Roles of Heparan Sulfate Sulfation in Dentinogenesis*

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Cell surface heparan sulfate (HS) is an essential regulator of cell signaling and development. HS traps signaling molecules, like Wnt in the glycosaminoglycan side chains of HS proteoglycans (HSPGs), and regulates their functions. Endosulfatases Sulf1 and Sulf2 are secreted at the cell surface to selectively remove 6-O-sulfate groups from HSPGs, thereby modifying the affinity of cell surface HSPGs for its ligands. This study provides molecular evidence for the functional roles of HSPG sulfation and desulfation in dentinogenesis. We show that odontogenic cells are highly sulfated on the cell surface and become desulfated during their differentiation to odontoblasts, which produce tooth dentin. Sulf1/Sulf2 double null mutant mice exhibit a thin dentin matrix and short roots combined with reduced expression of dentin sialophosphoprotein (Dspp) mRNA, encoding a dentin-specific extracellular matrix precursor protein, whereas single Sulf mutants do not show such defective phenotypes. In odontoblast cell lines, Dspp mRNA expression is potentiated by the activation of the Wnt canonical signaling pathway. In addition, pharmacological interference with HS sulfation promotes Dspp mRNA expression through activation of Wnt signaling. On the contrary, the silencing of Sulfs suppresses the Wnt signaling pathway and subsequently Dspp mRNA expression. We also show that Wnt10a protein binds to cell surface HSPGs in odontoblasts, and interference with HS sulfation decreases the binding affinity of Wnt10a for HSPGs, which facilitates the binding of Wnt10a to its receptor and potentiates the Wnt signaling pathway, thereby up-regulating Dspp mRNA expression. These results demonstrate that Sulfs-mediated desulfation of cellular HSPGs is an important modification that is critical for the activation of the Wnt signaling in odontoblasts and for production of the dentin matrix.

Sugar chain protein modification is a post-translational modification of genetic information, and the resultant sugar chain heterogeneity is associated with many diseases, immunity, and development. Heparan sulfate (HS) is a member of the glycosaminoglycan (GAG) family of polysaccharides, which attaches to core proteins to form proteoglycans and is ubiquitously present on the cell surface and in the extracellular matrix. It is also an essential regulator of cell signaling and development. HS traps many different proteins on the cell surface and basement membrane and regulates their functions. There is considerable interest in the structural variability generated by a series of modifications within HS, which are responsible for the binding and regulation of specific HS-interacting proteins. Such HS modifications, in particular in the form of its sulfation patterns, result in structural and functional heterogeneity, which are controlled in a tissue-specific and developmental manner. Here the specific and inhomogenous sulfation of the HS disaccharide building blocks (i.e. at the N-, O-, and 6-O-position of glucosamine and the 2-O-position of hexuronic acid) translates into dynamic docking sites for various ligands, among them important signaling molecules, such as FGF and Wnt.

Dentin is the mineralized portion of tooth tissue, and it is produced by odontoblasts differentiating from dental papilla mesenchymal cells. Dentinogenesis is regulated by sequential and reciprocal interactions with the dental epithelium mediated by epithelial-mesenchymal interactions, which direct both morphogenesis and differentiation. Odontoblasts are characteristically columnar polarized cells, and this cytological polarization specifically occurs in a single layer adjacent to the inner dental epithelium. Dentin sialophosphoprotein (Dsp) is a phosphorylated parent protein that is cleaved post-translationally into two dentin non-collagenous proteins: dentin sialoprotein (Dsp) and dentin phosphophosphoprotein (Dpp). In

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Situation hybridization and other experimental analyses have shown that *Dspp* is predominantly expressed in odontoblasts, transiently expressed in preameloblasts, and expressed at low levels in bone (13, 14). This suggests that the functional role of Dspp mainly involves tooth formation and mineralization. In humans, several mutations in *DSPP* have been identified in patients with dentinogenesis imperfecta, which is an autosomal dominant disorder of the tooth that specifically affects dentin biominalization (15–18). A similar phenotype is found in *Dspp* null mutant mice, which disrupts dentin mineralization without affecting bone (19). Hence, it is established that Dspp plays a specific and crucial role in the formation of mineralized dentin.

Wnt genes encode a large family of secreted signaling proteins that specify various cell lineage pathways during development. Wnt proteins are now recognized as one of the major functions performed by Wnt proteins are embryonic induction, the generation of cell polarity, and the specification of cell fate. The canonical Wnt pathway involves inhibition of the β-catenin degradation complex, allowing its interaction with the nuclear transcription factors LEF and TCF and the regulation of target gene expression (21). In early tooth development, various Wnt genes are expressed from the bud stage to the early bell stage (22). A recent study revealed that TOPGAL and Axin2 reporter activity are high in the odontoblast layer (23, 24), suggesting that dentinogenesis is associated with the activation of the canonical Wnt signaling pathway. These findings are in line with the human tooth phenotypes observed after the heterozygotic loss of *Axin2* function, which causes tooth agenesis and/or hypodontia (25). Among the numerous Wnt genes that are expressed in developing tooth germs, Wnt10a is specifically expressed in odontoblasts. Interestingly, the forced expression of Wnt10a induced *Dspp* expression when the transfected cells were cultured on Matrigel (26), indicating that Wnt10a is an upstream gene involved in *Dspp* expression. In addition, human WNT10A mutations were reported to be associated with hypodontia (27, 28). These findings strongly suggest that the Wnt10a-induced canonical Wnt pathway is involved in dentinogenesis, at least in part, by directly activating *Dspp* expression, although there is no direct in vivo or in vitro evidence to support this.

The Sulf proteins Sulf1 and Sulf2 were identified as a family of extracellular glucosamine-6-sulfatases that remove the sulfate groups from the 6-O-position of N-acetylgalactosamine (29, 30). Both Sulf1 and Sulf2 are endosulfatases that act extracellularly and generate structural heterogeneity along HS chains by modifying the sulfation pattern of HS postsynthetically. Sulf1/ Sulf2 double null mutant mice show numerous distinct phenotypes (31–37). In skeletal structures, malformations, including reduced bone length, premature vertebrae ossification, and fusion of sternebrae and tail vertebrae, are observed (35). Due to their activity, the Sulfs control HS proteoglycans (HSPGs) functioning as coreceptors for the signaling activities of multiple developmental ligands (32). In particular, Sulf1 activity has been shown to decrease the affinity of HS toward the Wnt ligand and promote the binding of Wnt to its cognate receptor Frizzled, thereby acting as a positive regulator of Wnt signaling (38). From these findings, together with those of our previous study (26), we hypothesized that Wnt ligands, especially Wnt10a, can be captured by cell surface or basement membrane extracellular matrix molecules to control the differentiation of pulp cells into odontoblasts and that such regulation of Wnt signaling is modulated by the Sulf enzymes.

This study presents the first molecular evidence for the functional roles of HSPG sulfation and desulfation in dentin formation. Here, we demonstrate that the desulfation of odontogenic cells progresses with differentiation and that the loss of the endosulfatases Sulf1 and Sulf2 results in defective dentin phenotypes. In particular, we show that the cell surface sulfation of HSPGs affects the Wnt canonical signaling pathway and consequently regulates *Dspp* expression in odontogenic cells. We also show that the binding affinity of HS for Wnt10a is directly dependent on its 6-O-sulfation status.

**EXPERIMENTAL PROCEDURES**

**Animals—**Sulf1 and Sulf2 mutant embryos and postnatal mice were used in this study. *Sulf1* and *Sulf2* knock-out mice were generated by the insertion of a neomycin resistance cassette into exon 2 of the murine *Sulf1* gene and exon 1 of the murine *Sulf2* gene, as described previously (37). Control mouse fetuses were also obtained from the ICR strain. The control group consisted of control or single-transgenic animals from the same litter. Animal experiments were performed under the research protocol approved by the Animal Research Committee at Bielefeld University and Okayama University.

**Cell Culture—**The mDP odontoblast-like cell line, which was derived from the embryonic mouse mesenchyme (39), was used in this study. The mDP cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were subcultured every 3–4 days, and 0.05% (w/v) EDTA was used to detach the cells from the culture dish. These cells were used for the real-time PCR experiments.

Sodium chlorate is a reversible inhibitor of glycosaminoglycan sulfation. mDP cells were treated to pharmacologically remove sulfated residues from their cell surface HSPGs (40). The chloride-treated cultures were incubated with 50 mM sodium chlorate for 72 h before being processed for immunocytochemistry and real-time RT-PCR analysis.

Lithium chloride (LiCl) is an inhibitor of glycogen synthase kinase-3 and mimics Wnt signaling by inhibiting glycogen synthase kinase-3 activity (41). mDP cell lines were treated with 50 mM LiCl for 5 days before being processed for immunocytochemistry and real-time RT-PCR analysis.

The cells were also incubated with 1 mM 4-nitrophenyl-β-D-xylopyranoside (Sigma) for 72 h. β-D-Xylose is a primer of GAG chain synthesis and inhibitor of proteoglycan assembly (42, 43). Consequently, at 1 mM β-D-xylose, HSPGs without core proteins are supposed to be released from the cells and may form a complex with Wnts in the conditioned medium. In the present study, we also confirmed that 1 mM β-D-xylose does not affect the proliferation of cells or type I collagen synthesis in odontoblast cell lines (data not shown). Therefore, we can rule out a toxic effect of β-D-xylose. Fur-
ther, mDP cells were incubated for 120 h with 5 units/ml heparin (Fuso Pharma, Osaka, Japan), which directly binds free Wnt proteins released into the culture medium.

**Microcomputed Tomography (Micro-CT)**—Three-dimensional images of tooth structure were analyzed using an inspeXio SMX-90CT Microfocus x-ray CT system, (Shimadzu, Kyoto, Japan). Briefly, image acquisition was performed at 90 kV and 110 mA. The resultant images were processed by the three-dimensional reconstruction software VG Studio MAX 2.0 (Volume Graphics, Heidelberg, Germany). The image reconstruction was carried out using appropriate cross-sections at a spatial resolution of 17 μm and was used to perform comparative measurements of tooth morphology.

The following parameters were defined: root length, distance between the apex and the cemento-enamel junction along the root canal for the mesial root of the first lower molar; crown length, anteroposterior distance between the mesial marginal ridge and the distal marginal ridge along the occlusal plane; dentin thickness, mean of the mesial and distal dentin thickness at the level of the cemento-enamel junction; enamel thickness, mean of the mesial and distal enamel thickness at the midlevel of crown height.

**Tissue Preparation and Histology**—Embryonic day 16.5 (E16.5) and postnatal day 0 (P0), P1, P7, and P28 heads were fixed in 4% paraformaldehyde or tissue fixative (Genostaff, Tokyo, Japan) at 4 °C overnight. P7 and P28 heads were decalciﬁed in 12.5% EDTA for 3 weeks. They were dehydrated, embedded in paraffin, and serially sectioned at 7 or 6 μm for hematoxylin and eosin staining and in situ hybridization. Frozen cross-sections (10 μm) were also prepared for in situ hybridization and immunohistochemistry. Those embryos were fixed in 4% paraformaldehyde at 4 °C overnight, incubated in 30% sucrose/PBS at 4 °C overnight, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and serially sectioned at 10 μm.

Predentin thickness was measured in frontal sections of P0 control and Sulf1/Sulf2 double null mutant lower molars. The maximum thickness of the predentin beneath the mesial cusps was measured in serial sections from each group.

**Immunohistochemistry and In Situ Hybridization**—In situ hybridization using digoxigenin-labeled RNA probes was performed as described previously (44). Mouse Sulf1 and Sulf2 probes were generated from a 461-bp Sulf1 fragment spanning the region between 124 and 584 in accession number AY101178 and a 459-bp Sulf2 fragment spanning the region between 124 and 584 in accession number AY101178. The preparation of Wnt10a, Dspp, and Axin2 RNA probes has been described previously (23, 26).

Cell proliferation was assessed by 5'-bromo-2-deoxyuridine (BrdU) labeling of dividing cells in the tooth germs of Sulf1 and Sulf2 mutants. E18.5 pregnant mice were intraperitoneally injected with 1.5 ml/100 g body weight of BrdU solution (Sigma) and killed after 2 h. BrdU staining was carried out in paraffin sections using a BrdU staining kit according to the manufacturer’s instructions (Zymed Laboratories Inc., South San Francisco, CA). Cell proliferation indices were determined by counting the BrdU-positive and -negative cells in defined areas of the cervical loop epithelium and mesenchyme.

Immunohistochemistry was performed on frozen sections of developing tooth germs and mDP cells. The sulfated HS distribution was visualized using 10E4 antibody (1:100; Seikagaku, Tokyo, Japan). HS distribution was visualized using the 3G10 antibody (1:100; Seikagaku). 3G10 identifies a neoeptope generated by heparinase III digestion of HS. Sections were pretreated with heparinase III (400 milliunits/ml for 2 h) immediately before immunohistochemistry.

Immunohistochemistry for Wnt10a (ab62051, Abcam (Cambridge, UK)) was also performed using mDP cells. Secondary antibodies conjugated with the appropriate fluorochrome, Cy3-conjugated AffiniPure goat anti-mouse IgG + IgM antibody (1:500; Seikagaku) were used.

Immunoreactivity to 10E4, 3G10, and Wnt10a was visualized with a FLUOVIEW FV500 confocal laser-scanning microscopy system (Olympus, Tokyo, Japan) equipped for differential interference contrast microscopy. The confocal laser-scanning microscopy system was coupled to an upright microscope (IX-71, Olympus) with a ×60 (numerical aperture = 1.4) oil immersion objective lens.

**RNA Extraction and Real-time RT-PCR Analysis**—Total RNA was extracted from the mDP cells using Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer’s protocol. Mandibular fragments containing tooth germs were also isolated from P0 transgenic and wild-type mouse pups and fixed overnight in buffered 4% paraformaldehyde. First molar tooth germs of the mandible were dissected in 0.1 m phosphate buffer (pH 7.4) under a stereomicroscope. Total RNA was isolated from formalin-fixed tooth germ using a RecoverAll total nucleic acid isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Total RNA (500 ng) was reverse-transcribed to cDNA using oligo(dt)20 primer (Takara, Shiga, Japan). For real-time RT-PCR analysis, the cDNA were amplified with Blend-Taq Plus (Toyobo, Osaka, Japan) and a regular thermal cycler. Quantitative real-time PCR was performed in duplicate for four independent sets of samples. The relative quantity of transcripts was determined using a standard curve and normalized in comparison with the expression of Gapdh mRNA. The sets of synthetic primers used for the amplification are as follows: mouse glyceraldehyde-3-phosphate dehydrogenase gene (Gapdh), 5’-GTCCCTGATGAAAATGTTG-3’ (sense) and 5’-CAATGAGGGGT CCTGTGTGATG-3’ (antisense); mouse Dspp, 5’-AGCCGTGGAGATGTCTTCTTATAA-3’ (sense) and 5’-ATCCAGTCTGCTGTCACCAC-3’ (antisense); mouse Axin2, 5’-CTCT CCACCAAAACCGGAATG-3’ (sense) and 5’-TTCCTGGCTGTTGCGTA-3’ (antisense). Each PCR was carried out and analyzed as described previously (45). Each amplification reaction was performed and checked to ensure the absence of nonspecific PCR products by melting curve analysis using a LightCycler™ system (Roche Applied Science). The relative cDNA copy numbers were computed using data from serial dilutions of representative samples for each target gene. The same pools of rat E17 head cDNA and mouse P0 tibia cDNA were used as calibrators.

**Overexpression and Suppression of Sulf**—For the overexpression of Sulf1 and Wnt10a, the pLPCX-based (pcI-neo-based) Sulf1-RGS-His6 expression vector (46) and the pCMV-based
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Wnt10a expression vector (26) were used, respectively. These vectors or empty vectors as a control, pMAX-GFP (Amaza, Koeln, Germany), were transfected into mDP cells using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocols.

For the suppression of Sulf2, the BLOCK-iT Pol II miRNA expression vector (Invitrogen) was used to silence mouse Sulf2. Two different double-stranded oligonucleotide duplexes encoding the desired miRNA sequences were selected using online software (BLOCK-iT RNAi Designer, Invitrogen) and ligated into the expression vector. The sequences of the two selected oligonucleotide duplexes were as follows: first Sulf2 miRNA, 5’-TGGTACGGCACTGACCTACTACCTAAACTCTCCCCCTTT-3′ (top sequence) and 5’-CTTGAAAGCGGGAGTAAAAAGAGTCTACGTAGTGCCACTGACCTACTACCTAAACTCTCCCCCTTT-3′ (bottom sequence); second Sulf2 miRNA, 5’-TGCCTTCTAGGGAGACATTCTCTGTTTGCCACTGACTGACAAGAATGTCTGCCATAGAA-3′ (top sequence) and 5’-CTTTGTTCTATGGCAGTCACATTCTTGTTTTGGCCACTGACTGACAAGAATGTCTGCCATAGAA-3′ (bottom sequence). Vectors containing the Sulf2-miRNA plasmid were transfected into mDP cells using FuGENE 6 according to the manufacturer’s protocols.

Statistical Analysis—Data are shown as the mean ± S.D. Statistical significance was evaluated using the Mann–Whitney U test for group comparisons, and p < 0.05 was considered significant. Statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL).

Affinity Chromatography—To investigate the affinity of Wnt10a toward HS, which had been either pretreated with active Sulf1 (termed HS-6S) or not (HS+6S), we used columns with immobilized HS-6S and HS+6S. Generation of these columns and characterization of their HS disaccharide composition has been described previously (47). Briefly, HS from porcine intestinal mucosa (Celsus Laboratories, Cincinnati, OH) was treated with purified, active Sulf1DHDC enzyme, reductively aminated, and covalently immobilized on a HiTrap NHS column. On each column, after equilibration with buffer A (20 mM Tris, pH 7.4), 35 μl of Wnt10a-containing cell lysate (Origene Technologies, Rockville, MD) was loaded and subjected to chromatography by using an ÄKTA Explorer chromatography system. After washing with 2 ml of buffer A, bound Wnt10a was eluted using a linear 10-ml gradient from 0 to 100% buffer B (20 mM Tris, pH 7.4, 1.5 M NaCl). Fractions of 1 ml were collected, concentrated 20-fold by using a 500-μl Spin-X concentrator (Corning Glass, 10,000 molecular weight cut-off), and analyzed by Western blotting using a polyclonal anti-Wnt10a antibody (Abcam, Cambridge, MA).

RESULTS

Specific Heparan Sulfate Modification during Tooth Development—To investigate the role of the sulfation status of heparan sulfate in tooth development, we examined the temporal-spatial changes of heparan sulfate sulfation, as specifically recognized by the 10E4 antibody (48–50). At E16.5 (Fig. 1A), we observed ubiquitous 10E4 staining in both tooth germs, including the dental mesenchyme (dm) and the surrounding bones (b). The staining was reduced in the dental epithelium, including the inner enamel epithelium (iee) and the stellate reticulum (sr). In contrast, it was intense in the basement membrane (bm). At P1 (Fig. 1B), polarized and differentiated odontoblasts were observed in the mesenchyme underlying the future cusp regions, and 10E4 staining was significantly reduced in these odontoblasts (asterisks in Fig. 1B), whereas 10E4 staining was retained in the dental mesenchyme (dm) of the future pulp. The 10E4 staining was significantly reduced in the differentiated ameloblasts (am), and intense 10E4 staining was present in the predentin (pd). This staining was continuous with that in the basement membrane (bm), and 10E4 staining was almost completely absent from the odontoblasts (od and asterisks) and ameloblasts (am) but was intense in the dental pulp (pu), s, stratum intermedium; Scale bars (A′, B′, and C′), 200 μm.

Expression of Sulf1 and Sulf2 in Developing Root—We evaluated the mRNA expression of Sulf1 and Sulf2 during tooth development. At E16.5, Sulf1 transcripts were present in the inner enamel epithelium (iee), stellate reticulum (sr), outer enamel epithelium (oeo), and the dental mesenchyme (dm) (Fig. 2A). At P0, Sulf1 transcripts were detected in the stratum intermedium (si) as well as the stellate reticulum (sr) and the outer enamel epithelium (oeo) (Fig. 2B). In the inner enamel epithelium (iee), Sulf1 transcripts were only present in the cervical regions of the inner enamel epithelium (iee) (Fig. 2, B and C), and they were not detected in the differentiated ameloblasts.

FIGURE 1. Analysis of the sulfated HS distribution during tooth development at E16.5 (A), P1 (B), and P7 (C). Immunofluorescent staining for 10E4 was visualized using the Cy3-conjugated secondary antibody, which is shown in red. Nuclear staining with Hoechst is shown in blue (A′–C′). A and A′, 10E4 staining was ubiquitously present in the dental mesenchyme and the surrounding bones (b). The staining was intense in the basement membrane (bm) and reduced in the dental epithelium, including the inner enamel epithelium (iee) and the stellate reticulum (sr). In contrast, it was intense in the basement membrane (bm). The 10E4 staining was significantly reduced in these odontoblasts (asterisks in Fig. 1B), whereas 10E4 staining was retained in the dental mesenchyme (dm) of the future pulp. The 10E4 staining was significantly reduced in the differentiated ameloblasts (am), and intense 10E4 staining was present in the predentin (pd). At P1 (Fig. 1B), polarized and differentiated odontoblasts were observed in the mesenchyme underlying the future cusp regions, and 10E4 staining was significantly reduced in these odontoblasts (asterisks in Fig. 1B), whereas normal 10E4 staining was retained in the dental mesenchyme (dm) of the future pulp. In addition, 10E4 staining was significantly reduced in the differentiated ameloblasts (am) but was intense in the dental pulp (pu). Scale bars (A′, A, B′, and C′), 200 μm.
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**Dentin Hypoplasia in Sulf Mutant Mice**—We next examined the phenotypes of teeth in the control (Sulf1<sup>+/+</sup>;Sulf2<sup>+/+</sup>), Sulf1 single null mutant (Sulf1<sup>−/−</sup>;Sulf2<sup>+/+</sup>), Sulf2 single null mutant (Sulf1<sup>+/+</sup>;Sulf2<sup>−/−</sup>), and Sulf1/Sulf2 double null mutant (Sulf1<sup>−/−</sup>;Sulf2<sup>−/−</sup>) mice. All lower molars had finished erupting in both the control and mutant littermates by P28, and the mutants did not show any tooth eruption delay. The roots of the Sulf1/Sulf2 double null mutant molars were short (Fig. 3D), but there were no differences in the size or shape of the Sulf1 or Sulf2 single null mice molars as compared with those of control mice (Fig. 3, A–C). These tooth phenotypes were further evaluated in detail by micro-CT scanning. The x-ray-opaque appearances of the enamel and dentin were not changed in the mutant teeth (Fig. 3, E–H). Tooth morphology was further evaluated on micro-CT-reconstructed images (Fig. 3, I–L). The anteroposterior length of the crown did not show any differences among the groups (Fig. 3J), whereas the root length was 30% shorter in the Sulf1/Sulf2 mutant teeth (Fig. 3J). The dentin thickness in the cement-enamel junction was also reduced by 30% in the double mutant mice (Fig. 3K). This tooth hypoplasia phenotype displayed 100% penetration with little variation in severity. In contrast, enamel thickness was not affected (Fig. 3L).

Then we evaluated the neonatal phenotypes of the Sulf1/Sulf2 double mutant mice in further detail. At P0, Sulf1 and Sulf2 deficiency resulted in a significant reduction of predentin (pd) thickness (Fig. 4, A–C). Next, we asked whether the deletion of Sulf1 and Sulf2 affects the sulfation state of cell surface HSPG in odontogenic cell regions. To answer this, we evaluated the changes in 10E4 and 3G10 immunoreactivity in odontoblasts and the surrounding cells. We have shown that the 10E4 immunoreactivity in the odontoblast layer decreased with differentiation (Fig. 1, A–C), and 10E4 staining was almost completely abolished in the odontoblast layer in the P0 control mice (arrowheads in Fig. 4D), whereas no such reduction in immunoreactivity was observed in the Sulf1 and Sulf2 double null mutant molars (Fig. 4E). These findings strongly suggested that Sulf proteins are involved in the desulfation of odontoblasts, and, as a consequence, their deficiency results in the oversulfation of these dentinogenic cells. To verify that the changed 10E4 staining in odontoblasts due to Sulf deficiency reflects a specific change in its HS sulfation status, we also examined the distribution of HS as recognized by the 3G10 antibody. 3G10 specifically identifies a neoeptope generated by heparinase III digestion of HS (50). We observed ubiquitous 3G10 staining in both the dental mesenchyme and the surrounding bones, similar to that of 10E4. There was less staining in the dental epithelium, including the stellate reticulum. Unlike 10E4 staining, which was significantly reduced in differentiated odontoblasts, 3G10 staining remained unchanged with differentiation of the odontoblast layer. In addition, the distribution of 3G10 immunoreactivity in odontoblasts and surrounding dental mesenchyme was not changed by Sulf1/Sulf2 deficiency (Fig. 4, F and G). These findings indicated that the HS distribution was not affected by odontoblast differentiation or by Sulf deficiency. Thus, the increase in 10E4 immunoreactivity, as specifically

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**FIGURE 2.** The expression pattern of Sulf1 (A–G) and Sulf2 (H–M) mRNA in frontal sections of developing teeth. A, at E16.5, Sulf1 transcripts were present in the inner enamel epithelium (iie), stellate reticulum (sr), outer enamel epithelium (oee), and the dental mesenchyme (dm). B, at P0, Sulf1 transcripts were detected in the stratum intermedium (s) as well as the stellate reticulum, the outer enamel epithelium, and the cervical regions of the inner enamel epithelium (G) but not in the differentiated ameloblasts (am) (D). E, at P10, Sulf1 expression was present in the dental pulp (pu), and the signals were particularly intense in the cells underlying the differentiated odontoblasts (F). At the growing root apex, Sulf1 mRNA expression was intense in the epithelial root sheath (er) (G). H, Sulf2 transcripts were intensely detected throughout the dental mesenchyme at E16.5. Sulf2 signals were also present in the inner enamel epithelium. I, at P0, Sulf2 transcripts were present in the dental mesenchymal cells, including odontoblasts (od) (J). K, at P10, Sulf2 expression was retained in the pulpal mesenchymal cells, except in the differentiated odontoblasts (L). Sulf2 signals were detected in the differentiating odontoblasts overlying the epithelial sheath (M). Scale bars, 200 μm (A, B, H, and I), 20 μm (C, D, F, G, J, L, and M), and 500 μm (E and K).
observed in Sulf-deficient odontoblasts, reflects changes in the HS sulfation status of these dentinogenic cells.

Epithelial and mesenchymal proliferation in the developing tooth germs was assessed by BrdU immunohistochemistry. Many BrdU-positive cells were identified in the first molars of the mutants and control, and there was no difference in BrdU incorporation in the epithelial or mesenchymal tissue between them (Fig. 4, H and I). Then we asked why thinner dentin walls and shortened roots were produced in the Sulf1/Sulf2 double mutant mice. Because Dspp is a tooth-specific extracellular matrix molecule and Dspp null mutant mice display similar tooth phenotypes as those observed in our Sulf1/Sulf2 mutant mice (reduced dentin thickness and shortened roots), we asked whether the aberrant expression of mutant mice (reduced dentin thickness and shortened root accompanied by the inhibition of desulfation in the differentiated odontoblast layer and the down-regulation of Dspp expression.

Desulfation Affects Dspp mRNA Expression through Wnt Signaling Pathway—To investigate the mechanisms underlying Sulf regulation of Dspp expression, we utilized in situ hybridization and biochemical and cell culture assays. Recent TOP-GAL staining data and Axin2 expression data suggested that the Wnt canonical pathway is activated in dentinogenesis (23, 24). This pathway is known to be promoted through Sulf activity (38). In the control molars at P0, Axin2 mRNA signal was detected in odontoblasts and slightly in the periodontal ligaments (Fig. 4, L and L’), whereas a reduction in Axin2 was observed in the Sulf1/Sulf2 null mutant molars (Fig. 4, M and M’). Real-time PCR also revealed a significant reduction in
Axin2 mRNA expression in Sulf1/Sulf2 null mutant teeth (p < 0.01; Fig. 4O).

We then tested whether Wnt signaling modulates Dspp expression in the odontoblast-like mDP cell line. LiCl, an inhibitor of glycogen synthase kinase-3, was used to activate Wnt signaling (41). Mimicking Wnt signaling with 5 or 50 mM LiCl treatment for 5 days resulted in the up-regulation of Dspp expression in a dose-dependent manner (Fig. 5A), which strongly suggests that Dspp mRNA expression is regulated, at least in part, by the Wnt canonical signaling pathway.

We evaluated the possible changes of 10E4 immunoreactivity in mDP cells (Fig. 5B). All cells displayed cell surface 10E4 immunoreactivity, which was reduced upon transfecting these cells with Sulf1 (Fig. 5B). In contrast, there was no difference in 3G10 immunoreactivity between transfected and control cells (Fig. 5C). These findings indicated that the sulfation status but not the amount and distribution of HS was affected by Sulf1.

We then attempted to confirm the physiological functions of Sulf proteins related to the tooth phenotype in vitro and to investigate the mechanism by which Sulf-mediated desulfation promotes Wnt signaling and the subsequent modulation of Dspp expression in vitro. Our in vivo findings showed that differentiating odontoblasts express both Sulf1 and Sulf2; however, the mDP cells predominantly express Sulf2 mRNA with little Sulf1 expression. Hence, to confirm the functional roles of endosulfatases in vitro, we transfected cells with a plasmid encoding an miRNA to down-regulate Sulf2 expression and GFP to enable identification of the transfected cells expressing
the Sulf2 miRNA. Using immunofluorescence, we verified that 120 h after the transfection, almost 95% of the mDP cells were GFP-positive. Real-time PCR demonstrated that miRNA against mouse Sulf2 decreased the mRNA expression levels of Sulf2 by 90% relative to the mock vector-treated control (Fig. 5D). Real-time PCR also demonstrated that Sulf2 miRNA treatment attenuated Dspp mRNA expression together with Axin2 expression (Fig. 5D).

Sodium chlorate is a competitive inhibitor of the formation of the sulfate donor required for sulfation during HS biosynthesis (40), and we confirmed that chlorate treatment resulted in a marked reduction in the binding of 10E4 antibody to mDP cells.
(Fig. 5E). In addition, chlorate treatment up-regulated Dspp mRNA expression and also expression of Axin2 mRNA (Fig. 5F). Taken together, the pharmacological interference with cell surface HSPG sulfation activates the Wnt canonical signaling pathway and consequently up-regulates Dspp mRNA expression; this could be further studied using Wnt10a-transfected cells.

Sulfation State of Cell Surface HS Modulates Wnt Canonical Signaling Pathway Induced by Wnt10a in Dentinogenesis—In a previous study, Ai et al. (38) demonstrated that QSulf1 removes 6-O-sulfates from HS chains to promote the formation of low affinity HS-Wnt complexes that can functionally interact with Frizzled receptors as a means of initiating Wnt signal transduction. In addition, we found that Wnt10a expression is found in odontoblast layers and also showed that Wnt10a directly induces Dspp expression in pluripotent mesenchymal cell lines. Taken together, we hypothesized that Sulf proteins function in dentinogenesis to control the affinity of the Wnt10a protein for HS in the extracellular matrix as a means of facilitating the binding of Wnt10a to its receptor and, consequently, of regulating the Wnt canonical signaling pathway.

To confirm the distribution of 10E4 immunoreactivity in mDP cells, 10E4 immunoreactivity was evaluated using a confocal laser-scanning microscope. Tomographic images, taken at 0.25-μm intervals, demonstrated that 10E4 immunoreactivity was detected on the cell surface (Fig. 5G). Wnt10a is specifically expressed in odontoblasts and differentiating ameloblasts (Fig. 6A). We found that the overexpression of Wnt10a induced the expression of Dspp mRNA in mDP (Fig. 6B), as shown previously in 10T1/2 cells, which are pluripotent mesenchymal cell lines (26). The application of sodium chlorate further up-regulated Dspp and Axin2 mRNA expression (Fig. 6C), indicating that reducing HS sulfation by chlorate treatment sensitizes Wnt10a, thereby inducing the Wnt canonical signaling pathway and promoting Dspp mRNA expression.

Wnt10a immunoreactivity was identified in the cytoplasm and on the cell surface of mDP cells (Fig. 6D). Chlorate treatment inhibits sulfation, and β-D-xyloside uncouples GAG synthesis from proteoglycan linkage and hence surface anchoring. Both treatments reduced the cell surface immunoreactivity of Wnt10a. These findings indicate that Wnt10a associates with sulfated cell surface GAGs (Fig. 6D).

Acting as an artificial primer of GAG chain synthesis β-D-xyloside may lead to release of HS without core proteins (42, 43). In fact, β-D-xyloside treatment significantly down-regulated Dspp expression in mDP cells. Likewise, exogenously added heparin significantly down-regulated Dspp expression in mDP cells (Fig. 6E). These findings suggest that released HS and also added heparin bind Wnt in the conditioned medium.

Wnt10a Interaction with HS Is Sensitive to 6-O-Desulfation in Vitro—To directly evaluate the specificity of Sulf-mediated 6-O-desulfation of HS on Wnt10a binding, we employed affinity chromatography on immobilized HS that had been pretreated or not with recombinant Sulf1. Earlier experiments, we could show that this treatment specifically removed the 6-O-sulfate groups located in the trisulfated UA(2S)-GlcNS(6S) disaccharide units of HS (47). Recombinant Wnt10a, loaded as a total lysate of producer cells (Fig. 7A) on the untreated HS matrix, showed strong binding, as evidenced by a high salt resistance of this interaction. In a linear NaCl gradient, Wnt10a eluted from HS at a concentration of ~460 mM (Fig. 7B), as calculated from the conductivity measured for each eluted fraction. By contrast, Wnt10a showed a significantly reduced affinity toward Sulf1-pretreated HS, eluting at an NaCl concentra-

\begin{figure}
\centering
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\caption{Sulfation of cell surface HSPG modulates the Wnt canonical signaling pathway induced by Wnt10a. A, at P0, Wnt10a mRNA was intensely detected in the odontoblast (arrows) and differentiating ameloblast (arrowheads) layers. B, the overexpression of Wnt10a potentiates Dspp mRNA expression in mDP cells, as revealed by quantitative RT-PCR (p < 0.05). C, chlorate treatment further up-regulated Dspp and Axin2 mRNA expression. D, Wnt10a immunoreactivity was identified in the cytoplasm and the cell surfaces of mDP cells. Both chlorate treatment and β-D-xyloside treatment reduced cell surface immunoreactivity for Wnt10a. E, in addition, treatment with β-D-xyloside and also with heparin reduced Dspp mRNA expression (p < 0.05). Scale bars, 500 μm (A) and 20 μm (D). Error bars, S.D.}
\end{figure}
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FIGURE 7. Affinity of Wnt10a toward heparan sulfate. A, Wnt10a could be specifically detected in a total lysate of producer cells by Western blotting. B, upon loading this lysate to immobilized heparan sulfate (HS + 6S), all Wnt10a bound to this matrix and was eluted in a linear salt gradient at the indicated NaCl concentration given below each elution fraction (FT, flow-through; W1 and W2, wash fractions; E1–E5, elution fractions). C, the salt resistance of Wnt10a binding to heparan sulfate was significantly reduced when loading the lysate on immobilized heparan sulfate that had been enzymatically 6-O-desulfated (HS-6S) by pretreatment with Sulf1. The given NaCl concentrations were calculated from conductivity measurements in each of the elution fractions.

tion of ~280 mM (Fig. 7C). Thus, Sulf1 directly affects Wnt10a binding to HS.

DISCUSSION

This study presents the first molecular evidence for the functional roles of HS sulfation in dentinogenesis. We found that the cell surface HS/HSPGs of odontoblasts are desulfated during odontoblast differentiation. The endosulfatases Sulf1 and Sulf2 are secreted to selectively remove 6-O-sulfate groups from cell surface HS/HSPGs, thereby modifying their affinity toward signaling molecules. We demonstrate that the loss of endosulfatases results in degenerative phenotypes elicited by disturbed dentin matrix formation. We found that the postsynthetic removal of sulfate moieties from HS/HSPGs modifies the Wnt canonical signaling pathway in cells of the odontogenic lineage, which regulates the expression of Dspp, a dentin-specific matrix protein. We also found that HS/HSPGs provide binding sites for Wnt10a and that specific desulfation regulates the binding affinity between Wnt10a and HS/HSPGs as a means of modulating the canonical Wnt signaling pathway.

Desulfation and Odontoblast Differentiation—HSPGs are found at the cell surface and also associated with the basement membrane (51). Tooth development is regulated by epithelial-mesenchymal interactions in the basement membrane, which is located between differentiating odontoblasts and ameloblasts and provides a scaffold for their differentiation. Our 10E4 immunocytochemistry indicated that HS/HSPGs are ubiquitously sulfated both at the cell surface and the basement membrane in developing tooth germs during early tooth development.

In odontogenesis, odontoblasts gradually lost their immunoreactivity for 10E4 during their maturation, whereas pulp cells retained their positive immunoreactivity, indicating that HS/HSPGs are specifically desulfated during odontoblast differentiation. The 10E4 antibody has been used previously to demonstrate Sulf-mediated HS desulfation (38, 52, 53); however, it is unclear which epitope the 10E4 antibody recognizes. If 10E4 recognizes HS in general, the decreased 10E4 immunostaining levels might indicate that 6-O-desulfation affects the turnover of cell surface HS/HSPGs. Therefore, we also evaluated 3G10 immunoreactivity in the control and mutant tooth germs and odontoblast cell lines. 3G10 antibody identifies a neoepitope generated by heparinase III digestion of HS (50), and the 3G10 immunocytochemical study clearly showed that distribution of HS/HSPGs in odontoblasts was changed neither by its differentiation nor by Sulf1/Sulf2 deficiency in vivo or by forced expression of Sulf1 in vitro. Therefore, decreased 10E4 immunostaining levels in the odontoblast layer obviously are due to 6-O-desulfation of HS/HSPGs.

Our in vitro study confirmed the presence of 10E4 immunoreactivity on the cell surfaces of odontoblast cell lines and showed that the forced expression of Sulf1, which selectively desulfates 6-O-sulfated residues, almost completely diminished the immunoreactivity to 10E4. Furthermore, gene ablation of Sulf1 and Sulf2 led to the suppression of differentiation-associated HSPG desulfation in odontoblasts. Taken together, Sulf1 and Sulf2 are essential for temporally and spatially regulating the 6-O-desulfation of odontogenic cells during their differentiation.

In contrast to the specific desulfation of odontoblast cell surface HS/HSPGs during differentiation, 10E4 immunoreactivity on the basement membrane was not affected by odontoblast differentiation or by Sulf1 or Sulf2 deficiency. During progressive tooth development, the direct interaction between the epithelium and mesenchyme is degraded when these cells start to secrete the matrix on the basal membrane side, and the basement membrane is replaced with predentin. Actually, a previous study showed that immunoreactivity to perlecan, an extracellular matrix HSPG, is intense in the basement membrane and is continuously detected in areas of predentin during the production of the dentin matrix (54). As in the basement membrane, 10E4 immunoreactivity was also present in the predentin regions, but it was not affected by odontoblast differentiation or by Sulf1 or Sulf2 deficiency. These findings indicate that matrix HS/HSPGs in the basement membrane are highly sulfated, whereas predentin shows little sulfation during tooth development. Therefore, it is likely that their functional roles differ from the differentiation-associated regulatory role of cell surface HS/HSPGs.

Defective Phenotypes of Sulf1- and Sulf2-deficient Molars—Sulf1/Sulf2 double mutant mice characterizedly showed dentin and predentin thinning and shortening of their roots. Because the morphology and size of the molars and enamel thickness were not affected by Sulf1 and Sulf2 deficiency, tooth
phenotypes are odontogenic lineage cell specific. Significantly, Sulf proteins are not essential for odontoblast differentiation itself because odontoblast cells in Sulf1/2 null mutant tooth also exhibit a columnar shape with preodontin formation. On the other hand, the Sulf protein-defective phenotypes are similar to Dspp null mutant phenotypes. Human DSPP mutations also cause hypodontia and/or oligodontia. Our in vitro study also confirmed the functional roles of Sulf proteins in odontoblasts, and actually, the down-regulation of Dspp by RNAi suppressed Dspp expression in odontoblastic cell lines. The down-regulation of Dspp and Axin2 mRNA expression was also observed in vivo in Sulf1/2 null mutant molars by in situ hybridization. Real-time PCR also confirmed significant down-regulation of Dspp and Axin2 mRNA expression in the mutant tooth germs. These data provide the first direct genetic evidence that the Sulf enzymes are specifically involved in dentinogenesis in odontoblasts. Importantly, we found that the Sulfs play regulatory, but not obligatory, roles in Dspp expression during dentinogenesis.

Because the individual knock-out of Sulf1 or Sulf2 did not produce altered tooth phenotypes, Sulf1 and Sulf2 display functional redundancy in dentinogenesis. This redundancy was also evident in skeletonogenesis (35).

Activation of Wnt Canonical Signaling Pathway in Dentinogenesis—Dspp expression is influenced by various growth signaling molecules, such as BMP, FGF, and Wnt, in a complexed manner in vivo. Sulf proteins also modulate the function of heparan sulfate by altering the binding sites for these signaling molecules. It is established that Sulf proteins are negative regulators of FGF signaling and positive regulators of Wnt signaling. Our mutant phenotypes revealed that Sulf proteins are positive regulators of dentinogenesis, and previous studies have demonstrated that the Wnt canonical signaling pathway is activated in dentinogenesis. Although FGF signaling is also activated in dentinogenesis, we hypothesize that Wnt signaling is the main target of Sulf-mediated HSPG modification during dentinogenesis.

The activation of the canonical Wnt signaling pathway can be achieved with LiCl. Our attempt to mimic Wnt signaling with LiCl clearly indicated the functional importance of Wnt canonical signaling in Dspp mRNA induction in odontoblasts. Although the findings suggested that the Wnt canonical signaling pathway is involved in dentinogenesis, our results are the first to provide direct evidence that Wnt canonical signaling is involved in the promotion of Dspp mRNA expression.

In our study, chlorate treatment, which pharmacologically blocked sulfation of cell surface HSPGs (40), up-regulated Dspp mRNA expression in odontoblasts and induced Axin2 mRNA expression. On the contrary, the Sulf2 RNAi-induced down-regulation of Dspp expression was accompanied by the suppression of Axin2 expression. In other developmental systems, it has been established that Sulf enzymes function as positive regulators of Wnt signaling (29, 38). Our results indicating that Sulf proteins promote Wnt signaling during dentinogenesis are in line with those previous studies.

Sulf Modification of Wnt10a-induced Signaling in Dentinogenesis—In the present study, pharmacological interference of HSPG sulfation by chlorate treatment augmented canonical Wnt signaling in mDP cells and subsequently up-regulated Dspp expression in these cells. We also detected Wnt10a immunoreactivity on the cell surfaces of odontoblastic cell lines, and chlorate treatment decreased this immunoreactivity. Similarly, Wnt10a protein disappeared from the odontoblast cell surface upon a 72-h treatment with xyloside. β-D-Xyloses linked to hydrophobic glycones act as artificial primers of GAG chain synthesis and block proteoglycan assembly (42, 43). Therefore, HS would be released without core proteins and could form a complex with Wnts, including Wnt10a, in the conditioned medium. Like β-D-xyloses treatment, exogenously added heparin also significantly down-regulated Dspp expression in mDP cells in the present study, suggesting that released HS, like heparin, provides binding sites for Wnt10a. Finally, our affinity chromatography data provide clear biochemical evidence for a direct physical interaction between Wnt10a and HS and demonstrate that the binding affinity of HS toward Wnt10a is reduced by 6-O-desulfation; it should be noted that the 6-O-desulfated HS used here had been generated through pretreatment of HS with recombinant Sulf1 in vitro, and its specific desulfation had structurally and functionally been characterized earlier (47). Taken together, these findings indicate that HSPGs on the cell surface provide binding sites for Wnt10a in odontoblasts and that specific sulfation patterns are edited post-synthetically by the Sulf enzymes, thereby regulating the binding affinity between Wnt10a and HSPGs as a means to modulate the canonical Wnt signaling pathway in dentinogenesis.

A previous study showed that Sulf proteins function autonomously to remodel the sulfation state of cell surface HS chains and promote Wnt signaling. However, there are some discrepancies among developmentally dynamic Sulf mRNA expression, 10E4 immunoreactivity, and TOPGAL reporter expression (23). Indeed, Wnt10a mRNA expression and TOPGAL reporter expression are only localized in the odontoblast layer. In contrast, Sulf1 and Sulf2 are only slightly expressed in odontoblasts but are intensely expressed in the dental pulp cells overlying odontoblasts. Interestingly, desulfated regions, which are identified by their negative immunoreactivity to 10E4, were expanded in the odontoblast layer as well as the Sulf1- and Sulf2-expressing layer. Hence, it is likely that the Sulfs, which are secretory proteins, also possess non-cell-autonomous or paracrine activity (36) and that odontoblasts are exposed to Sulf...
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proteins, which are released from the overlying pulpal cells, in a paracrine manner.

In conclusion, we found that the 6-O-desulfation of extracellular HSPGs is an important postsynthetic modification that is critical for the activation of Wnt signaling in odontoblasts and subsequent dentin matrix production. Notably, the loss of the Sulf 6-O-endosulfatasas results in degenerative phenotypes elicited by disturbed dentin matrix formation. Our findings indicate that the Sulf enzymes catalyze HS 6-O desulfation at the cell surface of odontoblast cells and that such postsynthetic modification of the HSPG sulfation status induces Wnt10a-mediated activation of odontoblast differentiation (Fig. 8).

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