Lacrimal Gland Development and Fgf10-Fgfr2b Signaling Are Controlled by 2-O- and 6-O-sulfated Heparan Sulfate*

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Heparan sulfate, an extensively sulfated glycosaminoglycan abundant on cell surface proteoglycans, regulates intercellular signaling through its binding to various growth factors and receptors. In the lacrimal gland, branching morphogenesis depends on the interaction of heparan sulfate with Fgf10-Fgfr2b. To address if lacrimal gland development and FGF signaling depends on 2-O-sulfation of uronic acids and 6-O-sulfation of glucosamine residues, we genetically ablated heparan sulfate 2-O and 6-O sulfotransferases (Hs2st, Hs6st1, and Hs6st2) in developing lacrimal gland. Using a panel of phage display antibodies, we confirmed that these mutations disrupted 2-O and/or 6-O but not N-sulfation of heparan sulfate. The Hs6st mutants exhibited significant lacrimal gland hypoplasia and a strong genetic interaction with Fgf10, demonstrating the importance of heparan sulfate 6-O sulfation in lacrimal gland FGF signaling. Altering Hs6st caused a much less severe phenotype, but the Hs2st;Hs6st double mutants completely abolished lacrimal gland development, suggesting that both 2-O and 6-O sulfation of heparan sulfate contribute to FGF signaling. Combined Hs2st;Hs6st deficiency synergistically disrupted the formation of Fgf10-Fgfr2b-heparan sulfate complex on the cell surface and prevented lacrimal gland induction by Fgf10 in explant cultures. Importantly, the Hs2st;Hs6st double mutants abrogated FGF downstream ERK signaling. Therefore, Fgf10-Fgfr2b signaling during lacrimal gland development is sensitive to the content or arrangement of O-sulfate groups in heparan sulfate. To our knowledge, this is the first study to show that simultaneous deletion of Hs2st and Hs6st exhibits profound FGF signaling defects in mammalian development.

Heparan sulfate is a cell-surface glycosaminoglycan playing important roles in the transport and signaling of multiple growth factors, including Hedgehog, Wnt, bone morphogenic protein (BMP), and fibroblast growth factor (FGF) (1–3). Heparan sulfate is first synthesized from the activated monosaccharides, UDP-glucuronic acid and UDP-N-acetylgalactosamine, by an Ext copolymerase complex to form a copolymer of glucuronic acid and N-acetylgalactosamine. Polymerization is followed by N-deacetylation/N-sulfation of subsets of N-acetylgalactosamine residues by N-deacetylasen-sulfotransferase (Ndst)2 enzymes (4). Because of the incomplete processing by the Ndst enzymes, the polysaccharide backbone is divided into stretches of variable length of N-sulfated disaccharides (NS domains) and N-acetylated disaccharides (NA domains). A portion of the D-glucuronic acid residues in the NS domains is next converted by glucuronil C5-epimerase (Hsepi) into l-iduronic acids. A 2-O-sulfotransferase (Hs2st) transfers a sulfate group to the C-2 carbon of the iduronic acids and less frequently to glucuronic acid. Finally, 6-O-sulfotransferases (Hs6st) and more rarely 3-O-sulfotransferases (Hs3st) add sulfate groups to the C-6 and C-3 carbon of the glucuronic residues, respectively. These reactions do not go to completion, leading to great structural complexity within the NS domains. Additional complexity of heparan sulfate can be generated at the cell surface, as membrane-bound Sulf1 and Sulf2 specifically remove a subset of 6-O sulfates from heparan sulfate, whereas extracellular heparanase can cleave and release fragments of the heparan sulfate chain.

The interaction of heparan sulfate with FGF and FGF receptors is still under considerable scrutiny (5–14). At least in vitro, the FGF family proteins and their receptors often bind with increasing affinity to more highly sulfated heparan sulfates, suggesting a lack of specificity with respect to the precise arrangement of N-, 2-O, and 6-O sulfate groups (11, 15, 16). In Drosophila, tracheal branching morphogenesis depends on FGF signaling mediated by the interaction of the FGF homolog branchless with the FGF receptor, breathless. Mutating individually Hs2st and Hs6st genes apparently causes minimum consequence in trachea branching morphogenesis (17). However, strong trachea defects are revealed in Hs2st;Hs6st double mutants, demonstrating that the 2-O-sulfate and 6-O-sulfate groups are individually dispensable for heparan sulfate function in FGF signaling in this system. These observations and the

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2 The abbreviations used are: Ndts, N-deacetylase-N-sulfotransferase; NS domain, N-sulfated disaccharide domain; NA domain, N-acetylated disaccharide domain; LACE, ligand and carbohydrate engagement; MEF, mouse embryonic fibroblast; FGFR, FGF receptor.
related finding that many organs that depend on heparan sulfate appear to develop normally in mutants lacking individual sulfotransferases (18–21) have led to the idea that the interaction of FGFs and FGF receptors with heparan sulfate shows little specificity.

In counterpoint to this conclusion, other studies have pointed to specificity in the interaction of some FGF family members with their receptors. Binding studies of oligosaccharides to FGFs showed that Fgf10 requires 6-O-sulfate but not 2-O-sulfate groups, whereas Fgf2 requires only 2-O-sulfate and N-sulfate groups (22). The selectivity of FGFs becomes even more pronounced when presented in a complex with FGFR and endogenous heparan sulfate in mouse embryos. Using a ligand and carbohydrate engagement assay (LACE), Allen and Rapraeger (23) have shown that 2-O-sulfation of heparan sulfate is required for Fgf1-Fgfr2b binding but not for Fgf1 or Fgf1-Fgfr2c binding. Heparan sulfate 2-O and 6-O-sulfation was found to promote Fgf10-mediated end budding or duct elongation in submandibular gland culture, and distinct organ specific phenotypes were observed in vivo when murine 2-O and 6-O-sulfation genes, Hs2st and Hs6st1, were mutated (18, 20, 24–27). Similarly, we have shown in lens development that a lack of heparan sulfate N-sulfation disrupts its interaction with Fgf8b-Fgfr3c but not with the Fgf8b-Fgfr3b pair (28). We recently showed that N-sulfated heparan sulfate is highly enriched in the lacrimal gland bud, which potentiates a restricted activation of FGF signaling during lacrimal gland outgrowth (29).

Taken together, these findings suggest that FGF signaling is sensitive to the positional distribution of sulfate groups in the heparan sulfate chains.

In this study we took a similar approach taken by Kamimura et al. (17) in their study of FGF signaling in Drosophila and investigated the role of heparan sulfate sulfation sequence in lacrimal gland development in mice by deleting Hs2st and Hs6st genes. In contrast to the lack of branching morphogenesis defects in the Drosophila Hs6st mutants, we showed that the loss of murine Hs6st significantly disrupted lacrimal gland formation. The Hs2st mutant phenotype was much weaker, but the Hs2st/Hs6st double mutants completely abolished lacrimal gland development. These results demonstrated that vertebrate FGF signaling in the lacrimal gland indeed depends on specific sulfation of the chains.

**EXPERIMENTAL PROCEDURES**

**Mice**—Hs6st1flox and Hs2sflox mice were obtained from Lexicon Genetics via Mutant Mouse Regional Resource Centers (MMRRC:011715-UCD). Fgfr3c—mice were kindly provided by Dr. Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel) and Dr. Richard Lang (Children's Hospital Research Foundation, Cincinnati, OH) (31). All experiments were performed in accordance with institutional guidelines.

**Immunohistochemistry and RNA in Situ Hybridization**—Immunohistochemistry was performed on cryosections or paraffin sections as previously described (28, 29). For phospho-ERK staining, the Tyramide Signal Amplification kit (TSA™ Plus System, PerkinElmer Life Sciences, Waltham, MA) was used to amplify the signal (32). The antibodies used were anti-phospho-ERK1/2 (#9101, Cell Signaling Technology, Beverly, MA) and anti-phosphohistone H3 (#06–570, Upstate Biotechnology, Temecula, CA). TUNEL assays were performed on 10-μm paraffin sections following the manufacturer’s instructions in the In Situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN). Cell proliferation and apoptosis rates were calculated as the ratio of phosphohistone H3 or TUNEL-positive cells against DAPI-positive cells, and results were analyzed by one-way analysis of variance.

A series of phage-display derived antibodies with VSV-tag were used to detect specific modifications of heparan sulfate in tissue sections (33). Cryo-sections were hydrated in PBS for 10 min, quenched of peroxidase activity by 3% H2O2 and 10% methanol in PBS solution, and then blocked with 2% BSA in PBS at room temperature for 1 h followed by incubation with phage-display-derived antibodies (1:1–1:5 diluted with 0.2% BSA/PBS) at 4 °C overnight. After PBS washing 3 times, sections were incubated with rabbit anti-VSV antibody (#563, MBL, Woburn, MA) for 2 h at room temperature and rinsed in PBS before a 1-h incubation with HRP-labeled anti-rabbit antibody. The signal was amplified and detected using Tyramide Signal Amplification kit. The total heparan sulfate was detected using 3G10 antibody (Seikagaku, Tokyo, Japan) after the section was treated with heparitinase I.

RNA in situ hybridization on cryosections was performed according to a standard protocol (29). The following probes were used: Erm (from Dr. Bridget Hogan, Duke University Medical Center, Durham, NC), Hs6st1, Hs6st2, and Hs6st3 (34). Hs2st probe was generated from a full-length cDNA clone (IMAGE: 6849136, Open Biosystems, Huntsville, AL). At least three embryos of each genotype were analyzed for each probe.

**FGF-FGFR Complex Binding Assay**—The binding of the FGF-FGFR complex with heparan sulfate was examined using the LACE assay as previously described (28, 29). Briefly, 10 μm paraffin sections of the mutant embryos and their matched littermates were deparaffinized and rehydrated followed by incubation in 0.5 mg/ml sodium borohydride for 10 min and in 0.1 M glycine for 30 min and then blocked with 2% BSA for 1 h at room temperature. Next, the sections were incubated with a mixture of 20 μM FGF and 20 μM human FGFR-Fc chimera (both from R&D Systems, Minneapolis, MN) in RPMI 1640 with 10% FBS at 4 °C overnight. After rinsing three times with PBS, the slides were incubated with cy3-labeled anti-human Fc IgG antibody for 2 h at room temperature. The fluorescent signal was examined using a Leica DM5000 fluorescent microscope.

**Explant Culture**—Lacrimal gland explant cultures were carried out with E13.5–E14.5 embryos as described (29). Briefly, 80–120-μm diameter heparin acrylic beads (Sigma) were washed in PBS and incubated with 250 μg/ml recombinant FGF10 (R&D Systems) or 5 mg/ml BSA at 4 °C overnight. The whole eye with ectoderm and surrounding mesenchyme was dissected and laid flat on a filter paper (Nitrocellulose Membrane Black Gridded, 0.45 μm pore, Millipore, Billerica, MA). After FGF10- or BSA-soaked beads were punched into the periocular mesenchyme by forceps, the tissue...
on top of the filter was floated in the culture medium (CMRL-1066 supplemented with 10% FBS, 4 mM L-glutamine, 0.1 mM nonessential amino acids, and antibiotics (Invitrogen, Carlsbad, CA). Explants were cultured for 48 h in a 37 °C humidified incubator with 5% CO₂, and the GFP expressing lacrimal gland buds were examined and photographed under a Leica MZ16F dissecting microscope.

Mouse Embryonic Fibroblast (MEF) Cells—Primary MEF cells were isolated from embryos at the E13.5 to E14.5 stage. Briefly, the uterine horns were dissected from pregnant females and rinsed in 70% (v/v) ethanol before transfer into sterilized PBS. After the heads and the internal organs were cut away, the trunks were washed with fresh PBS to remove blood cells, finely minced into small pieces in a minimal amount of PBS, and digested in 1–2 ml of 0.25% trypsin-EDTA for 10 min at 37 °C under gentle agitation. The supernatant was combined with 2 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium vanadate) with protease and phosphatase inhibitors (Pierce). After centrifugation at 10,000 × g for 15 min at 4 °C, 30 μg of cell lysate protein was analyzed by SDS-PAGE (12% gels) and transferred to Millipore Immobilon FL PVDF membranes (Millipore). The membranes were blocked in Odyssey blocking buffer for 1 h with gentle shaking followed by an overnight incubation in the mixture of mouse anti-Phospho-ERK1/2 (sc-7383, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-ERK1/2 (#4695, Cell Signaling Technology) antibodies. After rinsing with PBS-T (0.1% Tween 20 in PBS) 3 times at 4 °C, the blots were incubated with IRDye-linked anti-mouse and anti-rabbit secondary antibodies for 1 h and washed 3 times with PBS

twice with PBS. The membrane was scanned in an Odyssey SA scanner (LICOR Biosciences, Lincoln, NE), and band intensities were quantified using the Odyssey software.

Heparan Sulfate Disaccharide Compositional Analysis—Disaccharide analysis of heparan sulfate extracted from primary MEFs was carried out using procedures previously described (37). Briefly, heparan sulfate was extracted from MEF cell pellets by exhaustive digestion with Pronase (Sigma) in PBS at 37 °C for 24 h followed by anion-exchange chromatography (DEAE-Sephasel, GE Healthcare). Heparan sulfate was eluted with μl of buffer containing 40 mM ammonium acetate and 3.3 mM calcium acetate, pH 7. Heparan sulfate disaccharides were then tagged with [13C6]aniline. For compositional analysis, each sample was mixed with an equimolar amount of [13C6]aniline-tagged disaccharide standards. Aniline-derivatized disaccharides were separated on a C18 reversed-phase column (Phalanx 1 × 150-mm micro-bore, 5 μm, Higgins Analytical) with 5 μl concentrations of the ion pairing agent dibutylationine (Sigma) and 8 mM acetic acid, operated at a flow rate of 50 μl/min. Disaccharides with higher sulfation were eluted with increasing methanol concentration using a step gradient produced on a Dionex U3000 capillary HPLC and analyzed in tandem by a LTQ Orbitrap mass spectrometer (Thermo, San Jose, CA). Data were analyzed with the Xcalibur software supplied with the mass spectrometer.

RESULTS

Hs2st and Hs6st Are Required for Lacrimal Gland Development—Lacrimal gland development is an excellent model for studying the role of heparan sulfate in FGF signaling (29, 38, 39). Arising from the conjunctival epithelium at the temporal side of the eye, the budding of the lacrimal gland is critically dependent on Fgf10 expressed in the periocular mesenchyme (40, 41). Heparan sulfate 2-O sulfotransferase (Hs2st) and two of the heparan sulfate 6-O sulfotransferases (Hs6st1 and -2) were detected in the E14.5 lacrimal gland bud by RNA in situ hybridization using their antisense probes (Fig. 1, A–D). As a control, no signal was observed with the corresponding sense probes (data not shown). We, thus, generated the lacrimal gland-specific ablations of Hs2st and Hs6st1/2 genes using the Le-Cre driver, which we have previously shown to express both Cre recombinase and GFP reporter in the lacrimal gland epithelium (29). At E14.5, when lacrimal gland buds became visible in the wild type embryos (Fig. 1E), no obvious defects were observed in the Le-Cre;Hs2st CKO (hereafter referred as Hs2st CKO) (Fig. 1F), the Le-Cre;Hs6st1 CKO, and the Hs6st2 CKO (hereafter referred as Hs6st2 CKO) mutant embryos exhibited stunted (47%) or even no (17%) lacrimal gland bud (Fig. 1, G and M). The most severe phenotype was observed in the Le-Cre;Hs2st CKO; Hs6st1 CKO; Hs6st2 CKO (hereafter referred as Hs2st CKO; Hs6st1 CKO; Hs6st2 CKO) mutants, where the majority (61%) of the embryos lack lacrimal gland bud at E14.5 (Fig. 1, H and M). At birth, 50% of the Hs2st CKO mutants also exhibited diminutive lacrimal
glands with few side branches, whereas 59% of the Hs6stCKO and 100% of the Hs2stCKO;Hs6stCKO completely lost lacrimal glands (Fig. 1, I–M). Therefore, ablation of Hs6sts resulted in significantly more severe phenotype than that of Hs2st, but Hs2st and Hs6st also exhibited genetic synergy in lacrimal gland branching morphogenesis.

Disruption of Heparan Sulfate O-Sulfation in the Hs2st and Hs6st Mutants—To determine whether the mutations affected heparan sulfate composition in situ, we stained tissue sections with mAb 3G10 after heparinase digestion, which creates a neo-epitope in place of each chain. 3G10 antibody staining was unchanged in all lacrimal gland buds (Fig. 2, A–D), indicating that altering sulfation per se did not affect the number of heparan sulfate chains in the tissue. We next used a panel of phage-display antibodies to examine the sulfation patterns of heparan sulfate in the mutants. The first step in heparan sulfate modification catalyzed by Ndst is the formation of N-sulfated glucosamine residues, which can be recognized by the HS4E4V antibody (42). As expected, the HS4E4V staining was preserved in the Hs2st and Hs6st mutants (Fig. 2, E–H). In contrast, the AO4B08 staining, which was reported to require N-, 2-, and 6-O-sulfated heparan sulfate, was abolished in all Hs2st and Hs6st mutants (Fig. 2, I–L) (42). Therefore, altering Hs2st and Hs6st resulted in loss of 2-O and 6-O sulfation but not N-sulfation. EV3C3V antibody was reported to require N- and 2-O-sulfated heparan sulfate epitopes (43), and it strongly stained the basement membrane of the wild type and Hs6stCKO lacrimal gland buds but not that of the Hs2stCKO and Hs2stCKO; Hs6stCKO mutants (Fig. 2, M–P). Finally, using RB4EA12, which was reported to be specific for N- and 6-O-sulfate groups (43),
we observed that heparan sulfate 6-O sulfation was lost in the Hs6st<sup>cko</sup> and the Hs2st<sup>cko</sup>;Hs6st<sup>cko</sup> mutants but strongly elevated in the Hs2st<sup>cko</sup> mutants (Fig. 2, Q–T). These results showed that Hs2st and Hs6st mutations indeed disrupted the addition of 2-O and 6-O sulfate groups and that there was an increase in 6-O sulfation in the Hs2st<sup>cko</sup> mutant.

Hs6st Genetically Interacts with Fgf10 in Lacrimal Gland Budding—Both human and mouse Fgf10 mutations can cause lacrimal gland aplasia similar to that observed in Hs6st<sup>cko</sup> newborn pups, suggesting that Fgf10 may genetically interact with Hs6st in lacrimal gland development (41, 44). To test this idea, we crossed Fgf10 with Hs6st<sup>cko</sup> mutant mice and collected embryos at E14.5. Although Fgf10<sup>−/−</sup> animals were previously reported to lack lacrimal gland at birth, we observed that these mutants retained either normal (3 of 12) or stunted (9 of 12) lacrimal gland buds at E14.5 (Fig. 3, B and E). The majority of the Hs6st<sup>cko</sup> embryos (10 of 12) also exhibited some degree of lacrimal gland budding (Fig. 3, C and E; also Fig. 1M). In contrast, 10 of 12 Fgf10<sup>−/−</sup>;Hs6st<sup>cko</sup> embryos did not show any lacrimal gland buds (Fig. 3, D and E), strongly suggesting Fgf10 and Hs6st operate in the same genetic pathway in lacrimal gland development.

Defective Fgf10 Signaling in the Hs2st and Hs6st Mutants—To investigate the mechanism of Hs6st function in Fgf10 signaling, we next carried out the LACE assay to examine the in situ binding of lacrimal gland heparan sulfate with Fgf10-Fgfr2b, the known FGF ligand/receptor pair in lacrimal gland development (23, 28). In the following experiments, we took care to select the Hs2st<sup>cko</sup>/Hs6st<sup>cko</sup> mutants that exhibited at least a small lacrimal gland bud, although similar results were also observed in the conjunctival epithelium from the more severe mutants that lacked any lacrimal gland budding (data not shown). As we have shown previously, soluble Fgf10 and Fgfr2b can bind the lacrimal gland sections through their interaction with endogenous heparan sulfate in wild type embryos (Fig. 4A). As a control, such binding was eliminated in sections pretreated with heparan sulfate-degrading enzyme heparitinase I (data not shown). Finally, Fgf10-Fgfr2b binding was weakened in the Hs2st<sup>cko</sup> and the Hs6st<sup>cko</sup> mutants and abolished in the Hs2st<sup>cko</sup>;Hs6st<sup>cko</sup> mutants (Fig. 4, B–D, arrow), suggesting that heparan sulfate O-sulfation was essential for Fgf10-Fgfr2b interaction on lacrimal gland cell surface.

Fgf10 signaling is known to induce ERK phosphorylation and expression of Erm transcription factor in the lacrimal gland bud (28, 29). Although RNA in situ hybridization analysis showed that Fgf10 and Fgfr2b expression were unchanged (data not shown), phospho-ERK and Erm levels were reduced in the Hs2st<sup>cko</sup> and the Hs6st<sup>cko</sup> mutants and completely lost in the remnants of the Hs2st<sup>cko</sup>;Hs6st<sup>cko</sup> mutant buds (Fig. 4, E–L, arrows). Consistent with this finding, the Hs2st<sup>cko</sup>;Hs6st<sup>cko</sup> mutants exhibited much reduced phosphohistone H3 (pH3) labeling for cell proliferation and increasing TUNEL staining for apoptosis (Fig. 5, A–J). These results demonstrated that Hs2st and Hs6st mutations disrupted Fgf10-dependent downstream signaling and lacrimal gland outgrowth.

Combinatorial Contribution of Heparan Sulfate 2-O and 6-O Sulfation in Lacrimal Gland Fgf10 Signaling—Having established the importance of heparan sulfate 2-O and 6-O sulfation in lacrimal gland Fgf10 signaling, we next turned to the Hs2st and Hs6st MEF cells to extend our analysis to cellular level. MEF cells isolated from Hs2st<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> and Hs6st<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>;Hs6st<sup>−/−</sup> embryos were first immortalized with a retrovirus expressing SV40 large T antigen followed by infection of a Cre-expressing adenovirus to convert the floxed alleles to null alleles. After clonal selection, the Hs2st<sup>−/−</sup> and Hs6st<sup>−/−</sup> cells were propagated indefinitely in culture to ensure complete turnover of heparan sulfate that was present before adenoviral infection. As expected, disaccharide analysis of heparan sulfate from Hs2st<sup>−/−</sup> cells showed a complete loss of disaccharides containing 2-O-sulfate groups (D0S0). Less dramatic 2-O-sulfation (D0S<sup>−</sup>) was noted in each of the single
When total sulfation of heparan sulfate was determined by summation of N- and O-sulfates, the overall level of sulfation did not differ significantly in the single and double mutants compared with wild type (Fig. 6B). In general, the alterations in heparan sulfate composition in the fibroblasts resembled the changes inferred by in situ assay of tissue sections from the lacrimal gland with single chain antibodies (Fig. 2). We next examined Fgf10 signaling in the Hs2st<sup>-/-</sup> and Hs6st<sup>-/-</sup> cells by measuring its downstream ERK phosphorylation. As shown by quantitative Western blot, wild type MEF cells responded to Fgf10 stimulation with a rapid increase in ERK phosphorylation, whereas the peak level of such response was only slightly reduced in the Hs2st<sup>-/-</sup> and Hs6st<sup>-/-</sup> cells (Fig. 6, C and D). Importantly, the Hs2st<sup>-/-</sup>;Hs6st<sup>-/-</sup> cells

**FIGURE 3.** Genetic interaction between Hs6st and Fgf10. A–D, lacrimal gland bud was present in both Fgf10<sup>-/-</sup> and Hs6st<sup>CKO</sup> mutants at E14.5 but lost in the Fgf10<sup>-/-</sup>;Hs6st<sup>CKO</sup> compound mutants (arrow). E, quantification of the lacrimal gland phenotype is shown.

**FIGURE 4.** Hs2st/Hs6st mutants disrupted Fgf10/Fgfr2b signaling in lacrimal gland development. A–D, in situ binding (LACE assay) of Fgf10/Fgfr2b on lacrimal gland sections were reduced in the Hs2st<sup>CKO</sup> and Hs6st<sup>CKO</sup> single mutants and abolished in the Hs2st<sup>CKO</sup>;Hs6st<sup>CKO</sup> double mutants (arrow). E–L, downstream of FGF signaling, phospho-ERK and Erm expression was also reduced in the Hs2st<sup>CKO</sup> and Hs6st<sup>CKO</sup> single mutants and lost in the Hs2st<sup>CKO</sup>;Hs6st<sup>CKO</sup> double mutants (arrow).
exhibited dramatic reduction in Fgf10 response, consistent with the critical role of heparan sulfate 2-O and 6-O sulfations in Fgf10 signaling.

Finally, we performed explant culture experiments to assay Fgf10 signaling response directly in the Hs2st and Hs6st lacrimal glands. After culturing for 2 days, wild type eye rudiments readily grew endogenous lacrimal glands, which were unper- turbed by BSA-soaked beads (Fig. 7A, arrow). Ectopic lacrimal gland budding, however, was only observed in response to Fgf10-soaked beads implanted around the eye (Fig. 7B, arrowhead). Interestingly, Fgf10 beads also induced ectopic lacrimal glands in the Hs2stCKO and the Hs6stCKO explants, suggesting that these mutants could still respond to high levels of exogenous Fgf10. In contrast, both endogenous and ectopic lacrimal gland budding were lost in the Hs2stCKO;Hs6stCKO mutant cultures (p = 0.0005, Fisher’s test). Therefore, ablations of both Hs2st and Hs6st activities were necessary to completely abolish Fgf10 signaling in lacrimal gland budding.

DISCUSSION

All cells synthesize heparan sulfate and, therefore, must express at least a core set of biosynthetic enzymes required for chain polymerization (Ext1, Ext2, and Ext13), and processing (one or more Ndsts, Hsepi, and Hs2st and one or more Hs6sts and Hs3sts). However, it has long been recognized that the levels of the various enzymes are expressed in a dynamic pattern in different tissues. For example, Hs6st1 is strongly expressed in epithelial cells, whereas Hs2st and Hs6st2 are found expressed more strongly in mesenchymal compartments (18, 34). It is, therefore, surprising that the Hs6st1 systemic knockouts lack major organogenesis defects, Hs6st2 and Ndst2 mice are viable and fertile (19–21), and mutants altered in Ndst1 or Hs2st exhibit specific organ and tissue abnormalities. These observations suggest that heparan sulfate, although essential for organismal growth and development, plays different roles in different tissues.

It has been known since 1991 that members of the FGF family of growth factors and their receptors interact with heparan sulfate, which serves as a coreceptor by facilitating the formation and stability of FGF-FGFR complexes. Despite extensive biochemical studies in support of a role for heparan sulfate in FGF signaling, none of the murine Hs2st and Hs6st knock-out phenotypes has been conclusively attributed to FGF signaling defects. We previously showed that lacrimal gland develop- ment requires specific modification of heparan sulfates by Ndst genes in the epithelial cells at the tip of the lacrimal gland bud.

FIGURE 5. Cell proliferation and apoptosis defects in the Hs2st/Hs6st mutants. A–D, the Hs2stCKO;Hs6stCKO mutants exhibited reduced expression of cell proliferation marker phosphohistone H3 (pHH3). E–H, although the wild type control lacrimal gland showed no TUNEL staining, all Hs2stCKO and Hs6stCKO mutant lacrimal gland primordia contained TUNEL-positive cells. I and J, quantification of phosphohistone H3 and TUNEL staining (*, p < 0.01).
In this system, Ndst1-modified heparan sulfate serves as a coreceptor enabling the formation of Fgf10-Fgfr2b complexes and downstream signaling via phosphorylation of Shp2 and Erk. Reduction of Ndst1 causes reduced N-sulfation, which is prerequisite for all downstream reactions, including 2-O-sulfation and 6-O-sulfation. Thus, the role of O-sulfation in Fgf10-Fgfr2b signaling and lacrimal gland development remained an open question.

In this study we have generated combinatorial ablations of Hs2st, Hs6st1, and Hs6st2 in the lacrimal gland, which led to specific loss of heparan sulfate 2-O or 6-O sulfation and distinct developmental defects. In support of heparan sulfate O-sulfation in promoting FGF signaling, we showed that loss of Hs6st decreased lacrimal gland development and that Hs6st1 genetically interacted with Fgf10 in lacrimal gland budding. Because 2-O-sulfation increases somewhat in the absence of Hs6st, the contribution of 6-O-sulfation may be somewhat underestimated. Compounding the mutants resulted in complete loss of signaling and potentiated the loss of lacrimal gland development. Furthermore, Hs2st/Hs6st mutations disrupted cell surface Fgf10-Fgfr2b assembly and downstream ERK signaling. The profound effect of compounding these mutations suggests that both 2-O-sulfation and 6-O-sulfation of heparan sulfate contribute to Fgf10-Fgfr2b signaling. To our knowledge, this is the first report of a genotype that completely disrupts Fgf10-Fgfr2b signaling.

**FIGURE 6. Disruption of Fgf10 signaling in the Hs6st/Hs2st knock-out MEF cells.** A, immortalized MEF cells were clonally selected to generate the Hs2st and Hs6st null (Hs2st+/−, Hs6st+/−, and Hs2st−/−Hs6st−/−) cells. The disaccharide composition of heparan sulfate derived from the cells was determined by enzymatic depolymerization and liquid chromatography/mass spectrometry. Each disaccharide is defined by a structure code: D0H0, ΔUA-GlcNH2; D0H6, ΔUA-GlcNH2-6S; D2H0, ΔUA2S-GlcNH2; D0A0, ΔUA-GlcNAc; D0S0, ΔUA-GlcNS; D2H6, ΔUA2S-GlcNH2-6S; D0A6, ΔUA-GlcNAc6S; D0S6, ΔUA-GlcNS6S; D2A6, ΔUA2S-GlcNAc6S; D2S0, ΔUA2S-GlcNS; D2A6, ΔUA2S-GlcNAc6S; D2S6, ΔUA2S-GlcNS6S, where ΔUA = 4,5-unsaturated uronic acid generated during the heparin lyase reaction (47). *, one-way analysis of variance: p < 0.05 for the comparison with the wild type. The data reflected three independent experiments for each cell line and were confirmed in two independently cloned cells for each genotype. B, the amount of N-acetyl groups and sulfate groups at various positions was calculated from the data in panel A. *, one-way analysis of variance: p < 0.05 for the comparison with the wild type; N.S., not significant. C, the Hs2st and Hs6st knock-out MEF cells were stimulated with 10 ng/ml Fgf10, and the time course of ERK phosphorylation was determined by fluorescent Western blot. D, the relative level of phospho-ERK was significantly reduced in the Hs2st−/−;Hs6st−/− cells. Three independent experiments were performed for each cell line, and the results were confirmed in two independently cloned cells for each genotype. *, p < 0.01 for the comparison with the wild type.
first study to show that simultaneous deletion of Hs2st and Hs6st exhibits profound FGF signaling defects in mammalian development.

In Drosophila trachea branching morphogenesis, Kamimura et al. (17) have previously shown that Hs2st and Hs6st single mutants abolished the corresponding sulfation reactions, but the charge density of Drosophila heparan sulfate remained constant due to increases in sulfation at other positions. Because FGF signaling was unaffected in Hs2st and Hs6st single mutants but abolished in Hs2st/Hs6st double mutants, these authors concluded that the function of heparan sulfate depends on the absolute amount but not the specific pattern of sulfation. According to this model, mutant heparan sulfate chains should support FGF signaling as long as they maintain normal levels of overall sulfation (17). Mechanistically, any model must take into account the fact that the binding of ligands to heparan sulfate usually occurs via a short oligosaccharide of ~5–12 residues (12, 45). Thus, compensation can take place only if the binding sites for heparan sulfate in the ligand and/or the receptor can accommodate sulfate groups located at different positions and the heparan sulfate chain can reorient to position the sulfate groups appropriately. Structural studies of Fgf10-Fgfr2b complexes with defined heparan sulfate oligosaccharides are needed to critically evaluate this issue.

As in other systems, deletion of O-sulfotransferases involved in heparan sulfate processing results in enhanced sulfation at other positions. Thus, deletion of Hs2st in CHO cells, Drosophila, and mice results in enhanced N-sulfation and 6-O-sulfation, whereas deletion of Hs6st results in increased N-sulfation and 2-O-sulfation. The mechanism that underlies these changes is unknown and might reflect intrinsic properties of the enzymes with respect to substrate preference. Alternatively, changes in the level of expression or organization of the biosynthetic enzymes might occur. In either case, the assembly process appears to be coordinated to buffer changes in composition, which in turn prevents deleterious effects on receptor signaling, at least in the context of FGF reception. Whether these changes in heparan sulfate structure also result in activation or repression of other signaling systems is an interesting possibility that should be considered.

Given the large number of FGF and FGFR family members (46) and variation in the amount and composition of heparan sulfate expressed in different cells and tissues, it is not surprising that the requirement for heparan sulfate might vary considerably for different FGFs and FGR receptors. Here, we show a preference for 6-O-sulfate groups with contribution of 2-O-sulfate groups for FGF10-FGFR2b signaling that enables lacrimal gland development. Our findings are consistent with prior binding studies showing a requirement for 6-O-sulfation for oligosaccharide binding to Fgf10 but differ in that 2-O-sulfate groups were dispensable for binding (22). Presumably, the different results reflect the experimental systems under study and emphasize the importance of evaluating these interactions in vivo to determine the biological relevance of biochemical measurements.

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