC ACG CCA CCG CAC CCA C 3'
(3)SB3: Sp1(-176/-154) 5' CTG TCC CCG CCC CGC GCG CTC T 3'
SM3: Sp1(-176/-154) 5'CTG TCC ACG CAC CGC GCG CTC T 3'









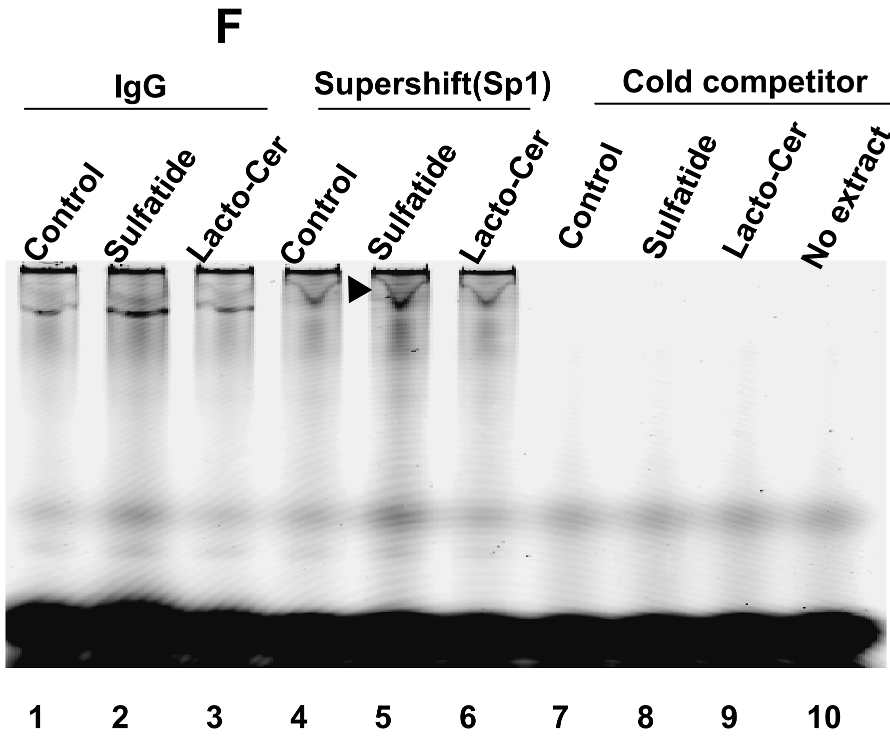


Figure 7

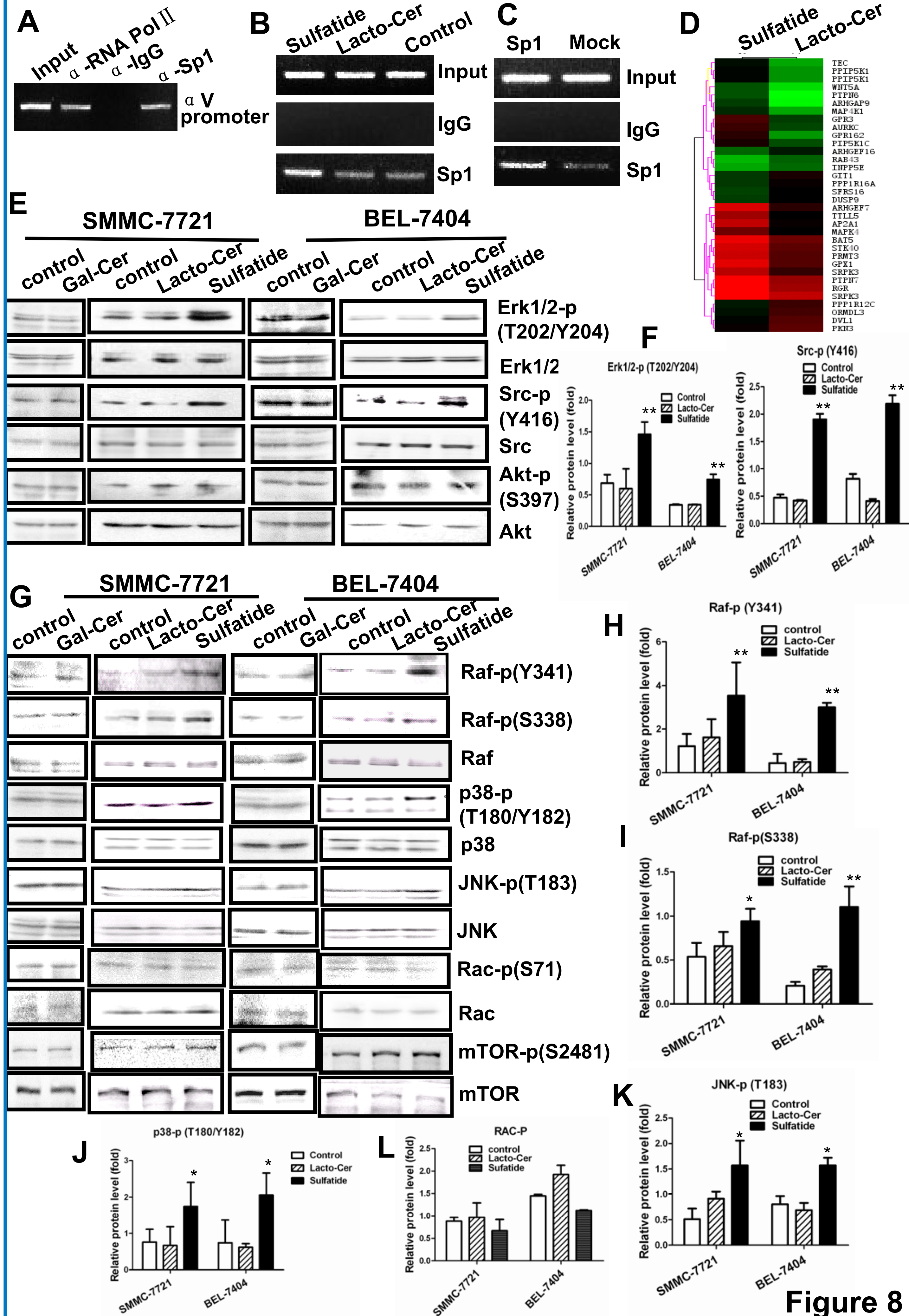


Figure 8

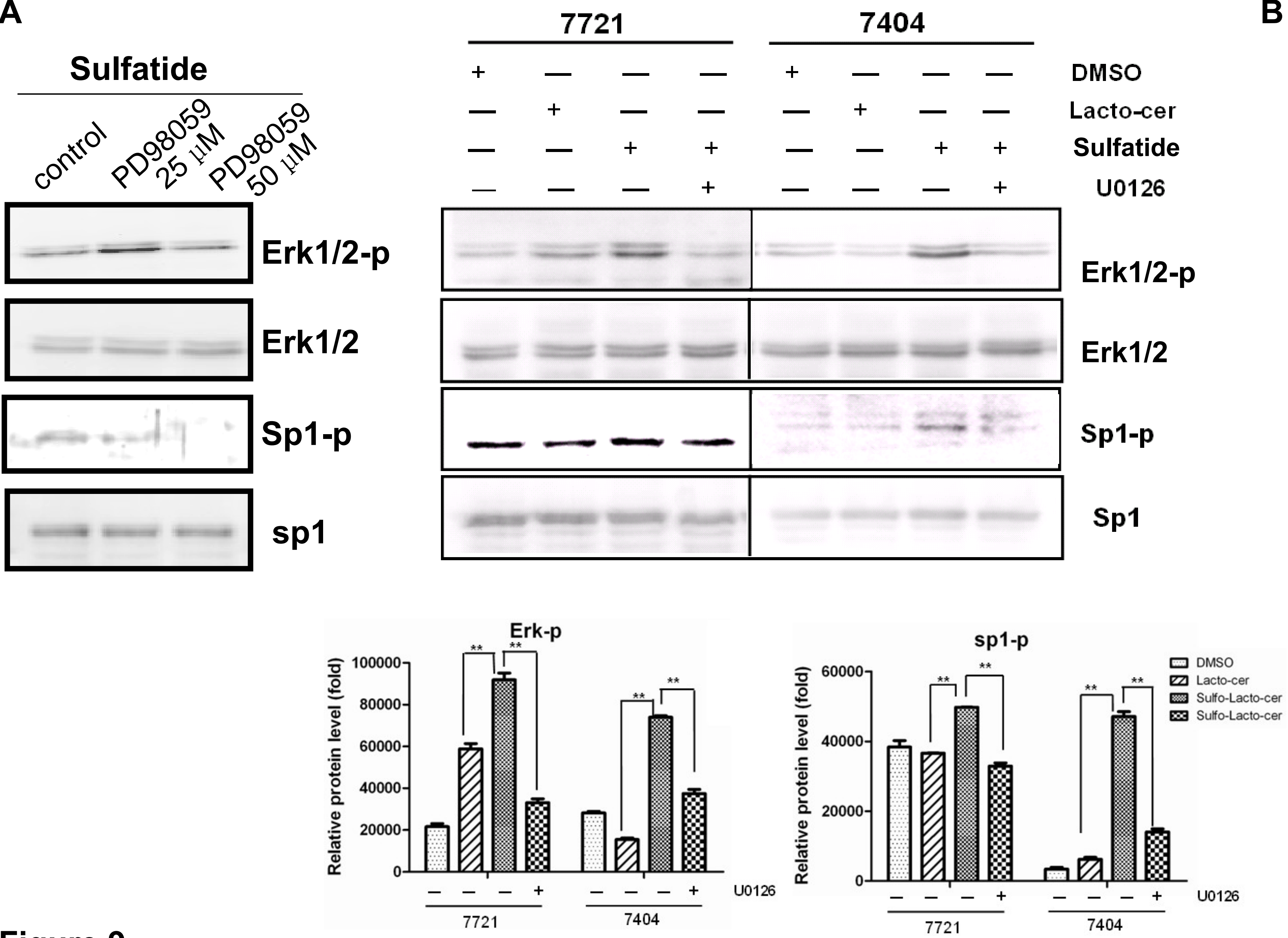


Figure 9