Organ-specific Sulfation Patterns of Heparan Sulfate Generated by Extracellular Sulfatases Sulf1 and Sulf2 in Mice*§

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Satoshi Nagamine1,2, Michiko Tamba3, Hisako Ishimine4, Kota Araki5, Kensuke Shiomii, Takuya Okada1, Tatsuyuki Ohto6, Satoshi Kunita7, Satoru Takahashi1, Ronnie G. P. Wismans8, Toin H. van Kuppevelt7, Masayuki Masu3, and Kazuko Keino-Masu1,4

From the 1Department of Molecular Neurobiology, 2Department of Pediatrics, Faculty of Medicine, and the 3Laboratory Animal Resource Center, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan and the 4Department of Matrix Biochemistry, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands

Background: Extracellular endosulfatases Sulf1 and Sulf2 hydrolyze 6-O-sulfate in heparan sulfate.

Results: Disaccharide analysis showed that 2-O-, 6-O-, and N-trisulfated disaccharide units in heparan sulfate were increased to different degrees in different organs in Sulf1 and Sulf2 knock-out mice. These findings indicate that Sulf1 and Sulf2 differentially contribute to the generation of organ-specific sulfation patterns of heparan sulfate.

Significance: This may indicate differences in activity between Sulf1 and Sulf2 in vivo.

Heparan sulfate endosulfatases Sulf1 and Sulf2 hydrolyze 6-O-sulfate in heparan sulfate, thereby regulating cellular signaling. Previous studies have revealed that Sulfs act predominantly on UA2S-GlcNS6S disaccharides and weakly on UA-GlcNAc6S disaccharides. However, the specificity of Sulfs and their role in sulation patterning of heparan sulfate in vivo remained unknown. Here, we performed disaccharide analysis of heparan sulfate in Sulf1 and Sulf2 knock-out mice. Significant increases in ΔUA2S-GlcNS6S were observed in the brain, small intestine, lung, spleen, testis, and skeletal muscle of adult Sulf1−/− mice and in the brain, liver, kidney, spleen, and testis of adult Sulf2−/− mice. In addition, increases in ΔUA-GlcNS6S were seen in the Sulf1−/− lung and small intestine. In contrast, the disaccharide compositions of chondroitin sulfate were not primarily altered, indicating specificity of Sulfs for heparan sulfate. For Sulf1, but not for Sulf2, mRNA expression levels in eight organs of wild-type mice were highly correlated with increases in ΔUA2S-GlcNS6S in the corresponding organs of knock-out mice. Moreover, overall changes in heparan sulfate compositions were greater in Sulf1−/− mice than in Sulf2−/− mice despite lower levels of Sulf1 mRNA expression, suggesting predominant roles of Sulf1 in heparan sulfate desulfation and distinct regulation of Sulf activities in vivo. Sulf1 and Sulf2 mRNAs were differentially expressed in restricted types of cells in organs, and consequently, the sulfation patterns of heparan sulfate were locally and distinctly altered in Sulf1 and Sulf2 knock-out mice. These findings indicate that Sulf1 and Sulf2 differentially contribute to the generation of organ-specific sulfation patterns of heparan sulfate.

Heparan sulfate (HS) is a long linear carbohydrate chain covalently attached to the core proteins of proteoglycans (1–7). It consists of repeating disaccharide units each composed of an uronic acid (UA); glucuronic acid (GlcA) or iduronic acid and a glucosamine (N-acetylgalactosamine (GlcNAc), N-sulfated glucosamine (GlcNS), or unsubstituted glucosamine). Each disaccharide has potential sulfation at the 2-O-position of UA and the 3-O-, 6-O-, or N-positions of glucosamine (1–7). Because the potential sites are not necessarily all sulfated, HS chains

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1 Both authors contributed equally to this work.

2 Present address: Dept. of Neurology, Tokyo Metropolitan Neurological Hospital, 2-6-1 Murashidai, Fuchu, Tokyo 183-0042, Japan.

3 To whom correspondence may be addressed. Tel.: 81-29-853-3249; Fax: 81-29-853-3498; E-mail: mmasu@md.tsukuba.ac.jp.

4 To whom correspondence may be addressed. Tel.: 81-29-853-3249; Fax: 81-29-853-3498; E-mail: kazumasa@md.tsukuba.ac.jp.
show enormous structural heterogeneity. Typically, HS contains low sulfated regions rich in GlcNAc (NA domain), highly sulfated regions containing contiguous GlcNS (NS domain), and transition zones that contain alternating GlcNAc and GlcNS units (NA/NS domain). After the synthesis of a GlcA-GlcNAc disaccharide polymer, N-deacetylase/N-sulfotransferases and HS 2-O-, 3-O-, and 6-O-sulfotransferases add sulfate groups to specific sites in the sugar backbone to form complex sulfation patterns (1–7). HS binds to growth factors, enzymes, receptors, and extracellular matrix molecules, thereby regulating many biological processes (1–7). Previous biochemical and genetic studies have shown that specific sulfation patterns of HS are important for the binding to and signaling of these bioactive molecules. Furthermore, distinct sulfation patterns in different tissues at different developmental stages and in different pathological conditions have potential roles in the regulation of cellular signaling (1–7).

Extracellular sulfatases, sulfatase 1 (Sulf1), and sulfatase 2 (Sulf2) catalyze hydrolysis of the sulfate ester bond at the C6 position of glucosamine residues in heparin and HS (8–13). By removing 6-O-sulfates in HS, Sulf1 and Sulf2 activate Wnt, Shh, bone morphogenetic protein (BMP), and glial cell line-derived neurotrophic factor (BDNF) and attenuate the signaling of FGF, VEGF, hepatocyte growth factor (HGF), and heparin-binding EGF-like growth factor (HB-EGF) (8, 11, 14–21). Therefore, Sulf1 and Sulf2 are thought to be key regulators of cell proliferation, differentiation, and migration and are also implicated in cancer progression and metastasis (22, 23). Biochemical studies have demonstrated that Sulf1 and Sulf2 are most active in the UA2S-GlcNS6S trisulfated disaccharide unit, which is present in the NS domain (9, 11, 13, 16, 24, 25). In addition, weaker 6-O-desulfation activity is also detectable in the UA-GlcNS6S-disulfated disaccharide unit (11, 24, 25). Subsequent disaccharide analysis of embryonic fibroblasts from Sulf1 and Sulf2 knock-out mice revealed changes suggestive of the specificity of Sulfs toward UA2S-GlcNS6S and UA-GlcNS6S disaccharide units (26). However, how many changes, if any, occur in the disaccharide compositions of HS in vivo and whether such changes occur to differing degrees in different organs remain unknown.

The physiological roles of Sulfs in vivo have been tested by targeted disruption of Sulf genes. Neither Sulf1- nor Sulf2-deficient mice showed obvious abnormalities despite abundant expression of Sulf1 and Sulf2 mRNA in embryonic and adult tissues and the crucial roles HS plays in development and in organ physiology (20, 28, 29). In contrast, double knock-out mice showed neonatal lethality associated with subtle skeletal abnormalities and kidney hypoplasia (20, 28, 29). Defects in esophageal innervation, muscle regeneration, and spermatogenesis were also reported in Sulf1/2 double knock-out mice (20, 30, 31). Recently, by using Sulf1/2 double knock-out mice that survived to adulthood (probably due to differences in genetic background), it was reported that aged double knock-out mice developed proteinuria and showed abnormal renal morphology (32).

In this study we performed systematic disaccharide analysis of HS and chondroitin sulfate (CS) from eight organs of adult Sulf1 and Sulf2 knock-out mice. We also determined the expression of Sulf1 and Sulf2 mRNA by using RT-PCR and in situ hybridization. These analyses revealed changes in HS disaccharide composition in each organ and their relationship with Sulf mRNA expression levels in wild-type mice. Our data provide evidence that Sulf1 and Sulf2 contribute differentially to the generation of organ-specific sulfation patterns of HS in vivo.

**Experimental Procedures**

**Materials**—Unsaturated HS/HEP-disaccharide mixture (HMix), unsaturated Chondro-disaccharide kit (C-Kit), heparin lyase II (heparitinase II; *Flavobacterium heparinum*), heparin lyase III (heparitinase I; *F. heparinum*), chondroitinase ABC (*Proteus vulgaris*), chondroitinase ACII (*Arthrobacter aureus*), hylauronidase (*Streptomyces hyaloideus*), CS-A (sturgeon notochord), CS-A (whale cartilage), CS-B (pig skin), CS-C (shark cartilage), CS-D (shark cartilage), CS-E (squid cartilage), and hyaluronic acid (pig skin) were purchased from Seikagaku Biobusiness (Tokyo, Japan). Two standard unsaturated HS disaccharides (UAUA2S-GlcNAc and UAUA2S-GlcNAc6S6), which are not included in the HMix, were purchased from Dextra Laboratories (Reading, UK). Heparin lyase I (heparitinase I; *F. heparinum*), protease type X (S. griseus), and benozonase were purchased from Sigma. Tetra-n-butylammonium hydrogen sulfate and 2-cyanoacetamide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Generation of Sulf-deficient Mice**—Gene targeting vectors were constructed by inserting the mouse genomic DNA fragments flanking exon 5 of Sulf1 or Sulf2 into a TC3 vector (a gift from R. Kageyama) that contained a cassette of stop-ires-lacZ-poly(A), a neomycin-resistant gene, and the diphtheria toxin A fragment gene (supplemental Fig. S1). The linearized targeting vectors were electroporated into 129/Ola-derived E14 ES cells, and neomycin-resistant colonies were selected. Recombinants were identified by PCR, and the correct homologous recombination was then confirmed by Southern blotting. The ES cells obtained were injected into C57BL/6N (CLEA Japan, Tokyo, Japan) blastocysts, and chimeric mice were mated with wild-type C57BL/6N mice. Offspring of mice backcrossed to C57BL/6N for 5 successive generations (N5 generation) were used. Genotyping was done by PCR using primer sets of T-GTC TGT CCA TCA CGC TCA TCC ATG-3′ and T-ACC ATC AGG CGA GGG ACTT TTG TG TC-3′ for Sulf1 and T-GCT TGG TAA GCC GGC ACA AG-3′ and T-GAG CTG ATG TGG GTT TGC TG-3′ for Sulf2 in combination with a neo primer (5′-GCC TAC CCG GTA GAA TTC GAT ATC-3′). All the experiments using animals were approved by the Animal Care and Use Committee of the University of Tsukuba and performed under its guidelines.

**Extraction of Glycosaminoglycans**—After induction of deep anesthesia by intraperitoneal injection of pentobarbital, 8–10-week-old mice were transcardially perfused with phosphate buffered saline (PBS) to remove blood cells. The brain, lung, liver, spleen, small intestine, kidney, testis, and muscle were isolated and weighed. The organs were then subjected to 3 cycles of homogenization in cooled acetone and centrifugation (2000 × g for 30 min at 4 °C). The precipitates were dried.
and treated with 10× the volume of the protease solution (0.8 mg/ml protease type XVI from *S. griseus* in 50 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, 1% Triton X-100, 0.1% BSA) at 55 °C overnight. After heat inactivation of the protease at 95 °C for 5 min, the solutions were treated with 125 units of Benzonase in the presence of 2 mM MgCl₂ at 37 °C for 2 h. After heat inactivation (95 °C for 2 min) and centrifugation (20,000 × g for >30 min at 4 °C), the supernatants were filtered with Ultrafree-MC (0.22 μm; Millipore), vacuum-dried, suspended in 10 mM ammonium bicarbonate (pH 6.0, 0.1% BSA) at 37 °C overnight. After heat inactivation (95 °C for 2 min) and centrifugation (20,000 × g for >30 min at 4 °C), the supernatants were filtered with Ultrafree-MC Biomax-5 spin columns (5,000 nominal molecular weight limit; Millipore, Billerica, MA) and purified with an anion-exchange column (Vivapure D Mini M; Sartorius, Göttingen, Germany). The eluates were desalted and concentrated using Ultrafree-MC Biomax-5 spin columns. The retained solution was vacuum-dried and suspended in 10 μl of H₂O.

**Heparin and Chondroitin Lyase Digestion**—For HS analysis, 8 μl of the purified glycosaminoglycans was treated with heparinase I (0.5 units), heparitinase I (1 mIU), and heparitinase II (1 mIU) in 15 μl of a digestion buffer (30 mM sodium acetate, pH 7.0, 3 mM calcium acetate, 0.1% BSA) at 37 °C overnight. For CS analysis, 2 μl of the purified glycosaminoglycans was treated with chondroitinase ABC (50 mIU) and chondroitinase ACII (50 mIU) in 15 μl of a digestion buffer (300 mM Tris acetate, pH 8.0, 0.1% BSA) at 37 °C overnight. In some experiments, for removal of hyaluronic acid, the glycosaminoglycans were treated with hyaluronidase (500 Turbidity Reducing Unit (TRU)) in 20 μl of a digestion buffer (30 mM phosphohosphate buffer, pH 6.0, 0.1% BSA) at 37 °C overnight. After heat inactivation at 95 °C for 2 min, the digested materials were treated with Ultrafree-MC Biomax-5 spin columns (5,000 nominal molecular weight limit; Millipore), vacuum-dried, suspended in 10 μl of H₂O, and subjected to CS analysis.

**Ion-pair Reversed-phase Chromatography**—Unsaturated disaccharides produced by the enzymatic digestions were analyzed by ion-pair reversed-phase chromatography as described above. A gradient was applied at a flow rate of 1.1 ml/min on a Senshu Pak Docosil column (4.6 × 150 mm, particle size 5 μm; Senshu Scientific, Tokyo, Japan) at 55 °C using an HPLC system ( Alliance 2695 Separations Module; Waters Corporation, Milford, MA). The eluents used were as follows: A, H₂O; B, 0.2 M NaCl; C, 10 mM tetra-n-butylammonium hydrogen sulfate; D, 50% acetonitrile. The gradient program was as follows: 0–10 min, 1–4% eluent B; 10–11 min, 4–15% eluent B; 11–20 min, 15–25% eluent B; 20–22 min, 25–53% eluent B; 22–29 min, 53% eluent B; equilibration with 1% B for 20 min. The proportions of eluents C and D were constant at 12 and 17%, respectively. Aqueous 0.5% (w/v) 2-cyanoacetamide solution and 0.25 M sodium hydroxide were added to the effluent at the same flow rate of 0.35 ml/min using Reagent Managers (Waters). The mixtures were passed through a reaction coil kept at 125 °C using a dry reaction temperature-controlled bath (Post-Column Reaction Module; Waters) and Temperature Control Module II (Waters). The effluent was fluorometrically monitored using a multiwavelength fluorescence detector (Waters 2475 detector: excitation 346 nm, emission 410 nm; Waters). Disaccharide peaks were identified and quantified by comparison with authentic unsaturated disaccharide markers. The chromatograms were analyzed using Empower 2 software (Waters).

**Statistical Analysis**—Statistical significance of the differences in the disaccharide compositions between the control and single knock-out mice was analyzed using Student’s *t* test. First, the *F* test was used to determine whether the variances between the two groups were equal. When the variances were equal (*p* > 0.05), an unpaired form of the *t* test was used. When the variances were unequal (*p* < 0.05), Welch’s *t* test was used. To analyze the differences among three or more groups, analysis of variance was performed using PRISM software (GraphPad Software, La Jolla, CA).

**RT-PCR**—Expression of *Sulf1* and *Sulf2* mRNA in adult mouse organs was determined using quantitative RT-PCR. After decapsulation, the brain, lung, liver, spleen, intestine, kidney, testis, and muscle were dissected. Total RNAs were extracted using Sepasol I (Nacalai Tesque, Kyoto, Japan) and purified using an RNaseasy kit (Qiagen, Hilden, Germany). Total RNAs (5 μg) were subjected to reverse-transcription using oligo(dT)₁₂–₁₈ and Superscript II (Invitrogen). Quantitative PCR was carried out using a LightCycler and LightCycler Fast-Start DNA Master SYBR Green I reagent (Roche Diagnostics). The copy number of each cDNA in the RT solution was determined using standard template DNAs of the predetermined concentrations. Expression levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. The primers used in this study were as follows: *Sulf1* forward, 5′-CCA TGC TCA CTG GGA AGT ACG TG 3′; *Sulf1* reverse, 5′-CTT CCT CTT GAT GCC GTT GCG A-3′; *Sulf2* forward, 5′-AGT GGG TCG GCC TAC TTA AGA ACT C-3′; *Sulf2* reverse, 5′-ATA GAT CGT CT CTC ATG GCA GTA A-3′; *Gapdh* forward, 5′-CAA TGT GTC GTG CCT GGT GAC-3′; *Gapdh* reverse, 5′-CTG TTG AAG TCG CAG GAG GAC ACC-3′.

**Endosulfatase Assay**—Endosulfatase activities were measured essentially as described previously (34). The 293EBNA cells (Invitrogen) were transfected with pCEP4-*Sulf1-FLAG* or pCEP4-*Sulf2-MycHis* with pCEP4-*Sumf1* using Lipofectamine 2000 (Invitrogen). After the cells were cultured in Opti-MEM I (Invitrogen) without fetal bovine serum for 3 days, the conditioned medium was concentrated 30-fold using a Microcon YM-30 filter (Millipore). To measure HS endosulfatase activity, the concentrated conditioned medium (5 μl) was incubated with 10 μg of heparin in a total volume of 10 μl of 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 10 mM MgCl₂ at 37 °C for 24 h. The mixture was heated at 95 °C for 2 min and then incubated with 1 μlU heparinase I, 1 μlU heparitinase I, and 1 μlU heparitinase II in 10 μl of 40 mM HEPES-NaOH, pH 7.0, and 2 mM calcium acetate at 37 °C for 24 h. To measure CS endosulfatase activity, the concentrated conditioned medium (5 μl) was incubated with 10 μg of heparin in a total volume of 10 μl of 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 10 mM MgCl₂ at 37 °C for 24 h. The mixture was heated at 95 °C for 2 min and then incubated with 1 μlU heparinase I, 1 μlU heparitinase I, and 1 μlU heparitinase II in 10 μl of 40 mM HEPES-NaOH, pH 7.0, and 2 mM calcium acetate at 37 °C for 24 h. To measure CS endosulfatase activity, CS-A, CS-B, CS-C, CS-D, or CS-E was incubated with the concentrated conditioned medium and subsequently subjected to digestion by chondroitinase ABC and chondroitinase ACII. After the digestion was stopped by heating at 95 °C for 2 min and the mixture cleaned using an Ultrafree-MC filter (Millipore), unsaturated disaccharides were analyzed by ion-pair reversed-phase chromatography as described above.

**In Situ Hybridization**—*In situ* hybridization was performed essentially as described previously (10). After induction of deep anesthesia, male mice were transcardially perfused with 4% paraformaldehyde in PBS. Dissected organs were incubated in...
the same fixative and subsequently in 30% sucrose, PBS at 4 °C overnight. After being embedded in OCT compound (Sakura Finetek, Tokyo, Japan), 10-μm-thick sections were cut using a cryostat (CM1850; Leica Microsystems, Wetzlar, Germany). For the lung, snap-frozen tissues were used. The sections were hybridized with 1 μg/ml digoxigenin-labeled antisense RNA probe for Sulf1 or Sulf2 in a hybridization solution (50% formamide, 5× SSC, pH 4.5, 1% SDS, 50 μg/ml heparin, 50 μg/ml yeast RNA) at 65 °C for 16 h. The slides were washed with 50% formamide, 5× SSC, and 1% SDS at 65 °C for 30 min, with 50% formamide and 2× SSC at 65 °C for 30 min 3 times, and with Tris-HCl, pH 7.6, containing 0.8% NaCl, 0.02% KCl, and 0.1% Tween 20 (TBST) at room temperature for 5 min 3 times. After blocking with 0.5% blocking reagent (Roche Diagnostics) in TBST at room temperature for 1 h, the slides were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; Roche Diagnostics) in 0.5% blocking reagent in TBST at 4 °C for 16 h. After washing with TBST for 20 min 3 times and with 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2, and 0.1% Tween 20 (TBST) at 4 °C for 16 h, the slides were incubated with anti-Myc (1:200; Cell Signaling Technology, Danvers, MA) or anti-VSV-G antibodies (1:200; MBL, Nagoya, Japan) for 60 min. Finally, the slides were incubated with Alexa568-conjugated anti-rabbit IgG (Invitrogen) for 60 min and mounted with coverslips using Fluoromount-G (Southern-BioTech, Birmingham, AL). The images were obtained by means of laser confocal microscopy (LSM510; Carl Zeiss, Jena, Germany). To compare the signal intensities among the different samples, the parameters for image acquisition were kept constant.

RESULTS

Disaccharide Compositions of HS in Adult Mouse Organs—To examine the disaccharide compositions of HS in vivo, we performed disaccharide analysis of HS in mouse organs (33). Crude extracts of glycosaminoglycans were prepared from the brain, lung, liver, small intestine, kidney, spleen, testis, and skeletal muscle of 8–10-week-old male mice. The extracts were exhaustively digested with a mixture of heparin lyases and subjected to ion-pair reversed-phase HPLC (33). This enzyme treatment yielded eight different unsaturated disaccharides (Fig. 1A), one unsulfated disaccharide (ΔUA-GlcNAc), three monosulfated disaccharides (ΔUA-GlcNS, ΔUA2S-GlcNC, and ΔUA-GlcNaC6S), three disulfated disaccharides (ΔUA2S-GlcNS, ΔUA-GlcNaC6S, and ΔUA2S-GlcNaC6S), and one trisulfated disaccharide (ΔUA2S-GlcNS6S). The unsaturated disaccharides were fluorometrically detected after post-column reaction with 2-cyanoacacetamide. The compositions of the eight unsaturated disaccharides were compared with the unsaturated HS disaccharide standards and thus quantitatively determined. 3-O-Sulfated disaccharide units were not detected because they were resistant to heparin lyase digestion. This method allowed sensitive and accurate determination of the sulfation patterns of HS in vivo.

The compositions of the HS disaccharides from eight organs of the wild-type mice were different according to the organ (Fig. 1B, Table 1). The percentages were relatively high (>10%) for ΔUA2S-GlcNS6S in the liver (16.7%) and kidney (10.7%), for ΔUA-GlcNS6S in the spleen (14.9%) and kidney (10.7%), for ΔUA2S-GlcNS in the lung (17.2%), brain (14.7%), and testis (13.7%), and for ΔUA-GlcNaC6S in the spleen (12.6%), kidney (12.2%), and liver (10.1%). These sulfation profiles of HS concur well with those previously reported in mouse and bovine organs (37–40).

Disaccharide Compositions of HS in Sulf Knock-out Mice—To examine the roles of Sulf genes in generating sulfation patterns of HS in vivo, we compared the disaccharide compositions of HS in wild-type and Sulf knock-out mice (Fig. 1C, data not shown). The percentages of trisulfated disaccharide ΔUA2S-GlcNS6S were significantly higher in the brain, small intestine, lung, spleen, testis, and skeletal muscle of Sulf1 knock-out mice (Table 1, Fig. 2A). Concomitantly, ΔUA2S-GlcNS decreased significantly in the brain, small intestine, lung, testis, and skeletal muscle of Sulf1 knock-out mice (Table 1). In addition, a statistically significant increase in ΔUA-GlcNaC6S was observed in the lung and small intestine of Sulf1 knock-out mice, and a significant increase was observed in ΔUA-GlcNaC6S in the small intestine (Table 1, Fig. 2A). Similar changes in the HS profiles were observed in Sulf2 knock-out mice, but the degree of the changes was smaller than in Sulf1 knock-out mice. A significant increase in ΔUA2S-GlcNS6S was observed in the brain, liver, kidney, spleen, and testis, whereas a significant decrease in ΔUA2S-GlcNS was observed in the liver, kidney, and spleen (Table 1, Fig. 2B). No increase in ΔUA-GlcNaC6S or ΔUA-GlcNaC6S was observed in Sulf2 knock-out mice (Table 2, Fig. 2B).

We also examined the disaccharide compositions of HS in Sulf knock-out mice. Exhaustive digestion of glycosaminoglycans with chondroitinase ABC and chondroitinase ACII yielded six CS unsaturated disaccharides (supplemental Fig. S2A), one unsulfated disaccharide (ΔDi-0S (ΔUA-GalNAc)), two monosulfated disaccharides (ΔDi-4S (ΔUA-GalNAc4S), ΔDi-6S (ΔUA-GalNAc6S)), two disulfated disaccharides (ΔDi-diS (ΔUA2S-GalNAc6S), ΔDi-diS (ΔUA-GalNAc6S)), and one trisulfated disaccharide (ΔDi-triS (ΔUA2S-GalNAc4S6S)). The eight organs showed unique CS disaccharide patterns (supplemental Fig. S2B). Quantitative comparison of the CS disaccharides between the Sulf1 or Sulf2 knock-out mice and the wild-type mice revealed a significant increase in ΔDi-6S and ΔDi-diS in some organs of the Sulf1 knock-out mice (supplemental Tables S1 and S2), but these changes appeared to be due to secondary effects, as discussed below.

Correlation between Sulf mRNA Expression and HS Sulfation Profiles—Our data indicate that the increase in ΔUA2S-GlcNS6S induced by Sulf gene disruption is large in organs
Sulfation Patterns of Heparan Sulfate in Sulf Knockout Mice

We first determined the Sulf mRNA expression in eight adult organs by quantitative RT-PCR. In each organ, Sulf1 mRNA in Sulf1 heterozygotes was about half that in wild-type mice and negligible in Sulf1 homozygotes, whereas Sulf2 mRNA in Sulf2 heterozygotes was about half that in wild-type mice and negligible in Sulf2 null mice (supplemental Fig. S3). In addition, Sulf1 mRNA levels were unchanged in the Sulf2 homozygotes except in the liver, whereas Sulf2 mRNA levels were unchanged in the Sulf1 homozygotes except in the liver (supplemental Fig. S3). In the liver, disruption of Sulf1 led to a 2.4-fold increase in Sulf2 mRNA (the effects of the Sulf1 gene disruption were compensated), whereas disruption of Sulf2 led to a 60% decrease in Sulf1 mRNA (the effects of the Sulf2 gene disruption were exaggerated). Such reciprocal regulation of Sulf expression may be attributable to the relatively small changes in ΔUA2S-GlcNS6S in the Sulf1-deficient liver and relatively large changes in ΔUA2S-GlcNS6S in the Sulf2-deficient liver.

Next we compared the levels of Sulf1 expression (normalized to Gapdh expression) in the wild-type mice and the increase in ΔUA2S-GlcNS6S in the Sulf1 knock-out mice in each organ. As shown in Fig. 3A, Sulf1 expression and ΔUA2S-GlcNS6S increase were proportional and highly correlated ($R = 0.88$). These findings indicate that high levels of Sulf1 mRNA expression lead to greater degrees of 6-O-desulfation. In contrast, no clear correlation was observed between Sulf2 expression and ΔUA2S-GlcNS6S increase in the Sulf2 knock-out mice (Fig. 3B). In this experiment we calculated the copy numbers of Sulf1 and Sulf2 mRNA, allowing the comparison of the absolute levels of Sulf1/2 mRNA expression. As shown in Fig. 3, the overall mRNA expression levels of Sulf2 were higher than those of Sulf1. However, the changes in ΔUA2S-GlcNS6S were smaller in the Sulf2 knock-out mice than in the Sulf1 knock-out mice. These results suggest that Sulf2 is less active in 6-O-desulfation of HS despite higher mRNA expression levels and that Sulf1 predominantly contributes to the generation of the sulfation patterns of HS in many adult organs.

Disaccharide Compositions of HS in Sulf Double Knock-out Mice—Given that both Sulf1 and Sulf2 have HS endosulfatase activity in vitro, they may be functionally redundant in vivo. To test this, we analyzed the disaccharide compositions of HS in Sulf1/2 double knock-out mice. Because the double knock-out mice die within 1 day of birth, we used neonatal mice. We analyzed the lung, liver, and kidney because these organs from 1 or 2 neonatal mice gave sufficient HS and CS for the disaccharide analysis. Significant increases in ΔUA2S-GlcNS6S were observed in the lung of Sulf1 single knock-out mice and in the lungs. The Sulf1/−/− lung contains higher ΔUA2S-GlcNS6S (peak 8) and lower ΔUA2S-GlcNS (peak 6) than those from wild-type controls. a.u., arbitrary units.
In the double knock-out mice, \( \text{UA2S-GlcNS} \) was significantly and more robustly increased in the lung, kidney, and liver as compared with in the single knock-out mice, indicating that \( \text{Sulf1} \) and \( \text{Sulf2} \) are redundant in vivo (Fig. 4A, Table 3). The percentages of \( \text{UA-GlcNAc6S} \) were increased in the lung of \( \text{Sulf1} \) single knock-out and \( \text{Sulf1} / \text{Sulf2} \) double knock-out mice (Fig. 4A, Table 3). However, contrary to the prediction made based on the lack of 6-O-desulfation activity in \( \text{Sulf} \) knock-out mice, the percentages of \( \text{UA-GlcNS6S} \) and \( \text{UA-GlcNAc6S} \) were decreased in the liver of the double knock-out mice (Fig. 4A, Table 3). These changes are not simply explained by the disruption of 6-O-endosulfatase activities and thus can be attributed to secondary changes induced by the disruption of \( \text{Sulf1} / \text{Sulf2} \) genes.

We next examined the sulfation patterns of CS. Disaccharide analysis of CS showed that \( \text{Di-diSE} \) increased in the lung of the \( \text{Sulf1} \) single knock-out and \( \text{Sulf1} / \text{Sulf2} \) double knock-out mice, whereas \( \text{Di-6S} \) increased in the lung of the double knock-out mice and the kidney of the \( \text{Sulf2} \) single knock-out mice (Fig. 4B, Table 3). These results may imply that \( \text{Sulfs} \) can act on 6-O-sulfated disaccharide units in CS. We thus examined whether \( \text{Sulf1} \) and \( \text{Sulf2} \) have 6-O-endosulfatase activity toward CS in vitro. In agreement with the results obtained in previous studies including ours (9, 11, 13, 34), when heparin or HS was incubated with a conditioned medium of cells transfected with \( \text{Sulf1} \) or \( \text{Sulf2} \) expression constructs, decreases in \( \text{UA2S-GlcNS6S} \) and increases in \( \text{UA2S-GlcNS} \) were observed. In contrast, when CS was incubated with \( \text{Sulf1} \) or \( \text{Sulf2} \), no changes were observed in the compositions of CS disaccharides in any of the CS subtypes examined (CS-A, CS-B, CS-C, CS-D, and CS-E), indicating that \( \text{Sulf1} \) and \( \text{Sulf2} \) have no endosulfatase activity toward CS in vitro (supplemental Fig. S4; see also Refs. 9 and 13).

**Sulf mRNA Expression in Organs** —We wondered whether \( \text{Sulf} \) genes are broadly expressed and affect global sulfation patterns of HS or whether their expression is rather restricted to specific cell populations and affects local sulfation patterns in adult organs. To examine this, we performed *in situ* hybridization of \( \text{Sulf} \) mRNAs in tissue sections. By using antisense RNA probes, we found that \( \text{Sulf} \) mRNAs are expressed in various organs, including the lung, liver, kidney, spleen, testis, and muscle (Fig. 2A). The percentages of \( \text{UA-GlcNAc6S} \) and \( \text{UA-GlcNS6S} \) were increased in the lung of \( \text{Sulf1} \) single knock-out and \( \text{Sulf1}/\text{Sulf2} \) double knock-out mice (Fig. 4A, Table 3). This is consistent with the prediction based on the lack of 6-O-desulfation activity in \( \text{Sulf} \) knock-out mice, as the percentages of \( \text{UA-GlcNS6S} \) and \( \text{UA-GlcNAc6S} \) were decreased in the liver of the double knock-out mice. However, contrary to the prediction made based on the lack of 6-O-desulfation activity in \( \text{Sulf} \) knock-out mice, the percentages of \( \text{UA-GlcNS6S} \) and \( \text{UA-GlcNAc6S} \) were decreased in the liver of the double knock-out mice (Fig. 4A, Table 3). These changes are not simply explained by the disruption of 6-O-endosulfatase activities and thus can be attributed to secondary changes induced by the disruption of \( \text{Sulf1}/\text{Sulf2} \) genes.

**Sulf1 and Sulf2 in Knock-out Mice** —We next examined the sulfation patterns of HS. Disaccharide analysis of HS showed that \( \text{Di-diS} \) increased in the lung of the \( \text{Sulf1} \) single knock-out and \( \text{Sulf1}/\text{Sulf2} \) double knock-out mice, whereas \( \text{Di-6S} \) increased in the lung of the double knock-out mice and the kidney of the \( \text{Sulf2} \) single knock-out mice (Fig. 4B, Table 3). These results may imply that \( \text{Sulfs} \) can act on 6-O-sulfated disaccharide units in HS. We thus examined whether \( \text{Sulf1} \) and \( \text{Sulf2} \) have 6-O-endosulfatase activity toward HS in vitro. In agreement with the results obtained in previous studies including ours (9, 11, 13, 34), when heparin or HS was incubated with a conditioned medium of cells transfected with \( \text{Sulf1} \) or \( \text{Sulf2} \) expression constructs, decreases in \( \text{UA2S-GlcNS6S} \) and increases in \( \text{UA2S-GlcNS} \) were observed. In contrast, when HS was incubated with \( \text{Sulf1} \) or \( \text{Sulf2} \), no changes were observed in the compositions of HS disaccharides in any of the HS subtypes examined (HS-A, HS-B, HS-C, HS-D, and HS-E), indicating that \( \text{Sulf1} \) and \( \text{Sulf2} \) have no endosulfatase activity toward HS in vitro (supplemental Fig. S4; see also Refs. 9 and 13).
probes against *Sulf1* or *Sulf2*, we could detect specific signals, whereas sense probes yielded no signals (data not shown). In the lung, *Sulf1* mRNA was detected in the blood vessels (most likely the pulmonary arteries), whereas *Sulf2* mRNA was seen in the bronchial wall (Fig. 5, A and B). In the kidney, *Sulf1* mRNA was strongly detected in the glomeruli (Fig. 5 C and supplemental Fig. S5, A and C) as reported previously (32, 41). Weak *Sulf1* signals were observed in the blood vessels (supplemental Fig. S5 E). In contrast, *Sulf2* mRNA was seen in a portion of the renal tubules, which based on the morphological characteristics were most likely the distal renal tubules (Fig. 5 D). Moreover, marginal to weak signals of *Sulf2* mRNA were also observed in the glomeruli (supplemental Fig. S5, B and D). In the tests, both *Sulf1* and *Sulf2* mRNAs were seen in the Sertoli cells of the seminiferous tubules in a stage-dependent manner (Fig. 5, E and F), as reported previously (31). Therefore, *Sulf* expressions are restricted to particular cell types.

**Changes in Expression Patterns of HS Epitopes in Sulf Knockout Mice**—To examine possible changes in the sulfation patterns of HS at the cell level, we performed immunohistochemistry of HS by using a set of phage display-derived antibodies (35, 36). Biochemical and histological studies have shown that these antibodies recognize different epitopes in HS chains and are, therefore, useful for examining the heterogeneity of HS in vivo (35, 36). We selected two well-characterized antibodies, AO4B08 and RB4CD12. AO4B08 reacts with heavily O-sulfated NS domains composed of at least three disaccharide units (36).

![TABLE 2](https://www.jbc.org/content/287/12/9585)

| Disaccharides | Lung | Liver | Brain | Small Intestine | Kidney | Spleen | Testis | Muscle |
|---------------|------|-------|-------|----------------|--------|--------|--------|--------|
| **UA-GlcNAc** |      |       |       |                |        |        |        |        |
| *Sulf1*       | 0.51 | 0.51  | 0.51  | 0.51           | 0.51   | 0.51   | 0.51   | 0.51   |
| *Sulf2*       | 0.51 | 0.51  | 0.51  | 0.51           | 0.51   | 0.51   | 0.51   | 0.51   |
| **UA-GlcNS**  |      |       |       |                |        |        |        |        |
| *Sulf1*       | 0.51 | 0.51  | 0.51  | 0.51           | 0.51   | 0.51   | 0.51   | 0.51   |
| *Sulf2*       | 0.51 | 0.51  | 0.51  | 0.51           | 0.51   | 0.51   | 0.51   | 0.51   |
| **UA-GlcNAc** |      |       |       |                |        |        |        |        |
| *Sulf1*       | 0.51 | 0.51  | 0.51  | 0.51           | 0.51   | 0.51   | 0.51   | 0.51   |
| *Sulf2*       | 0.51 | 0.51  | 0.51  | 0.51           | 0.51   | 0.51   | 0.51   | 0.51   |

**FIGURE 3.** Correlation between *Sulf* mRNA expression and changes in HS sulfation patterns in *Sulf* knock-out mouse organs. The levels of *Sulf1* or *Sulf2* mRNA expression in eight organs of wild-type mice were quantitatively determined and normalized to Gapdh expression. Increase in ∆UA2S-GlcNS6S in *Sulf1* or *Sulf2* knock-out as compared with wild-type controls (%) was calculated. *Sulf1* expression in wild-type mice and increase in ∆UA2S-GlcNS6S in *Sulf1* knock-out mice (A) and *Sulf2* expression in the wild-type mice and increase in ∆UA2S-GlcNS6S in *Sulf2* knock-out mice (B) in eight organs are plotted. The insets show magnifications of the low expression regions. *Sulf1* expression was highly correlated with the increase in ∆UA2S-GlcNS6S in *Sulf1* knock-out mice (R = 0.88).
We first examined the localization of the HS epitopes in the kidney because previous studies have revealed that Sulf1/2 double knock-out led to renal hypoplasia in neonates and glomerular abnormalities and proteinuria in aged animals (28, 32). In the wild-type mice, RB4CD12 strongly stained the renal tubules and Bowman’s capsules and weakly stained the glomeruli (Fig. 6A). AO4B08 stained the renal tubules and Bowman’s capsules but not the glomeruli (Figs. 6D), as reported previously (36). Next we examined the staining patterns in the kidneys of Sulf1 and Sulf2 knock-out mice. In the Sulf1 knock-out mice, RB4CD12 staining was slightly increased in the glomeruli, whereas increases in AO4B08 staining in the glomeruli were small if any at all (Fig. 6, B and E). In one of the Sulf2 knock-out mice, strong punctate signals of AO4B08 were observed in the glomeruli (supplemental Fig. S6B). In the Sulf2 knock-out mice, neither of the two antibodies showed increases in the glomerular signals (Fig. 6, C and F). Given the specific and robust expression of Sulf1 mRNA in the glomeruli, these findings indicate that the localized changes in HS disaccharide composition occurred as a result of Sulf1 disruption. We could not see obvious increases in anti-HS staining intensity in the renal tubules of Sulf1 knock-out mouse probably because the staining in the renal tubules in the wild-type mice was so strong that it was hard to detect subtle changes in the staining intensity by immunohistochemistry. To detect possible changes in the renal tubules, we performed titration experiments. When stained by diluted antibodies (1:50 dilution instead of the 1:5 dilution used in other experiments), no obvious increases were observed in any regions of the Sulf knock-out kidneys except for increases in the AO4B08 signals in the blood vessels of Sulf1 knock-out mice (supplemental Fig. S7). In the lung, both RB4CD12 and AO4B08 staining appeared to increase in the blood vessels of the Sulf1 knock-out mice (supplemental Fig. S8), although precise quantitation of the change in the signal intensity was difficult.

Finally we examined the changes in HS staining in neonatal mice. In the lung, both RB4CD12 and AO4B08 staining appeared to increase in the blood vessels of Sulf1 knock-out mice and more robustly in those of double knock-out mice (Fig. 7). In the kidney of neonatal wild-type mice, both RB4CD12 and AO4B08 signals were observed in the glomeruli (supplemental Fig. S9, A and E). RB4CD12 staining appeared to be slightly increased in the double knock-out mice (supplemental Fig. S9D).

**FIGURE 4. Changes in 6-O-sulfated disaccharide units in neonatal Sulf knock-out mice.** Percentages of ΔUA-GlcNAc6S, ΔUA-GlcN5S6S, and ΔUA2S-GlcN5S6S in total HS (A) and percentages of ΔDI-6S and ΔDI-diS in total CS (B) in wild-type, Sulf1 knock-out, Sulf2 knock-out, and Sulf1/2 double knock-out mice are shown. Bars indicate the means ± S.E. Analysis of variance with the Bonferroni post hoc test was performed for each organ, and statistical significance was compared with the wild-type controls (*, p < 0.05; **, p < 0.01; ***, p < 0.001) is shown. Refer to Table 3 for the numbers of mice examined and values for each disaccharide composition.

**TABLE 3**

| HS Disaccharides | Lung (n = 8) | Kidney (n = 3) | Liver (n = 6) |
|------------------|-------------|---------------|-------------|
| ΔUA-GlcNAc       |             |               |             |
| ΔUA-GlcN5S6S     |             |               |             |
| ΔUA2S-GlcN5S6S   |             |               |             |
| ΔDi-6S           |             |               |             |
| ΔDi-diS          |             |               |             |

We here performed systematic disaccharide analysis of HS in Sulf1 and Sulf2 knock-out mice. As predicted from the in vitro activities of Sulfs, ΔUA2S-GlcN5S6S was increased, and ΔUA2S-GlcN5S6S was decreased in Sulf-deficient organs. How-
ever, the degree of change was different from organ to organ and between Sulf1 and Sulf2 knock-out mice. In general, the increase in \( /H9004 \) UA2S-GlcNS6S was large in organs that showed relatively low percentages of \( /H9004 \) UA2S-GlcNS6S and relatively high percentages of \( /H9004 \) UA2S-GlcNS in wild-type mice. These findings indicate that the HS disaccharide profiles that are characteristic to each organ, especially the low \( /H9004 \) UA-GlcNAc6S patterns, are attributable to HS 6-O-desulfation by Sulfs. Therefore, in addition to HS 6-O-sulfotransferases (43), Sulfs contribute to generating organ-specific sulfation patterns of HS.

In addition to the increase in \( \Delta UA2S-GlcNS6S \), \( \Delta UA-GlcNS6S \) was also increased to a lesser extent but significantly in the lung and small intestine of adult Sulf1 knock-out mice as well as in the lungs of neonatal Sulf1 knock-out and Sulf1/Sulf2 double knock-out mice. Given that Sulf1/2 can hydrolyze 6-O-sulfate in UA-GlcNS6S disaccharide units in HS \textit{in vitro} (11, 24, 25), these findings suggest the possibility that Sulfs act on UA-GlcNS6S disaccharide units in some organs. Because the Sulf1 expression is highest in the lung, high endosulfatase activity may lead to desulfation of UA-GlcNS6S disaccharide units. Or a specific oligosaccharide sequence that contains UA-GlcNS6S disaccharide units and undergoes desulfation by Sulfs may be abundant in the lung and small intestine.

We also noted changes in HS and CS disaccharide compositions that were not predicted from \textit{in vitro} studies. HS disaccharide \( \Delta UA-GlcNAc6S \) was increased in the small intestine of Sulf1 knock-out mice, and CS disaccharide \( \Delta Di-diSe \) was increased in the lungs of adult and neonatal Sulf1 knock-out mice as well as of Sulf1/Sulf2 double knock-out mice. These increases may simply mean that Sulfs have 6-O-endosulfatase activities toward these disaccharide units. However, given that HS 6-O-endosulfatase activity toward UA-GlcNAc6S units has never been detected \textit{in vitro} (9, 11, 13, 24, 25, 34) and that Sulfs show no endosulfatase activity toward CS (this study; see also Refs. 9, 13), these changes seem to have occurred as a secondary consequence of alteration of the HS sulfation patterns, although the possibility cannot be formally excluded that Sulfs acquire such activity in collaboration with an unknown factor(s) \textit{in vivo}. Interestingly, Sulf1 and Sulf2 have different

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**FIGURE 5.** \textit{In situ} hybridization of Sulf1 and Sulf2. Cryostat sections of the adult lung (A and B), kidney (C and D), and testis (E and F) were hybridized with digoxigenin-labeled RNA probes specific to Sulf1 (A, C, and E) or Sulf2 (B, D, and F). The signals were detected by BM purple. Arrows in C and D indicate expression of Sulf1 in glomeruli and Sulf2 in distal renal tubules, respectively. Asterisks in E and F indicate the seminiferous tubules containing Sertoli cells expressing Sulf1 and Sulf2, respectively. b, bronchus; p, pulmonary artery. Scale bar, 100 \( \mu m \).
degrees of impact on 6-O-sulfation states of HS in vivo, although they have indistinguishable activity in vitro. For example, although Sulf2 mRNA expression was about 3-fold higher than that of Sulf1 mRNA in the lung, increases in ΔUA2S-GlcNS6S in Sulf2 knock-out mice were trivial (5.5%) in contrast to large (126%) increases in ΔUA2S-GlcNS6S in Sulf1 knock-out mice. Although the specific activities of Sulf1 and Sulf2 (6-O-desulfation activity per unit protein) have not been determined, if we assume that they have the same specific activity, these data suggest that Sulf1 and Sulf2 function in a different fashion in vivo. The following are possible causative factors for the differences. First, Sulf2 may be more labile than Sulf1, and the steady state levels of Sulf2 may be low. The levels of Sulf1 and Sulf2 proteins should be determined and compared with the changes in HS composition in future. Second, the activity of Sulf1 protein may be more diffusible and thus able to desulfate more HS. Given that the cleavage of Sulf proteins by furin-type proteinases affects the accumulation of Sulf proteins in lipid-rich domains as well as Wnt activation (44), Sulf1 and Sulf2 may undergo different protein cleavage in vivo. Although Sulf1 and Sulf2 show overall sequence similarity, the hydrophilic domains in their middle portions are divergent (22). Because the hydrophilic domains are required for secretion and cell surface localization of Sulf proteins (13, 22), these sequences may give rise to the functional differences between these two Sulf proteins. Fourth, Sulf2 may be localized apart from the target HS. Fifth, native HS may contain certain oligosaccharide structures that are more vulnerable to desulfation by Sulf1. Future studies are required to elucidate the molecular mechanisms that lead to the functional differences between Sulf1 and Sulf2 in vivo.

We showed that the composition of HS changed at the cell level as a result of Sulf gene disruption. In the kidney glomeruli, the RB4CD12 epitope and (to a lesser extent) AO4B08 epitope increased in adult Sulf1 knock-out mice. Because both AO4B08 and RB4CD12 react with trisulfated disaccharide motifs in HS (45) and because Sulf1 mRNA is expressed specifically in the glomeruli of adult kidneys, these findings indicate that Sulf1 remodels sulfation profiles of HS locally. In the disaccharide analysis, however, increases in ΔUA2S-GlcNS6S were not significant in the Sulf1 knock-out kidney. This is likely because the increases in ΔUA2S-GlcNS6S in the glomeruli were masked when analyzed at the organ level. Conversely, in the Sulf2 knock-out mice, we could not see any obvious changes in anti-HS staining in the renal tubules, whereas increases in ΔUA2S-GlcNS6S were significant in the Sulf2 knock-out kidney. It is likely that strong anti-HS signals in the renal tubules hamper the detection of probable changes in the staining, although it is also possible that the antibodies used in this study did not recognize the increased ΔUA2S-GlcNS6S-containing HS domains. Thus, the combination of biochemical analysis of HS disaccharide profiles and immunohistochemical analysis by anti-HS antibodies would be useful for elucidating where and how HS regulates cell signaling and how Sulfs are involved in the processes in vivo. In the lung, increases in RB4CD12/AO4B08 staining were robust in the blood vessels of Sulf1

![Image of immunohistochemistry of HS in adult kidneys](image1)

![Image of immunohistochemistry of HS in neonatal lungs](image2)
knock-out mice as well as of double knock-out mice. Given that RB4C1D1 staining in wild-type mice is strong in the blood vessels of the mouse brain (46), Sulf1 may regulate vascular signaling in general. Therefore, future studies are necessary to unravel the possible roles of Sulf1 in the physiology and pathology of the vascular system.

Although accumulating evidence has suggested that Sulfs regulate multiple signaling pathways in vitro, the functional consequences of Sulf gene disruption are small. Mice deficient in either Sulf1 or Sulf2 are healthy and appear to be normal (20, 28, 29) except for some subtle phenotypes. The body weight of Sulf2 knock-out mice is smaller than those of wild-type and Sulf1 knock-out mice (28). Sulf2 mutant mice generated by gene trapping occasionally showed defects in the lung (27). In contrast, double knock-out mice die postnatally, indicating overlapping and essential roles of Sulf genes in mouse development. Double knock-out mice showed reduced body weight, kidney hypoplasia, and skeletal abnormalities (20, 28, 29). In embryonic kidneys, Sulf1 mRNA is expressed in the developing glomeruli, whereas Sulf2 mRNA is expressed in the nephron progenitors and tubules (32). Thus Sulfs likely regulate cell differentiation and/or proliferation in kidneys, although more studies are required to elucidate the molecular mechanisms by which Sulfs play roles in kidney development. Recently, by using double knock-out mice that survived to adulthood, it was shown that simultaneous disruption of Sulf1 and Sulf2 genes led to proteinuria and glomerular defects in aged animals (32). Clearly the phenotype seems to be associated with changes in HS sulfation in the glomeruli in Sulf1 knock-out mice, although we did not examine the HS profiles of adult double knock-out mice due to their neonatal lethality. Utilization of such double knock-out mice may facilitate the understanding of the roles of Sulf genes and 6-O-sulfation in vivo.

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