Loss of HSulf-1 Up-regulates Heparin-binding Growth Factor Signaling in Cancer*

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Emerging data suggest that signaling by heparin-binding growth factors is influenced by the sulfation state of N-acetylgalactosamine residues of heparan sulfate proteoglycans (HSPGs). Here we report that the recently identified protein HSulf-1, a heparin-degrading endosulfatase, encodes a cell surface-associated enzyme that diminishes sulfation of cell surface HSPGs. The message encoding this enzyme is readily detectable in a variety of normal tissues, including normal ovarian surface epithelial cells, but is undetectable in 5 of 7 ovarian carcinoma cell lines and markedly diminished or undetectable in ~75% of ovarian cancers. Similar down-regulation is also observed in breast, pancreatic, renal cells, and hepatocellular carcinoma lines. Re-expression of HSulf-1 in ovarian cancer cell lines resulted in diminished HSPG sulfation, diminished phosphorylation of receptor tyrosine kinases that require sulfation, and reduced downstream signaling through the extracellular signal-regulated kinase pathway after treatment with fibroblast growth factor-2 or heparin-binding epidermal growth factor. Consistent with these changes, HSulf-1 re-expression resulted in reduced proliferation as well as sensitivity to induction of apoptosis by the broad spectrum kinase inhibitor staurosporine and the chemotherapeutic agent cisplatin. Collectively, these observations provide evidence that HSulf-1 modulates signaling by heparin-binding growth factors, and HSulf-1 down-regulation represents a novel mechanism by which cancer cells can enhance growth factor signaling.

HSPGs are major constituents of the extracellular matrix, where they act as important mediators of adhesion as well as modulators of growth factor signaling and proteolysis (1, 2).

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‡‡ These abbreviations used are: HSPGs, heparan sulfate proteoglycans; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; EST, expressed sequence tag; FITC, fluorescein isothiocyanate; GFF, growth factor; FGFR, FGFR receptor; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; TRITC, tetramethylrhodamine isothiocyanate; LOH, loss of heterozygosity.

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way, including FGF and HB-EGF, have been seen frequently in ovarian cancers and other solid tumors (14–20). Moreover, activation of the ERK pathway has been implicated in enhanced proliferation as well as resistance to apoptosis (21, 22). As a result, there is considerable interest in understanding the factors that contribute to activation of this pathway as well as mechanisms that can reverse this activation.

In this report, we describe the widespread down-regulation of HSulf-1 in cancer cell lines and ovarian cancer specimens. To examine the functional consequences of this down-regulation, we re-expressed HSulf-1 in two ovarian cancer cell lines that lack this message. Results of this analysis suggest that HSulf-1 is a cell surface sulfatase that diminishes HSPG sulfation, thereby modulating signaling by HB-GFs. Consistent with this conclusion, we demonstrate diminished ERK pathway activation after treatment with FGF-2 and HB-EGF, diminished proliferation, and enhanced drug-induced apoptosis in ovarian cancer cell lines in which HSulf-1 is re-expressed. These observations not only provide new insight into the function of HSulf-1, but also suggest that its down-regulation is a previous unidentified mechanism by which cancer cells enhance growth factor signaling.

MATERIALS AND METHODS

Cell Culture—OV167, OV177, OV202, OV207, and OV266 ovarian cancer cell lines and the short term cultures of the normal ovarian epithelium were established at the Mayo Clinic (23). The ovarian cancer cell lines OVCA-5 and SKOV-3, as well as breast (BT474, MCF-7, MCF10A, MDA-MB-157, MDA-MB-361, MDA-MB-453, UACC828, and UACC983) pancreas (AsPC1, BxPC3, CAPAN 1, CAPAN 2, CFPAC-1, and Mia), kidney (HTB45, HTB49, and CRL 1633), and hepatocellular carcinomas (HepG2, HepG2, HUH7, HUH7, SNU182, SNU387, SNU423, SNU449, SNU475, SKHepl and PLC5) were from American Type Culture Collection (Manassas, VA). Normal human mammary epithelial Cells (HMEC) were from Clonetics Corp. (San Diego). All cells were grown according to the providers’ recommendations.

Drugs and Reagents—Staurosporine (Sigma) and UCN-01 (Drug Synthesis Branch, NCI, National Institutes of Health) were dissolved in Me2SO at a concentration of 1 mm, stored at −20 °C, and subsequently diluted with serum-free medium before use. In all experiments the concentration of Me2SO did not exceed 0.1%. Cisplatin (Sigma) was prepared immediately before use as a 1000-fold concentrated solution in Me2SO. Fibroblast growth factor, heparin-stabilized FGF, and heparin-binding epithelial growth factor (Sigma) were dissolved in water.

Cloning of HSulf-1 cDNA and Gene—BLAST search of the isolated sequence from SSSH libraries of early and late stage tumors identified ESTs homologous to KIAA1077 in the dbEST. The homologous ESTs were assembled into a contig with the use of the Vector NTI 3 software (Gene Codes Corp, Ann Arbor, MI). Additional 5′ sequences not present in KIAA1077 were obtained with electronic walking by assembling overlapping EST sequences in the genome BLAST server. The integrity of the full-length cDNA obtained by this electronic walking was confirmed by PCR analysis using PCR primers flanking each junction between EST clones.

For expression of truncation mutants of HSulf-1, the N-terminal portion of HSulf-1 (N-Sulf) containing only the sulfatase domain was amplified using primers NF (5′-ATTGACAAATACTAATAGG-3′) and NRFlg (ttaagctctagctgctgtagctGAAAGTATACCGCAAAAT), where the lowercase letters encode the FLAP tag. The C-terminal portion of HSulf-1 (C-Sulf) was amplified using primers CF (5′-CTGTGATTACCTGCTAGTGG), and CRFlg (ttaagctctagctgctgtagctACC-TCCCATCATCTCCCA), with a stop codon introduced after the epitope tag. The full-length HSulf-1 was amplified using primers NF and CRFlg using Expand™ Long Template PCR system (Roche Applied Science). All three products were cloned into GFP Fusion TOPO® TA Expression plasmid (Invitrogen). For generating a full-length HSulf-1/GFP fusion construct, the stop codon of CRFlg was not included. cDNAs generated from short term cultures of normal ovarian surface epithelial cells were used as a template for generating PCR products for cloning. The products of each PCR were resolved on a 1.6% agarose gel and purified using a gel extraction kit (Qiagen, Valencia, CA) for cloning into expression vectors.

Establishment of Stable Transfectants—Exponentially growing SKOV3 cells in 100-mm dishes were washed with serum-free medium and treated with a mixture containing 4 μg of plasmid, 30 μl of LipofectAMINE, and 20 μl of Plus reagent. After 3 h of incubation, complete medium with serum was added. G418 (400 μg/ml) was added 24 h later to select transfectants. Individual colonies were subsequently cloned using cloning rings. For controls, cells were similarly transfected with vector (pDNA3.1 GFP-CT), and stable clones were selected.

Semi-quantitative RT-PCR—Total RNA was extracted from 7 ovarian cancer cell lines and 30 primary ovarian tumors using the NuSsey mini kit (Qiagen). cDNA synthesis was performed as described (9). 50–100 ng of reverse-transcribed cDNA was used in a multiplex reaction with three different primer pairs Sulf-1F (5′-CCACCTTCTCATATG-GCTT-3′) and Sulf-1R (5′-CCTTGTACGTTCCAAACGTCG-3′), Sulf-2F (5′-CATATTATACCGCCGACG), and Sulf-2R (5′-CCTGCC-TCCTCTCTCCTT-3′), Sulf-3F (5′-GAGCATCATCTTACCACTCAGC), and Sulf-3R (5′-TCCACCAACTTATGGCAGCCGTA-3′), and GAPDH-F (5′-ACCAGACTCATACATCG-3′) and GAPDH-R (5′-TCCGACAGTGACTGACAC-3′) in separate reactions to yield 760-, 1260-, and 825-bp products, respectively. The primer mixes contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 400 μM concentration of each primer for HSulf-1 and 50 μM for the GAPDH primers, and 0.5 units of Taq polymerase (Promega) in a 12.5-μl reaction volume. The conditions for amplification are as follows: 94 °C for 3 min followed by 29 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s in a PerkinElmer Life Sciences 9600 Gene-Amp PCR system. The products of the reaction were resolved on a 1.6% agarose gel and photographed using the Gel Doc 1000 photo documentation system.

LOH Analysis—The 5 pairs of microsatellite markers within the HSulf-1 gene used in this study are listed in Table I along with their locations within the HSulf-1 gene. Amplifications were performed as described (9) except that annealing was performed at 52–57 °C, and reactions were run in a 96-well plate. After denaturation, PCR products were run on 6% polyacrylamide sequencing gels containing 8 μM urea. Gels were dried, subjected to autoradiography using multiple exposure

| Sequences | Location within the gene | Product size | % LOH (no. samples with LOH/ no. informative samples) |
|-----------|--------------------------|--------------|-----------------------------------------------------|
| CA1F-5′  | 5′ UTR                    | 379          | 46% (4/9)                                           |
| CAIR-5′  | 13 CA repeats            | 275          | 47% (7/15)                                          |
| CA2F-5′  | Intron 1                  | 247          | 53% (9/17)                                          |
| CA2R-5′  | 21 CA repeats            | 247          | 50% (4/7)                                           |
| CA3F-5′  | Intron 2                  | 212          | 50% (10/20)                                         |
| CA3R-5′  | Intron 2                  | 193          | 50% (10/20)                                         |
| CA4F-5′  | Intron 3-1                | 10 GAAG repeats |                                                  |
| CA4R-5′  | 24 CA repeats            |              |                                                    |
| CA5F-5′  | Intron 3-2                |              |                                                    |
| CA5R-5′  | 10 GAAG repeats           |              |                                                    |
times, and scored for LOH. Allele imbalance indicative of LOH was scored, and a >50% loss of intensity of one allele in the tumor sample with respect to the matched allele from normal tissue was observed.

**Northern Blot**—Total RNA (15 μg) was fractionated on 1.2% formaldehyde agarose gels and blotted in 1× SPMC buffer (20 mM NaHPO4, 2 mM CDTA (pH 6.8)) onto Hybond-N membranes (Amersham Biosciences). The probes were labeled using the random primer labeling system (Invitrogen) and purified using spin columns (100 TE) from Clontech. Filters were hybridized at 68°C with radioactive probes in a hybridization incubator and washed according to the manufacturer's guidelines.

**Analysis of Apoptosis**—Apoptosis was quantitated by assessing the number of cells containing nuclear changes indicative of apoptosis (chromatin condensation and nuclear fragmentation) after staining with DAPI. HSulf-1-transfected SKOV3 cells were seeded in 35-mm plates at a density of 2×10^5 cells/well. After incubation at 37°C for 24 h, the plates were washed and changed to serum-free medium. Staurosporine was added to a final concentration of 1 μM for 5 h. DAPI was then added to each well at a final concentration of 5 μg/mL. After a 20-min incubation in the dark at 20°C, cells were examined by fluorescence microscopy (Nikon Eclipse TE200; Nikon Corp., Tokyo, Japan) using excitation and emission filters of 380 and 430 nm. An individual blind was performed to the experimental conditions counted at least 300 cells in six different high power fields for each treatment. Each treatment was repeated at least three times, performed in triplicate each time. To inhibit apoptosis, the cells were pretreated with 10 μM 17β-estradiol (Sigma) or 40 μg/ml DEX (Sigma) for 24 h before harvest and the times indicated in individual figures. Following treatment, cells were rinsed with ice-cold PBS, scraped from the dishes, and lysed at 4°C in SDS sample buffer without bromophenol blue. Protein concentrations were determined with bicinchoninic acid (Pierce).

**Immunoblotting**—Equal amounts of protein (20 μg/lane) were separated by electrophoresis on an SDS gel containing a 4–12% acrylamide gradient and electrophoretically transferred to nitrocellulose. Blots were washed once with TBS, 0.2% Tween 20 (TBST) and blocked with TBST containing 5% non-fat dry milk for 1 h at 20°C. The blocking solution was replaced with a fresh solution containing a 1:500 dilution of rabbit anti-phospho-ERK (Cell Signaling Inc., Beverly, MA). The blot was subsequently washed three times for 10 min each in TBS, 0.1% (v/v) Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody in 5% milk/TBST at 20°C for 1 h. After washing three times in TBST, the proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). The blots were stripped and reprobed with antisera that recognize total ERK, EGFR phosphorylated on Tyr**1045** and/or Tyr**1068**, total EGFR (all from Cell Signaling Inc., Beverly, MA), or mouse monoclonal anti-actin (Sigma).

**Sulfatase Assay**—Confuent flasks of stable transfectants were washed in ice-cold PBS and lysed in SIE buffer (250 mM sucrose, 3 mM imidazole (pH 7.4), 1% ethanol) containing 1% (w/v) Nonidet P-40 and protease inhibitor mixture (Roche Applied Science). After cells were sheared by passage through a 22-gauge needle, protein concentration was determined using the Bradford assay. 100 μg of total cellular protein was preincubated with 10 μM estrone 3-O-sulfamate (Sigma) at 37°C for 1 h to inhibit steroid sulfatase. 4-MUS was then added to a final concentration of 10 μM in the presence of 10 μM lead acetate in a total of 500 μL SIE buffer. After incubation at 37°C for 30 min at 2°C, the substrate was terminated by addition of 1 mL of 0.5 M Na2CO3/NaHCO3 (pH 10.7). The fluorescence of the liberated 4-methylumbelliferone was measured using excitation and emission wavelengths of 360 and 460 nm, respectively. To assess the effects of transient transfection, SKOV3 cell lysates prepared 48 h after transfection with empty vector, full-length HSulf-1 cDNA, or mutant N-Sulf were assayed as described above except that the incubation was shortened to 3.5 h.

**HSulf-1 Localization**—SKOV3 cells seeded on glass coverslips were allowed to adhere overnight and then transfected with empty vector or cDNA encoding full-length HSulf-1 fused at its C terminus to GFP, 24 h after transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and mounted with Vectashield® mounting medium containing DAPI. The GFP fusion protein was visualized using a Zeiss LSM510 laser scanning confocal microscope. Alternatively, SKOV3 cells were transfected with 4 μg of plasmid encoding FLAG-tagged HSulf-1, incubated for 24 h, washed with PBS, fixed for 10 min in PBS containing 3.7% formaldehyde and 1% sucrose, washed with 0.1 M glycine in PBS, permeabilized with PBS containing 0.4% Triton X-100 and 2% bovine serum albumin for 20 min, and washed three times in washing buffer (PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100). After incubation for 1 h at 20°C with anti-EGFR antibody, cells were washed four times with washing buffer, incubated with 1:200 FITC-conjugated anti-rabbit IgG and 1:200 FITC-conjugated anti-mouse IgG, then washed twice in PBS, and viewed with an Axiovert 35 epifluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a 100-watt mercury lamp or a Zeiss LSM510 confocal microscope.

**HSulf-1 Distribution**—48 h after transfection of cDNA encoding GFP-tagged HSulf-1 into SKOV3 parental cells, cells and medium were collected separately. The cells were washed in ice-cold PBS, scraped, and lysed at 4°C in SDS buffer without bromophenol blue. Protein concentrations were determined with bicinchoninic acid (Pierce). Conditioned medium was concentrated in a Centricon 10 microconcentrator (Millipore Corp). The cell lysate and 50× concentrated medium were resolved on 7.5% SDS-polyacrylamide gels under reducing conditions. Immunoblotting was performed as described above using anti-GFP antibody (Santa Cruz Biotechnology).

**Sulfation State of Cell Surface HS-GAGs**—Cells growing on coverslips for 24 h were fixed in methanol for 10 min at −20°C, washed with PBS, and incubated for 1 h at 20°C with 1.30 dilution of mouse 10E4 antibody, which recognizes an epitope that includes the N-sulfated glucosamine residue (Seikagaku America, Falmouth, MA). After washing, cells were stained with FITC-conjugated anti-mouse IgG and examined by laser scanning confocal microscopy as described above. As a control, cells were stained with antibody 3G10, which recognizes non-glycosylated glycosaminoglycans. In brief, cells grown on coverslips were fixed in 4% paraformaldehyde at 4°C for 30 min, preincubated in buffer containing 50 mM sodium acetate (pH 7.0), 100 mM NaCl, and 3.3 mM CaCl2 at 37°C for 1 h, and followed by incubation with 20 milliunits/ml of heparitinase I (Sigma) for 1.5 h at 37°C. After thorough washing, cells were incubated with a 1:250 dilution of 3G10 monoclonal antibody (Seikagaku America) for 2 h at 37°C followed by FITC-conjugated anti-mouse IgG and examined as described above.

**RESULTS**

**Characterization of the HSulf-1 cDNA and Gene**—Differential screening of suppression subtraction cDNA libraries generated from primary ovarian tumors subtracted against normal ovarian epithelial cells previously identified an EST homologous to KIAA1077 (9) as a transcript that is down-regulated in ovarian cancer. In order to study the function of the corresponding polypeptide, the cDNA and gene were cloned as described under “Materials and Methods.”

After HSulf-1 was assembled into a contig using Sequencer3 software, a 5696-bp cDNA containing a single open reading frame encoding an 871-amino acid polypeptide was isolated (GenBank™ accession number AF545571). The predicted amino acid sequence is identical to that recently reported for HSulf-1 (8). Based on the computer algorithm Signal P version 1.1 at the Centre for Biological Sequence Analysis website (24), a 22-amino acid signal peptide (MKYSCCALVLAVL-GTELLGSLCST) is found at the N terminus, with the most likely cleavage site indicated by ↓. The transmembrane prediction program TMpred (25) predicts two additional membrane-spanning domains at amino acids 69–779. The cDNA contains five potential polyadenylation signals AATAAA at positions 4170, 4679, 4820, 4824, 5052, and 5678. The HSulf-1 gene spans −211-kb genomic fragment on chromosome 8q13.3. Our analysis of the cDNA and gene agree with that reported by Morimoto-Tomita et al. (8) with one notable exception. Morimoto-Tomita et al. (8) reported the presence of a 280-bp noncoding exon in the 5′-UTR that they called exon 1
(8), and we will denote exon 1A. In addition to this sequence, we have identified two other noncoding exons, one 5′ to this exon (314 bases, which we call exon 1) and another 3′ to this sequence (165 bases, which we call exon 2). To determine which of these exons, 1, 1A, or 2, are present in transcribed message, primers in exons 1 and 5 were utilized to amplify a 950-bp 5′-UTR from normal ovarian epithelial cells, HMEC, and normal kidney. All of the cDNAs amplified exon 1A. Instead, two different splice variants were amplified, a less abundant one lacking exon 1A but containing exons 1 and 2, and another more abundant one lacking both exon 1A and exon 2 (data not shown).

**HSulf-1 Is Widely Expressed in Normal Tissue**—Northern blot analysis using full-length HSulf-1 cDNA as a probe (Fig. 1D) revealed a 6.0-kb and a smaller 5.0-kb transcript. Expression was observed in all nonlymphoid tissues but was highest in small intestine, pancreas, and colon. Importantly, the blot shown in Fig. 1D contains message from total ovary. Further analysis (Fig. 2, A and B) demonstrated that HSulf-1 message was readily detected in purified human ovarian surface epithelial cells. Testis expresses tissue-specific transcripts.

**Decreased HSulf-1 Expression in Primary Ovarian Cancers**—Because KIAA1077 was initially identified as a downregulated transcript in SSH libraries of ovarian cancer, we next evaluated HSulf-1 expression ovarian cancer cell lines and primary tumor specimens. Although semi-quantitative PCR (Fig. 2, A, C, and E, lane 1) and Northern blotting (Fig. 2B) readily detected HSulf-1 mRNA in all normal ovarian surface epithelial samples examined, the message was undetectable in 5 of 7 ovarian cancer cell lines (Fig. 2, A and B). Moreover, semi-quantitative RT-PCR with overlapping primers spanning the entire HSulf-1 open reading frame (illustrated in Fig. 2C and summarized in Table II) demonstrated that HSulf-1 down-regulation is common in primary ovarian cancer specimens. In particular, HSulf-1 mRNA was undetectable in 40% of samples and extremely weak in another 37%. Among the histological subtypes of ovarian cancer, clear cell cancers have a particularly poor prognosis (26) and uniformly lack detectable HSulf-1 expression (Fig. 2C and Table II).

To begin to assess the mechanism by which HSulf-1 expression might be decreased in ovarian cancer, LOH analysis was performed. Genomic sequence analysis revealed microsatellite markers in the 5′-UTR, introns 1 and 2, and two within intron 3. The primers flanking these repeats are shown in Table I. Analysis in 30 primary ovarian tumor samples revealed that LOH of these markers ranged from 44 to 53% (Fig. 2D).

**Decreased HSulf-1 Expression in Other Tumor Types**—To assess the possibility that loss of HSulf-1 expression might be unique to ovarian cancer cells (Fig. 2, A–C), semi-quantitative RT-PCR was performed using cancer cell lines of breast, pancreas, kidney, and liver origin. For these studies, normal HMEC and normal kidney (Fig. 2E, lanes 1 and 16) served as positive controls. Results of this analysis revealed that the majority of cancer cell lines had either a complete loss or markedly diminished expression of HSulf-1 (Fig. 2E), raising the possibility that down-regulation of this transcript might be relatively widespread among epithelial malignancies. However, MCF10A cells derived from fibrocytic breast generally regarded as relatively normal (27) also had a complete loss of HSulf-1 expression, possibly implicating HSulf-1 loss as an early event in mammary carcinogenesis.

**HSulf-1 Encodes an Active Sulfatase**—To begin to analyze the function of HSulf-1, full-length cDNA was stably transfected into two lines lacking detectable message, SKOV3 and OV207, and multiple clones were isolated. Analysis by RT-PCR demonstrated varying levels of message within the clones (Fig. 3A). Both sets of lines were utilized in the analyses presented below.

To determine whether the stable transfectants exhibited sulfatase activity, cell lysates from clones displaying abundant HSulf-1 message were incubated with the fluorogenic substrate 4-MUS in the presence of estrone 3-O-sulfamate to specifically inhibit endogenous steroid sulfatase activity (Fig. 3, B and C). By using this nonspecific sulfatase substrate, an increase in activity comparable with that reported by Morimoto-Tomita et
was observed in HSulf-1-expressing clones compared with parental cells or clones transfected with empty vector. A comparable increase in sulfatase activity was also observed 48 h following transient transfection of full-length H-Sulf-1 cDNA into SKOV3 cells (data not shown).

**HSulf-1 Is Localized to the Cell Surface**—The avian ortholog of HSulf-1, QSulf1, was shown to localize to the cell surface (7). In contrast, Morimoto-Tomita et al. (8) reported that HSulf-1 is secreted to the medium after transfection into Chinese hamster ovary cells. To begin to address this apparent inconsistency, cDNA encoding HSulf-1 fused to GFP was transiently transfected into SKOV3 cells. Blotting with anti-GFP antibody readily detected the fusion protein in whole cell lysates but not in medium that was concentrated 50-fold (Fig. 4A). This is similar to observations made previously with the HSulf-1 homologs QSulf-1 and RsulfFP1 (7, 28).

Confocal microscopy of cells expressing the HSulf-1/GFP fusion protein demonstrated that HSulf-1 localized to the plasma membrane (Fig. 4B). Further analysis demonstrated co-localization with growth factor receptors such as EGFR at the cell surface (Fig. 4C).

**HSulf-1 Expression Is Associated with Decreased HSPG Sulfation**—To assess the possibility that HSulf-1 affects HSPG sulfation, clones transfected with empty vector or HSulf-1 were stained with 10E4 antibody, which specifically recognizes sulfated glucosamine in HSPGs (29). Intense staining was observed in parental and vector transfected cells (Fig. 5A). In contrast, HSulf-1-expressing clones showed...
reduced staining. Staining with anti-stub antibody 3G10 revealed that both HSulf-1-negative and HSulf-1-transfected cells express HSPGs equally (Fig. 5B).

To assess further the ability of HSulf-1 to modulate cell surface sulfation, OV202 cells, which endogenously express HSulf-1 (Fig. 2, A and B), were transiently transfected with HSulf-1 antisense cDNA. Cell surface staining for sulfated glucosamine in HSPGs was markedly increased (Fig. 5C). Collectively, these results provide preliminary evidence that HSulf-1 down-regulation increases HSPG sulfation and HSulf-1 re-expression decreases HSPG sulfation.

**HSulf-1 Modulates Signaling by HB-EGF but Not EGF**—To determine whether HSulf-1 modulates HB-GF signaling, we examined the action of HB-EGF in parental and HSulf-1-transfected ovarian cells. This growth factor was chosen because of its dependence on heparin binding for its action and because of its postulated role in ovarian carcinogenesis (30). Overexpression of HER2 and HER4, which mediate the effects of heparin-independent EGF and HB-EGF, respectively, is well documented in ovarian cancer cells (30, 31).

HB-EGF treatment of serum-starved parental and vector-transfected cells resulted in sustained phosphorylation of the EGFR on Tyr1068 (Fig. 6A, upper panels). Identical results were obtained with anti-Tyr992 antiserum (data not shown). Both Tyr1068 and Tyr992 have been implicated in EGFR-induced ERK pathway activation (32, 33). Consistent with the enhanced phosphorylation of these sites, ERK phosphorylation was demonstrated in these cells after HB-EGF treatment (Fig. 6A, lower panels). In contrast, HSulf-1-expressing clones demonstrated diminished EGFR phosphorylation and diminished ERK phosphorylation after HB-EGF treatment (Fig. 6A). These results suggest that HSulf-1 can modulate signaling by HB-EGF.

To determine whether this modulation was specific for HB-EGF, the same cells were treated with heparin-independent EGF. As indicated in Fig. 6B, there was no difference in either EGF-stimulated EGFR phosphorylation at Tyr1068 or ERK phosphorylation in HSulf-1-expressing clones compared with parental and vector-transfected controls.

**HSulf-1 Also Modulates FGF-2 Signaling**—Previous studies (4–6, 34–36) have shown that N-sulfation and 2-O-sulfation are critical for the interaction between HS-GAGs and FGF-2, whereas 6-O-sulfation is required for the interaction between HS-GAGs and FGFR1 in the formation of the FGF-2-HS-GAG-FGFR1 ternary complex. Conversely, it has been reported that cells containing GAG chains with reduced sulfation lose their proliferative response to FGF-2 (34, 35, 37). These observations, coupled with the demonstration of elevated FGF-2 and FGFR1 in ovarian cancer cells (38, 39), prompted us to examine the effect of HSulf-1 on FGF signaling.

As a read out for FGF signaling, we again measured mitogen-activated protein kinase pathway activation. Formation of the FGF-2-HS-GAG-FGFR1 ternary complex induces receptor dimerization, activation of the FGFR1 tyrosine kinase (36, 40), receptor autophosphorylation, and binding of the adaptor SNT/FRS, which then activates intracellular signaling pathways, including the ERK pathway (41).

When serum-starved vector-transfected cells were treated with FGF-2, sustained phosphorylation of ERK1 and ERK2 lasting >60 min in parental (not shown) and vector-transfected cells (Fig. 7A) was observed. In contrast, HSulf-1-expressing clones demonstrated lower basal levels of ERK phosphorylation and only transient elevation after FGF-2 treatment (Fig. 7A). Collectively, these results suggest that HSulf-1 not only downregulates the basal activation of ERK pathway activation, but also inhibits a sustained activation of this pathway that may be required for cell survival and proliferation.

Several controls pointed to sulfation of HSPGs as a critical factor in this HB-GF signaling modulation. First, replacement of FGF-2 with heparinated FGF (heparin-stabilized FGF) abrogated the ability of HSulf-1 to modulate FGF-initiated signaling (Fig. 7B). In addition, transient transfection of SKOV3 cells with the sulfatase domain N-Sulf also dampened FGF

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**Table II**

*Expression analysis in primary ovarian tumors*

| HSulf-1 mRNA was examined by semiquantitative RT-PCR as illustrated in Fig. 2. Relative levels were scored as absent (0), barely detectable (1 +), detectable but diminished compared with OSE (2 +), or equal to ovarian surface epithelial cells (3 +). |
|---|---|---|
| **Samples** | **Tested** | **HSulf-1 = 0** | **HSulf-1 = 1 +** |
| **Total** | 30 | 12 (40%) | 11 (37%) |
| **Clear cell** | 4 | 1 (25%) | 0 |
| **Endo** | 9 | 6 (67%) | 3 (11%) |
| **Stage I** | 5 | 4 (80%) | 1 (20%) |
| **Stage II** | 4 | 3 (75%) | 0 |
| **Stage III** | 17 | 1 (6%) | 16 (59%) |
| **Stage IV** | 5 | 1 (20%) | 4 (80%) |
| **Stage V** | 11 | 0 | 6 (55%) |

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**Fig. 3. Increased sulfatase activity after HSulf-1 re-expression.** A, RT-PCR of parental, vector-transfected, and HSulf-1-transfected clones obtained from SKOV3 (left) and OV207 (right) ovarian cancer cell lines. B, cell extracts from parental cells and stable SKOV3 clones were assayed for sulfatase activity using 10 μM 4-MUS in the presence of 10 μM estrone 3-O-sulfamate to inhibit endogenous steroid sulfatase activity. C, sulfatase activity in parental OV 207 cells and the indicated clones was measured as in B. No activity was detected in the absence of added substrate or lysate (data not shown). M, marker.
signaling, whereas mutation of two conserved cysteines at the active site of this domain abrogated the modulation (Fig. 7C). Collectively, the results in Figs. 6 and 7 demonstrate the ability of HSulf-1 to modulate signaling by two different HB-GFs and the dependence of this modulation on an intact sulfatase domain.

**HSulf-1 Modulates Proliferation and Apoptosis**—Previous studies have demonstrated that stimulation of the Mek/ERK pathway leads to enhanced proliferation and inhibition of apoptosis (reviewed in Refs. 21 and 22). Based on the ability of HSulf-1 to modulate signaling through this pathway, we predicted that HSulf-1 re-expression in HSulf-1-deficient cells would lead to inhibition of proliferation and enhancement of apoptosis.

To test the first of these predictions, parental or HSulf-1-transfected clones were plated at 100,000 cells/dish and counted at various times. As predicted HSulf-1-expressing clones proliferated more slowly than parental or empty vector-transfected clones (Fig. 8A).

To examine the effects of HSulf-1 re-expression on apoptosis, stable transfectants were treated for 24 h with 5 μM cisplatin, an agent that is widely used to treat ovarian cancer (42), or diluent. Cells were then stained with DAPI and examined for apoptotic morphological changes (nuclear fragmentation) by fluorescence microscopy (schematic, Fig. 8B). Results of this analysis indicated that cisplatin induced little apoptosis in parental or vector-transfected cells under these conditions. In contrast, cisplatin induced apoptosis in 25–40% of HSulf-1-transfected cells (Fig. 8B). HSulf-1 re-expression likewise sensitized OV207 clones to cisplatin (Fig. 8C). To rule out the possibility that these results were unique to cisplatin, the same cell lines were treated for 5 h with 1 μM staurosporine, a broad spectrum kinase inhibitor (43) that is widely used as a prototypic pro-apoptotic stimulus because it induces apoptosis in a wide variety of cells (44), or with UCN-01, a staurosporine analog currently in phase I clinical trials (45). Once again, HSulf-1-transfected clones were sensitized to the induction of apoptosis (Fig. 8D). Similar results were observed in the OV207 clones and in a second set of independently derived HSulf-1-expressing SKOV3 clones (data not shown). Further experiments revealed the typical biochemical hallmarks of apoptosis, including cytochrome c release from mitochondria and DNA.

**Fig. 4. HSulf-1 is a cell surface-associated polypeptide.** A, 48 h after transient transfection with cDNA encoding an HSulf-1/GFP fusion protein, whole cell lysates and 50-fold concentrated conditioned medium were subjected to immunoblotting with anti-GFP antibodies. An intense band was detected at 135 kDa, the predicted size of the fusion protein, in the cell extracts but not the conditioned medium. B, confocal images of SKOV3 cells obtained 24 h after transient transfection of cDNA encoding an HSulf-1/GFP fusion protein (left panel) or GFP alone (right panel). C, co-localization of HSulf-1 and EGFR. SKOV3 cells transiently transfected with FLAG-tagged HSulf-1 were fixed and stained with rabbit anti-EGFR and TRITC-conjugated anti-rabbit IgG as well as FITC-conjugated anti-FLAG antibody. The arrow points to the co-localization of HSulf-1 and EGFR to the cell membrane in the merged panel.

**Fig. 5. HSulf-1 expression is associated with decreased sulfation of cell surface HS-GAGs.** A, SKOV3 parental cells and the indicated stable, cloned transfectants were fixed and stained for sulfated HS-GAG using antibody 10E4, which recognizes native heparan sulfate with sulfated glucosamine residues (A) or antibody 3G10, which recognizes deglycosylated HS-GAG (B). C, OV202 cells, which express endogenous HSulf-1 (Fig. 2A), were transiently transfected with cDNA encoding full-length HSulf-1 in the antisense orientation (HSulf-1 AS) or empty vector and stained with antibody 10E4.
fragmentation, when HSulf-1-transfected clones were drug-treated (data not shown). Two aspects of these results deserve particular emphasis. First, HSulf-1 by itself did not induce apoptosis but instead modulated the sensitivity of cells to other stimuli (Fig. 8, B–D). Second, higher expression of HSulf-1 correlated with somewhat higher induction of apoptosis (Fig. 8, B–D).

To confirm that this modulation of apoptosis reflected the sulfatase activity of HSulf-1, SKOV3 cells were transiently transfected with two truncation constructs and an active site mutant. Transfection with the C-terminal half of HSulf-1 (C-Sulf) had little effect on the ability of staurosporine and UNC-01 to induce apoptosis (Fig. 8E). In contrast, an N-terminal fragment containing the entire sulfatase domain (N-Sulf) enhanced the ability of staurosporine to induce apoptosis. Importantly, site-directed mutagenesis of the putative catalytic cysteines Cys<sup>87</sup> and Cys<sup>88</sup> in N-Sulf (Mut-N-Sulf) abolished the ability of HSulf-1 to modulate apoptosis (Fig. 8E), providing evidence that sulfatase activity is required for this modulation.

**DISCUSSION**

In the present experiments, we have examined the activity, localization, and function of HSulf-1, a recently cloned member of the HSPG sulfatase family. Earlier studies by Dhoot et al. (7) demonstrated that QSulf-1, the avian homolog of this enzyme, is a cell surface protein that modulates signaling by the HB-EGF

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**FIG. 6.** HSulf-1 modulates HB-EGF signaling but not EGF signaling. A, effect of HB-EGF on EGFR phosphorylation and downstream signaling. After the indicated cell lines were cultured in serum-free medium for 12 h, 100 ng/ml HB-EGF was added for 15 and 60 min. Whole cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and sequentially probed with antisera that recognize phospho-Tyr<sup>1068</sup>-EGFR, total EGFR, phosphorylated ERK1/2, or total ERK1/2. B, effect of EGF on EGFR phosphorylation and downstream signaling. After cells were cultured in serum-free medium for 12 h, 10 ng/ml heparin-independent EGF was added. Whole cell lysates were prepared and probed as in A. In contrast to the diminished and unsustainable EGFR phosphorylation observed in HSulf-1-expressing clones after treatment with HB-EGF, receptor phosphorylation and signaling were robust and sustained after EGF treatment.

**FIG. 7.** HSulf-1 modulates FGF-2 signaling but not heparinated FGF-2 signaling. A, signaling by FGF-2. Whole cell lysates were probed with anti-phospho-ERK1/2 and anti-ERK1/2 as described in Fig. 6. B, signaling by heparinated FGF-2. The indicated cell lines were cultured in serum-free medium for 8 h and then treated with 10 ng/ml heparinated FGF-2 for 15, 30, and 60 min. FGF-2 induced sustained phosphorylation of ERK1/2 in parental and vector-transfected cells but not HSulf-1-expressing clones. In contrast, heparinated FGF-2 induced sustained signaling in all four cell lines. C, an active sulfatase domain is required to modulate signaling. SKOV3 cells were transfected with cDNA encoding the N-terminal domain of HSulf-1 (Fig. 1C), the C-terminal domain, or an N-terminal domain containing cysteine-alanine mutations of two conserved cysteines in the sulfatase domain (Mut-N-Sulf). After 12 h in serum-free medium, cells were incubated for 15 min with 10 ng/ml FGF-2. Phosphorylation of ERK1/2 was decreased in cells transfected with the N-Sulf construct but not the mutant construct.
Wnt during quail muscle cell differentiation. Our studies extend this work by showing that HSulf-1 is a cell surface polypeptide that diminishes HSPG sulfation, inhibits signaling by heparin-dependent growth factors, diminishes proliferation, and facilitates apoptosis in response to exogenous stimuli. Further experiments have shown that HSulf-1 expression is diminished in a variety of cancer cell lines and in clinical cancer specimens. These observations provide new insight into the potential importance of HSPG sulfation and its regulation.

While these studies were in progress, Morimoto-Tomita et al. (8) reported cloning of the HSulf-1 cDNA and gene. Our analysis has extended this previous report by demonstrating the presence of two previously unrecognized upstream exons (Table III), both of which appear to be included in transcripts from a variety of human cell lines and cells.

Further studies by Morimoto-Tomita et al. (8) indicated that HSulf-1 exhibits sulfatase activity with a preference for glucosamine 6-sulfate substrates. Interestingly, transfection of Myc-tagged HSulf-1 into Chinese hamster ovary cells resulted in secretion of the protein into the culture medium rather than retention in or on the cells (8). In our experiments, HSulf-1 fused to GFP or tagged with the FLAG epitope was transfected into ovarian cancer cell lines that express undetectable amounts of endogenous HSulf-1 message. These experiments not only confirmed the induction of sulfatase activity (Fig. 3) but demonstrated desulfation of cell surface HSPGs upon HSulf-1 re-expression (Fig. 5). As was the case with QSulf-1 (7), however, our localization studies suggested that a substantial fraction of HSulf-1 remains associated with the cell surface. In particular, HSulf-1/EGFP was detected by immunoblotting in whole cell lysates but not 50-fold concentrated conditioned medium (Fig. 4). Moreover, both HSulf-1/EGFP and FLAG-tagged HSulf-1 were localized to the cell surface by confocal microscopy (Fig. 4). Why these results differ from those of

Fig. 8. HSulf-1 modulates proliferation and apoptosis. A, effect of HSulf-1 expression on proliferation. The indicated cell lines were plated at 100,000 cells/dish in triplicate and examined daily after trypan blue staining to determine the number of viable cells/plate. B, effect of HSulf-1 expression on induction of apoptosis by cisplatin (CDDP) in SKOV3 clones. The indicated SKOV3 lines were treated with 5 μM cisplatin for 24 h and then stained with DAPI. Representative micrographs are shown in the schematic. Bars indicate the percentage of total cells that exhibit nuclear condensation and fragmentation typical of apoptosis. C, effect of HSulf-1 expression on cisplatin-induced apoptosis in OV207 clones. The indicated clones were treated for 24 h with 10 μM cisplatin before apoptosis was assessed by DAPI staining. D, effect of HSulf-1 expression on induction of apoptosis by staurosporine and UCN-01. The indicated SKOV3 lines were treated for 5 h with 1 μM staurosporine or UCN-01 prior to DAPI staining and analysis as depicted in B. E, requirement for an active sulfatase domain to modulate apoptosis. 24 h after transient transfection of empty vector or cDNA encoding N-Sulf, C-Sulf, and mutated (C87A,C88A) N-Sulf, cells were treated with 1 μM staurosporine or UCN-01 for 5 h before staining with DAPI and analysis as depicted in B.
Loss of HSulf-1 in Cancer

Morimoto-Tomita et al. (8) is at present unclear but might reflect the transfection of different cell lines or the use of different epitope tags.

Previous SSH analysis of two early and late stage tumors subtracted against normal ovarian epithelial brushings identified HSulf-1 as an mRNA that was diminished in several ovarian cancers compared with the cell of origin of these neoplasms (9). We have extended these studies by showing that HSulf-1 mRNA is markedly diminished or absent from 5 of 7 ovarian cancer cell lines and 23 of 30 epithelial ovarian cancers (Fig. 2). Further analysis has demonstrated that the HSulf-1 locus is subject to allelic loss in 50–60% of ovarian cancers (Fig. 2D). As indicated in Table II, the lack of detectable HSulf-1 expression was particularly common in clear cell ovarian cancers. This variant accounts for ~10% of all epithelial ovarian cancers, but these tumors have a worse prognosis and are resistant to platinum-based chemotherapy (36, 46, 47). Interestingly, HSulf-1 expression was undetectable in the clear cell cancers even though all four of the tumors analyzed were early stage tumors. Equally important, analysis of a series of cancer cell lines demonstrated that HSulf-1 down-regulation is common in hepatocellular, renal, pancreatic, and breast carcinomas (Fig. 2E). This early and apparent widespread occurrence of HSulf-1 down-regulation raises the possibility that HSulf-1 down-regulation might be important in the carcinogenic process.

In order to examine the functional consequences of HSulf-1 down-regulation, we re-expressed HSulf-1 in two ovarian cancer cell lines lacking detectable message. Studies of Dhoot et al. (7) previously implicated QSulf-1 in the regulation of Wnt signaling and alluded to the possibility that QSulf-1 might also potentially regulate FGF signaling, which is controlled by 6-O-sulfation of N-acetylgalcosamine in HSPGs (4). Based on the similarity of HSulf-1 to QSulf-1, we hypothesized that HSulf-1 down-regulation might modulate HB-EGF signaling. Consistent with this possibility, we observed altered signaling by HB-EGF in cells after HSulf-1 re-expression (Fig. 6). In particular, we observed diminished HB-EGF-induced phosphorylation of at least two important sites on the EGFR, Tyr1068, a binding site for Grb2 (32), and Tyr992, a binding site for phospholipase C-γ (33). In previous studies (48), the interaction of HB-EGF with cell surface HSPGs was indirectly demonstrated by showing that sodium chlorate, which inhibits HS-GAG sulfation, and heparinase abrogate HB-EGF signaling. A subsequent report (49) indicated that 125I-HB-EGF binding is diminished in Chinese hamster ovary cells deficient in HSPG. The present studies extend these earlier results by demonstrating that HSulf-1 re-expression, which diminishes cell surface HS-GAG sulfation (Fig. 5), interferes with HB-EGF signaling through the EGFR (Fig. 6). Consistent with these results, we observed diminished HB-EGF-induced activation of the ERK pathway (Fig. 6A). Importantly, these results were not seen with EGF (Fig. 6B), which lacks an HSPG binding domain and is thought to signal independent of the HS-GAGs.

The effect of HSulf-1 re-expression was not limited to HB-EGF. A variety of previous studies has demonstrated that FGF-2 binds to its receptor and to HSPGs, which act as coreceptors and promote formation of a ternary complex that is essential for cell proliferation and angiogenesis (50, 51). As indicated in the Introduction, previous reports have also demonstrated that sulfation of specific sites on the HS-GAGs is critical for this interaction. This interaction of FGF-2 with FGFR and HS-GAGs leads to receptor dimerization, activation, and autophosphorylation followed by activation of downstream signaling pathways, including the mitogen-activated protein kinase pathway (4, 37). Our experiments demonstrated that HSulf-1 also modulated FGF-2 signaling. In particular, FGF-2 induced sustained ERK phosphorylation in cells lacking detectable HSulf-1 expression but only transient ERK phosphorylation at 15 min in the HSulf-1 transfected clones (Fig. 7A). These observations are consistent with a recent report that chlorate treatment, which inhibits GAG sulfation, results in cells that exhibit a transient early (15 min) phosphorylation of ERK in response to FGF-2 but no sustained ERK phosphorylation (37). Once again the modulation of signaling by HSulf-1 was not observed when cells were treated with heparinized FGF-2 (Fig. 7B), which would be expected to signal independent of HS-GAGs. In summary, our results indicate that HSulf-1 transfection abrogates the sustained activation of ERK1 and ERK2 required for cell survival and proliferation.

Because sustained ERK phosphorylation has been implicated in proliferation (52) as well as resistance to apoptosis (22, 53, 54), we next examined the effect of HSulf-1 re-expression on these processes. Results of this analysis demonstrated that HSulf-1 re-expression diminished proliferation of SKOV3 and OV207 clones (Fig. 8A and data not shown). Moreover, HSulf-1 re-expression enhanced the sensitivity of these cells to a number of pro-apoptotic stimuli, including cisplatin, which is widely used to treat ovarian cancer (55), and staurosporine, a broadly active kinase inhibitor that is widely employed as a stimulus of the mitochondrial apoptotic pathway (44, 56). These results are consistent with previous studies (57, 58) demonstrating ERK phosphorylation and ability of the Mek1/2 inhibitor PD98059 to modulate cisplatin sensitivity in ovarian cancer cells. Further analysis demonstrated that an N-terminal fragment containing an intact sulfatase domain is responsible for this HSulf-1-induced modulation of apoptosis (Fig. 8E). Because HSulf-1 modulates sensitivity of cells to induction of apoptosis by cisplatin, which is considered the single most active agent currently available for the treatment of ovarian cancer, future studies to evaluate the relationship between HSulf-1 expression and clinical response in ovarian cancer appear to be warranted.

Although there is ample evidence that differences in the sulfation state of HS-GAGs introduced by altering sulfotransferase activity can modulate growth factor signaling in vitro (35, 59–62), there is little precedent for the idea that variations in sulfatase activity can alter survival signaling in human cancer. The functional results described above suggest that

### Table III

Genomic structure of HSulf-1

| Exon | Amino acids | Size | Intron size |
|------|-------------|------|-------------|
| 1    | 5′-UTR      | 314  | 22,939      |
| 1A   | 5′-UTR      | 284  | 2917        |
| 2    | 5′-UTR      | 165  | 5994        |
| 3    | 5′-UTR      | 95   | 61,570      |
| 4    | 5′-UTR      | 73   | 304         |
| 5    | 5′-UTR, 1–57| 232  | 11,821      |
| 6    | 58–137      | 240  | 10,147      |
| 7    | 138–188     | 152  | 2463        |
| 8    | 189–257     | 170  | 11,461      |
| 9    | 258–295     | 151  | 900         |
| 10   | 296–354     | 176  | 1382        |
| 11   | 355–397     | 129  | 277         |
| 12   | 398–416     | 57   | 1146        |
| 13   | 417–459     | 130  | 16,102      |
| 14   | 460–531     | 217  | 2690        |
| 15   | 532–617     | 256  | 3012        |
| 16   | 618–649     | 97   | 465         |
| 17   | 650–681     | 95   | 304         |
| 18   | 682–703     | 66   | 1267        |
| 19   | 704–761     | 176  | 8822        |
| 20   | 762–809     | 143  | 90          |
| 21   | 810–850     | 124  | 1949        |
| 22   | 851–862     | 34   | 17,656      |
| 23   | 863–893 3′-UTR | 2408 |             |
HSulf-1 is a cell surface enzyme that desulfates critical sulfated moieties in HS-GAG, thereby inhibiting the action of heparin-dependent growth factors at their receptors and diminishing their ability to activate the ERK pathway. Because expression of HSF1 is undetectable or markedly attenuated in >75% of ovarian cancers relative to normal ovarian surface epithelium, these observations appear to outline a novel mechanism by which proliferative and anti-apoptotic signaling by heparin-dependent growth factors can be augmented during the process of carcinogenesis.

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Additions and Corrections

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Loss of HSulf-1 up-regulates heparin-binding growth factor signaling in cancer.

Jinping Lai, Jeremy Chien, Julie Staub, Rajeswari Avula, L. Eddie Greene, Tori A. Matthews, David I. Smith, Scott H. Kaufmann, Lewis R. Roberts, and Viji Shridhar

Page 23107: The footnote indicating grant support was incomplete. The first sentence should read:

“This work was supported by Department of Defense Grant DAMD17-99-1-9504 (to V. S., D. I. S., and S. H. K.), a John W. Anderson Foundation grant (to V. S.), National Institutes of Health Grant CA82862 (to L. R. R.), an Industry Research Scholar award from the Foundation for Digestive Health and Nutrition (to L. R. R.), and the Mayo Foundation.”

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Constitutive and agonist-dependent self-association of the cell surface human lutropin receptor.

Ya-Xiong Tao, Nathan B. Johnson, and Deborah L. Segaloff

Page 5911, Fig. 10: The labeling in panel B of this figure is incorrect. A corrected figure is shown below:

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.