A mutant-cell library for systematic analysis of heparan sulfate structure-function relationships

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Heparan sulfate (HS) is a complex linear polysaccharide that modulates a wide range of biological functions. Elucidating the structure-function relationship of HS has been challenging. Here we report the generation of an HS-mutant mouse lung endothelial cell library by systematic deletion of HS genes expressed in the cell. We used this library to (1) determine that the strictly defined fine structure of HS, not its overall degree of sulfation, is more important for FGF2–FGFR1 signaling; (2) define the epitope features of commonly used anti-HS phage display antibodies; and (3) delineate the fine inter-regulation networks by which HS genes modify HS and chain length in mammalian cells at a cell-type-specific level. Our mutant-cell library will allow robust and systematic interrogation of the roles and related structures of HS in a cellular context.

Here we report the development of an HS-mutant mouse lung endothelial cell (MLEC) library that enabled us to address some of the aforementioned fundamental HS biology issues, and which represents a novel test platform for systematic interrogation of the roles and related structures of HS in a cellular context.

Results

Generation of an HS-mutant MLEC library. To generate a comprehensive HS-mutant MLEC library that targeted all the HS-expressing genes in the cells, we first carried out qRT-PCR analysis to profile HS-gene expression in primary MLECs. In mammals, there are as many as 20 HS-specific genes that function to biosynthesize HS, as well as two genes involved in postsynthetic remodeling. Among these genes, 12 are expressed in mice: the chain polymerization genes exostosin 1 (Ext1) and Ext2; the modification genes N-deacetylase/N-sulfotransferase 1 (Ndst1) and Ndst2; glucuronyl C5-epimerase (Glce); HS 2-O-sulfotransferase 1 (Hs2st1); HS 6-O-sulfotransferase 1 (Hs6st1) and Hs6st2; HS (glucosamine) 3-O-sulfotransferase 1 (Hs3st1) and Hs3st2; and the remodeling genes HS 6-O-endosulfatase 1 (Sulf1) and Sulf2 (Fig. 1b).

We targeted all these HS-expressing genes. By means of cell immortalization and cloning (Supplementary Fig. 1), we derived MLEC lines directly from 8–10-week-old Ext1 WT, Ndst1 WT, Ndst1 Δ/Δ, Hs2st1 WT, Hs6st1 WT, Hs6st2 WT, and Sulf1 Δ/Δ, Sulf2 WT mice. We further subjected these cell lines to transient Cre recombinase expression to derive the corresponding mutant Ext1 Δ/Δ, Ndst1 Δ/Δ, Ndst1 Δ/Δ; Ndst2 Δ/Δ, Hs2st1 Δ/Δ, Hs6st1 Δ/Δ, and Hs6st2 Δ/Δ mice.

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Sulf1−/−; Sulf2−/− cell lines. All seven directly derived MLEC lines expressed endothelial cell markers CD31 and VEGFR2, which confirmed their endothelial cell identity (Supplementary Fig. 2).

The HS-gene expression patterns in the Ext1f/f, Ndst1f/f, Hs2st1f/f, Hs6st1f/f, and Sulf1f/f; Sulf2f/f MLEC lines were similar to those in primary MLECs (Fig. 1b,c and Supplementary Fig. 3), which indicated that the derived mutant cell lines might closely reflect HS structure alterations in vivo after targeted gene deletion. We did not collect the mice deficient for Glce, Hs3st1, or Hs3st4 because of their embryonic lethality or unavailability. Instead, we transiently cotransfected the Ndst1f/f MLEC line with Cas9 and guide RNA (gRNA) specific for Glce, Hs3st1, or Hs3st4 (Supplementary Figs. 1 and 4). After screening for induced genomic insertion/deletion (indel) mutations and confirming the indels by sequencing the gRNA-targeted regions, we obtained Glce−/−, Hs3st1−/−, Hs3st4−/−, and Hs3st1−/−; Hs3st4−/− MLEC lines (Supplementary Fig. 4). By deleting HS-expressing genes individually or in combination, we developed an HS-mutant MLEC library containing a total of 18 cell lines and harboring alterations of all HS modification types (Table 1).

Characterization of HS structure expression in mutant MLECs.

We next characterized the generated mutant cells to understand HS alteration after HS-gene deletion. As we reported previously10–12, Ext1 deletion decreases HS expression, as reflected by decreased staining for anti-HS (clone 10E4) (Fig. 2a). The other HS-mutant cell lines all expressed HS. We analyzed them for HS disaccharide

Fig. 1 | Expression of HS biosynthetic and remodeling genes in MLECs. a, HS structure and the biosynthetic/remodeling genes that affect various aspects of that structure. Each sugar residue is depicted by a symbol according to the key. GlcNH2, glucosamine; GlcNAc, N-acetylglucosamine; GlcA, glucuronic acid; IdoA, iduronic acid; Gal, galactose; Xyl, xylose. b, c, Expression patterns of HS polymerization, modification, and remodeling genes in primary (b) and immortalized (c) MLECs as determined by qRT-PCR analysis. The data are representative of 3 independent experiments and are presented as mean ± s.d.
composition by digesting isolated HS with heparinases I–III and separating the resulting disaccharide with an anion-exchange column in an HPLC system (Supplementary Data). For the Cre-loxP-derived HS mutants, we used the corresponding floxed cell lines as controls (the floxed control cell lines are referred to as wild-type controls here for convenience). For the Ndst1f/f, Ndst2f/f, and Cre-loxP cell lines that were originally derived from conventional gene-knockout mice, we used data pooled from the five ‘wild-type’ MLEC lines (Extf/f, Ndst1f/f, Ndst2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f) as the control (WTa, where “a” indicates the average). We generated the Glcef/f, Hs3tf/f, Hs3tf/f, Hs3tf/f, Hs3tf/f, and Hs3tf/f, Hs3tf/f, Hs3tf/f, Hs3tf/f, Hs3tf/f, and Hs3tf/f, Hs3tf/f lines via CRISPR-Cas9 technology, and used the parent Ndst1f/f line as the wild-type control.

We first examined samples with alterations in Ndst-family genes. HS in Ndst1−/− cell lines showed a 40–60% reduction in NS, 2S, and 6S compared with levels in the control (NS 58.7%, 2S 17.3%, and 6S 16.4% for Ndst1f/f versus NS 72.5%, 2S 6.8%, and 6S 33.5% for Glcef/f) (Fig. 2d, Table 1). Ndst2f/f: HS had a normal disaccharide composition, whereas Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and Hs6st1f/f, Hs6st2f/f, and...
Fig. 2 | HS expression in the generated mutant MLEC lines. a, Cell-surface binding of anti-HS 10E4 as assessed by flow cytometry. b-i, HS disaccharide composition analysis. HS isolated from mutant MLECs and their corresponding controls was digested with heparinases I–III, and the resulting disaccharides were separated by HPLC and quantified. b, Ndst1f/f, Ndst1−/−; c, WTa, Ndst1f/f;Ndst2−/−; Ndst1−/−;Ndst2−/−. d, Ndst1f/f, GlcE−/−. e, Hs2st1f/f, Hs2st1−/−; f, Hs6st1f/f, Hs6st1−/−; g, WTa, Hs6st1f/f;Hs6st2−/−. Hs6st1−/−;Hs6st2−/−. h, Sulf1f/f;Sulf2f/f, Sulf1−/−;Sulf2−/−. i, Ndst1f/f, Hs3st1−/−, Hs3st4−/−, Hs3st1−/−, Hs3st4−/−. We combined same-type sulfate groups, including NS, 2S, and 6S, of the separated disaccharides to assess the level of each type of sulfation modification. The data are summarized from 3 independent experiments and are presented as mean ± s.e.m. WTa (‘wild-type’ control) data were summarized from five similarly generated MLEC lines (ExtP, Ndst1f/f, Hs2st1f/f, Hs6st1f/f, and Sulf1f/f,Sulf2f/f). We calculated the total number of sulfate groups by adding up NS, 2S, and 6S. j, k, Cell-surface antithrombin binding. Control (Ndst1f/f) and Hs3st-mutant MLECs were stained with biotinylated antithrombin, and cell-surface-bound antithrombin was quantified by flow cytometry after further staining with fluorescein-tagged streptavidin. Representative histograms from 3 independent experiments are shown (j). The data for quantitation of mean fluorescence index are summarized from 3 independent experiments and are presented as mean ± s.d. (k). Statistical analyses were performed by two-sided Student’s t-test.
showed slight reductions in 6S levels (NS 58.7%, 2S 17.3%, 6S 16.4% for Ndst1f/f; NS 55.0%, 2S 20.1%, 6S 17.0% for Hs3st1f/f; NS 55.7%, 2S 18.0%, 6S 10.7% for Hs3st4f/f; NS 51.3%, 2S 18.9%, 6S 11.3% for Hs3st4f/f; Hs6st3f/f) (Fig. 2i, Table 1). We were not able to detect 3S in the disaccharide-composition assay and assessed it on the basis of binding of antithrombin, a ligand that strictly requires HS for binding. Hs3st1f/f and Hs3st4f/f MLECs both showed reduced antithrombin binding compared with that in controls, and this effect was even more pronounced in Hs3st1f/f;Hs3st4f/f−/− cells (Fig. 2j,k), indicating that Hs3st1 and Hs3st4 deletion each reduce 3S, Hs3st4f/f−/− and Hs3st1f/f−/−;Hs3st4f/f−/− HS, but not Hs3st1f/f−/− HS, showed slightly reduced overall sulfation compared with that in controls (Hs3st4f/f−/−, −10.6%; Hs3st1f/f−/−;Hs3st4f/f−/−, −12.3%) (Fig. 2i). Hs6st1f/f−/−, Hs6st1f/f−/−;Hs6st2f/f−/−, Hs6st1f/f−/−;Hs6st2f/f−/−, and Sulf1−/−;Sulf2−/− HS had normal overall sulfation compared with that in the respective control lines. Thus, the combined examination of cells with mutations in Hs6st, Sulf, and Hs3st genes could allow researchers to determine and differentiate the importance of 3S, 6S, and overall sulfation for ligand binding and downstream signaling activation in these cells.

As a whole, the generated MLEC library harbors alterations of all HS modification types and might allow for determination of the necessity of specific modifications and overall HS sulfation levels for ligand binding and subsequent downstream signaling activation.

The importance of fine structure versus sulfation level of HS for FGF2–FGFR1 signaling. HS functions as a coreceptor for FGF by interacting with FGF and FGFR to form functional FGF–HS–FGFR ternary complexes on the cell surface. FGF2 functions as a coreceptor for FGF binding was increased on Hs3st4f/f−/− MLECs but remained unchanged on Hs3st1f/f−/− and Hs3st4f/f−/− MLECs, and all the Hs3st-mutated MLECs showed normal Erk1/2 phosphorylation after FGF2 stimulation (Fig. 3f, Table 2, Supplementary Figs. 8 and 9). These results show that 3S is dispensable for HS facilitation of FGF2 signaling and demonstrate again that increased cell-surface FGF2 binding does not positively correlate with enhanced FGF2 signaling activation.

Our results demonstrate that NS, IdoA, and 2S are essential for HS binding to FGF2, but only in the presence of proper 6S modification can this binding effectively facilitate FGF2–FGFR1 signaling activation. Our data support the idea that HS fine structure is more important than overall sulfation for FGF2–FGFR1 signaling.

Characterization of anti-HS phage display antibody epitopes. Anti-HS phage display antibodies are commonly used to probe HS structure expression in tissue in situ. However, their putative epitopes have been determined only on the basis of their binding capacity for chemically modified heparins and a limited number of synthetic HS oligosaccharides. To address this, we examined our mutant library using the common anti-HS phage display antibodies AO4B08, EV3C3V, RB4EA12, and HS4C3 (Fig. 4a). We analyzed the mutant MLECs for antibody binding to cell-surface HS by flow cytometry. We observed positive staining for all four antibodies in Ext1f/f cells (Fig. 4b). Epitopes recognized by AO4B08 and EV3C3 are more abundant than the ones recognized by HS4C3 and RB4EA12. Compared with binding to Ext1f/f cells, antibody binding to Ext1−/− cells was largely lost for EV3C3, HS4C3, and RB4EA12, and was partially lost for AO4B08 (Fig. 4b), which shows that these antibodies indeed bind native HS.

Previous biochemical studies established that the AO4B08-binding epitope contains NS, 6S, IdoA, and an internal 2-O-sulfated IdoA residue. In agreement with these reports, we observed that binding of AO4B08 was reduced on Ndst1f/f−/−.
**Fig. 3 | Effects of HS structure alteration on FGF2 binding and downstream Erk1/2 activation.** The HS mutant MLECs and their controls were incubated with biotinylated FGF2, and the cell-surface-bound FGF2 was quantified by flow cytometry after staining with fluorescein-tagged streptavidin. In parallel, we stimulated the cells with FGF2, and assessed the resulting FGFRI signaling activation by measuring pErk1/2 levels. a, Ndst1+/+; Ndst1−/−; Ndst2+/+, Ndst1−/−; Ndst2−/−. b, Ndst1−/−, Glc3−/−; c, Hs2st1−/−, Hs2st1+/−; d, Hs6st1−/−, Hs6st1+/−; Hs6st2−/−, Hs6st1+/−; Hs6st2−/−; e, Sulf1+/−, Sulf2−/−; f, Ndst1−/−, Hs3st1−/−, Hs3st4−/−; Hs3st3−/−; Hs3st4−/−. The data are summarized from 3 independent experiments and are presented as mean ± s.d. Statistical analyses were performed by two-sided Student’s t-test. MFI, mean fluorescence index.

Ndst1−/−, Ndst2−/−, Ndst1−/−; Ndst2−/−, Glc3−/−; Hs2st1−/−, Hs2st1+/−, Hs6st1−/−, Hs6st1+/−, Hs6st2−/−, and Hs6st1−/−; Hs6st2−/− MLECs, whereas it was increased on Sulf1−/−; Sulf2−/− MLECs (Fig. 4c–i). The binding of AO4B08 to Hs3st1−/−, Hs3st4−/−, and Hs3st1−/−; Hs3st4−/− MLECs was also reduced (Fig. 4j), which indicates that the AO4B08 epitope contains 3S.

Biochemical studies have also shown that the EV3C3 epitope contains N5, IdoA, and 2S, and prefers a low level of 6S for good binding (8) (Fig. 4a). Compared with binding in controls, we observed reduced EV3C3 binding on Ndst1−/−, Ndst1−/−; Ndst2−/−, Ndst1−/−; Ndst2−/−, Glc3−/−, Hs2st1−/−, Hs6st1−/−, Hs6st1+/−; Hs6st2−/−, and Hs6st1−/−; Hs6st2−/− MLECs (Fig. 4c–h), which indicates that the EV3C3 epitope does contain NS, IdoA, 2S, and 6S. We noted increased EV3C3 binding to Sulf1−/−; Sulf2−/− MLECs, which expressed HS with increased levels of 6S (Fig. 4i), thus indicating that higher 6S levels do not interfere with EV3C3 binding. These observations suggest that 6S is an essential component of the EV3C3 epitope. Binding of EV3C3 to Hs3st1−/−, Hs3st4−/−, and Hs3st1−/−; Hs3st4−/− MLECs also was reduced (Fig. 4j), which indicates that the EV3C3 epitope contains 3S.
Biochemical studies have shown that the HS4C3 epitope contains NS, 2S, 6S, and 3S−/− (Fig. 4a). In agreement with these observations, we found that HS4C3 showed reduced binding on Ndst1−/−, Ndst1+/−;Ndst2−/−, Ndst1−/−;Ndst2−/−, Ndst1−/−;Ndst1−/−, Hs6st1−/−;Hs6st1−/−, Hs6st1−/−;Hs3st1−/−, Hs6st1−/−;Hs3st4−/−, and Hs6st1−/−;Hs3st4−/−;Hs4C3−/− MLECs, but increased binding on Sulf1−/−;Sulf2−/− MLECs (Fig. 4c,d,g–j). However, the binding of HS4C3 to HS2st1−/− MLECs was unchanged compared with that in controls (Fig. 4f), indicating that the HS4C3 epitope does not necessarily have to contain the 2S modification. In addition, the binding of HS4C3 to Glce−/− MLECs was reduced compared with that in controls (Fig. 4e), indicating that the HS4C3 epitope contains N-acetylated and N-sulfated glucosamine residues with 6S modification (Fig. 4a). This was supported in our study by reduced RB4EA12 binding on Ndst1−/−, Ndst1+/−;Ndst2−/−, Ndst1−/−;Ndst2−/−, Ndst1−/−;Ndst1−/−, Hs6st1−/−;Hs6st1−/−, Hs6st1−/−;Hs3st1−/−, Hs6st1−/−;Hs3st4−/−, and Hs6st1−/−;Hs3st4−/−;Hs4C3−/− MLECs and increased binding on Sulf1−/−;Sulf2−/− MLECs (Fig. 4c,d,g–j). Hs2st1−/− MLECs showed normal RB4EA12 binding compared with that in controls (Fig. 4f), indicating that the native HS epitope recognized by RB4EA12 does not necessarily have to contain 2S, in agreement with the reported biochemical analysis. RB4EA12 showed reduced binding to Glce−/−, Hs3st1−/−, Hs3st4−/−, and Hs3st1−/−;Hs3st4−/− MLECs (Fig. 4c–j), which suggests that the RB4EA12 epitope also contains Idoa and 3S.

Our HS-mutant cell-staining study determined the structural features of the native HS epitopes recognized by the four anti-HS phage antibodies, which, in most cases, were in good agreement with reported biochemical studies (Supplementary Table 1). However, we observed several discrepancies, including the requirement of 6S for EV3C3 and the lack of a requirement for 2S for HS4C3. In addition, we observed that 3S was a component of native HS epitopes for AO4B08, EV3C3, and RE4EA12. The 3S might have been additive, as the Hs3st1−/−, Hs3st4−/−, and Hs3st1−/−;Hs3st4−/− MLECs showed only reduced antibody binding. Furthermore, the epitopes for HS4C3 and RB4EA12 also contained Idoa.

Characterization of the HS modification network in HS fine structure expression. HS biosynthesis is generally referred to as a sequential process consisting of initiation by copolymerases Ext1 and Ext2 followed by modification reactions initiated by Ndst-family enzymes and proceeded by Glce, Hs2st, Hs6st-family, and Hs3st-family enzymes. The biosynthesized HS is further subjected to extracellular remodeling by Sulf-family enzymes. In HS composition analysis, we noticed that inactivation of an individual HS enzyme also affected upstream and/or downstream modifications that were not catalyzed by the mutant enzyme, which indicated that internal HS-modification network regulation exists in MLECs. We therefore correlated the disaccharide composition of the mutant HS with HS gene expression in the mutant cells to better understand the intermodification network regulation in MLECs.

Deletion of Ndst1 led to upregulation of Ndst3, Hs6st1, and Hs3st4 but downregulation of Hs2st1 (Fig. 5a). Ndst2 deletion did not affect the expression of other Ndst genes, but it led to upregulation of Hs2st1, Hs3st4, and Sulf1 (Fig. 5b), thus showing that Ndst1 and Ndst2 have distinct regulation effects on other modification genes. Ndst1 deletion reduced levels of NS and 6S but upregulated Ndst3 and Hs6st1. Similarly, Ndst2 deletion had no effect on 2S and 6S and upregulated Hs2st1 and Sulf1. These observations indicate that, at the NS step, the modification network regulation occurs at the transcriptional level but contributes minimally to HS fine structure expression. Furthermore, simultaneous deletion of Ndst1 and Ndst2 diminished the network regulation, except for slight Ndst3 upregulation (Fig. 5c, Supplementary Fig. 10a), indicating that the modification network regulation essentially depends on the expression of Ndst genes.

Glce deletion led to upregulation of Ndst1, Ndst2, Hs2st1, Hs6st1, and Sulf2, correlating with increased NS and 6S of HS (Fig. 5d), which indicates inhibitory regulation by Glce of NS and 6S via downregulation of Ndst1, Ndst2, and Hs6st1. Increased 6S in Glce−/− HS also indicated that the upregulation of Sulf2 contributes minimally to 6S modification.

Hs2st1 deletion led to upregulation of Ndst2, Glce, Hs6st1, Hs6st2, Hs3st1, Hs3st4, Hs6st6, Sulf1, and Sulf2 (Fig. 5e), thus revealing profound inhibition by Hs2st1 of other modification genes. Similar to Glce deletion, Hs2st1 deletion led to increased NS and 6S, indicating that Hs2st1 inhibits NS and 6S by downregulating Ndst2, Hs6st1, and Hs6st2, and that the contributions of upregulated Sulf1 and Sulf2 remain minimal.

Hs3st1 deletion upregulated Ndst1, Hs2st1, Hs3st1, Sulf1, and Sulf2 but did not affect NS and 2S (Fig. 5f). Hs6st2 deletion upregulated Hs6st1 and Hs3st1 but did not alter 6S, NS, or 2S (Fig. 5g, Supplementary Fig. 10b). However, simultaneous deletion of Hs6st1 and Hs6st2 led to upregulation of Ndst1, Ndst2, Hs2st1, Hs3st1, and Sulf2 (Fig. 5h), which correlated with increased NS and 2S, thus revealing inhibition of NS and 2S modification by Hs6st genes through suppression of Ndst1, Ndst2, and Hs2st1 expression.

Hs3st1 deletion led to slight upregulation of Hs2st2 but downregulation of Hs2st1 and Hs3st4 (Fig. 5i), and did not alter HS composition. Hs3st4 deletion led to slight upregulation of Ndst1, Ndst2, and Hs3st1; slight downregulation of Hs2st1; and high upregulation of Sulf2 (Fig. 5j), which correlated with reduced 6S. Simultaneous deletion of Hs3st1 and Hs3st4 showed regulatory effects similar to those of Hs3st4 deletion (Fig. 5k, Supplementary Fig. 10c–e), except for slight Hs6st1 upregulation, indicating that, within the Hs3st-family genes expressed in MLECs, Hs3st4 plays the major role in negative regulation of the modification network and has an overall role in increasing 6S.

Simultaneous deletion of Sulf1 and Sulf2 led to downregulation of Ndst1, Ndst2, and Hs3st1, and upregulation of Glce and Hs3st1 (Fig. 5i), which correlated with reduced NS and 2S, indicating positive regulation by Sulf genes of NS and 2S and negative regulation of epimerization and 3S. Furthermore, the increased 6S levels in Sulf1−/−;Sulf2−/− HS indicated that the overall 6S level is critically regulated by Sulf genes in MLECs.

In all, our systematic study reveals the following: (1) In MLECs, Ndst genes are essentially required in order for intermodification network regulation to occur, in good agreement with the modification-initiation function of Ndst genes in HS biosynthesis. (2) Glce,
HS2ST1, HS6ST1, and HS3ST genes. NDS1 genes reciprocally inhibit non-self-modification at the gene-expression and HS-composition levels. (3) SULF genes positively regulate NS and 2S, but negatively regulate epimerization and 3S.

Regulation of HS chain length by HS gene expression. NDS1, but not NDS1, was reported to regulate HS chain length. Currently we lack a systematic view of the HS chain-length regulation by HS genes. In PACE analysis of intact HS, we determined that NDS1 and HS3ST1;HS3ST4 HS had normal chain lengths compared with those in controls, whereas NDS2;GLCE;HS6ST1;HS6ST2, and HS3ST1 HS became shorter, and NDS1;NDS2;HS2ST1, HS6ST1;HS6ST2, and HS3ST4 HS became longer (Supplementary Fig. 11). A comparison of single- and
double-gene-deficient HS showed that for genes in the same family, including Ndst1 and Ndst2, Hs6st1 and Hs6st2, and Hs3st1 and Hs3st4, the two expressed genes had opposite regulation effects on HS chain length and reciprocally inhibited each other. All examined HS genes regulated HS chain length, and this regulation could be positive or negative, as well as direct or indirect.

**Discussion**

Examination of HS-mutant cells is an effective and straightforward approach for determining the function and structure–function relationships of native HS in a biological setting. This type of study was originally carried out with chemical-mutagenesis-generated HS-mutant CHO cell lines. Currently only four CHO HS-mutant cell lines are available. CHO cells do not express Hs3st genes and cannot be used to examine 3S-related modifications. CHO cells also lack endogenous expression of many HS-dependent signaling receptors, such as FGFR, receptors for vascular endothelial growth factor (VEGF), and Slit; very limited cellular function information has been obtained with these mutant CHO cells. Our examination of Ndst1−/− and Ndst1 f/f MLEC lines determined that NS is required for HS to function as a coreceptor for VEGF and Slit3 signaling. The success of previous studies and the FGF signaling coreceptor study described here demonstrate that our HS-mutant MLEC library represents a powerful platform for examining the roles and the structure–function relationship of HS in a cellular context, and findings could potentially be correlated with in vivo findings in corresponding mutant mice. In addition, our study demonstrates CRISPR–Cas9 technology as a highly efficient approach for generating HS-mutant cell lines.
to generate HS-mutant MLECs and is expected to be applicable to other cell types for the generation of HS mutants.

HS functions as a coreceptor for FGF signaling by interacting with both FGF and FGFR. A number of biochemical studies have shown that interactions of HS with FGFs require unique structures in which NS, 2S, and 6S contribute to the generation of specific sulfation patterns. Crystallographic studies showed that 2S and 6S form hydrogen bonds with heparin-binding residues of FGFs and/or FGFRs to induce dimerization of FGFRs. Meanwhile, other studies reported that FGF binding to HS is dictated primarily by the overall sulfation level, rather than by the precise positioning of various sulfate groups. We observed that MLECs with knockout of GlcE, Hs2st1, Hs6st-family genes, and Sulf-family genes, which express only FGFR1, showed altered FGFR binding and downstream signaling activation, even though the mutants had normal or slightly increased HS overall sulfation. Our results support the idea that HS fine structure is more important than overall sulfation for FGF2–FGFR1 signaling activation.

HS phase display antibodies have been widely applied to probe HS structure in various tissues in situ. However, their native HS epitope structures remain largely unclear. Using our HS-mutant cell library, we found that (1) the EV3C3 epitope involves 6S, and a natural increase in 6S levels does not inhibit antibody binding; (2) the HS4C3 epitope does not necessarily contain 2S but requires IdooA; and (3) the epitopes for AO4B08, EV3C3, and RB4EA12 also require 3S. The involvement of rare 3S suggests that the antibodies may function in the 'all-or-nothing' high-specific-binding mode, thus supporting the application of the antibodies as high-affinity probes for specific HS structures in situ. Further studies with a comprehensive, synthesized, 3S-containing HS library might help further define the epitope structures.

Studies of Hs2st1-deficient CHO cells and mouse fibroblasts revealed an inter-regulatory modification network in HS biosynthesis. Systematic perturbation of HS-modification genes in C. elegans led to the proposal of an inter-regulation model of modification enzymes in HS biosynthesis in metazoans: GlcE, Hs2st1, and Hs6st-family genes inhibit NS; GlcE stimulates both 2S and 6S; Hs2st1 and the Hs6st genes inhibit 6S and 2S, respectively; and 6S is inhibited by SulfI. We observed similar inter-regulation in MLECs, except for GlcE, which stimulated 2S but inhibited 6S, and SulfI genes, which also stimulated 2S. The discrepancy might be due to the fact that we examined only MLECs, whereas the C. elegans study examined the whole model organism. We also examined Ndst and Hs5st3 genes, and found that the inter-regulation network essentially depends on the expression of Ndst genes, and that Hs5st4 stimulates 6S. In addition, we also observed that HS genes generally regulated HS chain length, by either elongating or shortening the chain, and HS genes within same family normally acted reciprocally to regulate HS chain length. These studies provide a comprehensive and systematic view of the inter-regulation of HS genes in mammalian cells at specific cell-type, transcription, and HS-structure levels. HS expression is cell-type specific, and similar studies with other cell types might determine whether our observed inter-regulation of HS expression is common in mammalian cells.

In the current study, HS structure analyses determined disaccharide composition and chain length, but not sequence, which plays the central role underpinning the structure–function relationship of HS. This should be examined once capable technology becomes available in the future.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41592-018-0189-6.

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**Author contributions**
H.Q. and L.W. conceived and designed the research and wrote the manuscript. H.Q. generated all the cell lines. H.Q., S.S., L.L., X.L., G.L., S.A.A.-H., S.W., P.A., F.Z., and R.J.L. designed and performed disaccharide analysis. R.J.L. also contributed to manuscript preparation. H.Q., M.G., A.V.N., and K.W.M. performed the transcriptional analysis. T.H.v.K. provided their technical assistance.

**Competing interests**
The authors declare no competing interests.

**Additional information**

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CRISPR–Cas9 procedure to generate knockout cell lines. We designed gRNAs (Supplementary Fig. 4) and inserted them into the pCRISPR-LvS6G3 lasmide (Geneopera, USA). The gRNA and Cas9 plasmids were cotransfected into the Ndst1 MLEC line. The cells were selected by puromycin (10 μg/ml) for 3–7 d in culture and then were single-cell sorted into 96-well culture plates. The cell clones were screened for the targeted gene indel mutation by PCR amplification of the edited regions followed by enzyme mismatch assay after nuclease T7EI digestion52. The selected clones were further validated by sequencing of the targeted regions. The sequence chromatograms of the pooled PCR products of the targeted regions were decoded by either DSDecodeM® or CRISP-ID® software online.

FGF2 biotinylation. A heparin-Sepharose CL-6B column was pre-equilibrated with binding buffer PB150 (17.9 mM Na2HPO4, 2.1 mM NaH2PO4, 150 mM NaCl, 100 μg/ml heparin (Sigma-Aldrich)). The primary cells were immortalized by transfection of a plasmid encoding SV40 large T antigen and subjected to single-cell cloning culture. After 7 d, single cell clones were picked out and expanded for future use. A plasmid encoding Cre recombinase was introduced into the cells by transient transfection to knock out the floxed genes in the obtained cell clones. The knockout cell lines were obtained by single-cell cloning culture, and targeted gene deletion was confirmed by genomic DNA genotyping as reported previously48. The genotype condition and primers are listed in Supplementary Table 2.

Flow cytometry analysis. MLECs were collected after digestion with PBS supplemented with 2 μM EDTA and 1% BSA (PBS-EB), and adjusted at 106 cells/ml. For FGF2 binding, the dissociated cells were incubated with biotinylated FGF2 with binding buffer PB150 (17.9 mM Na2HPO4, 2.1 mM NaH2PO4, 150 mM NaCl, 100 μg/ml (w/v) phenol red and 25% (w/v) sucrose). All data generated or analyzed during this study are included in this article and/or its associated Supplementary Information files. The raw data files are available from the corresponding author upon request. Source data for Figs. 1–5 and Supplementary Figs. 3, 5, and 10 are available online.

Real-time polymerization chain reaction analysis. Cells in confluence were washed with PBS three times, flash-frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA isolation, cDNA synthesis, qRT-PCR reactions, and data analysis were performed as previously reported10. The gene ribosomal protein L4 (Rpl4) was included as an internal control for normalization of individual gene expression.

HS disaccharide analysis. Cells were cultured in a 10-cm dish in high-glucose DMEM supplemented with 10% FBS and penicillin (100 U/ml) and streptomycin (100 μg/ml)58. After being washed with PBS-EB, the cells were lysed with 1 ml of 0.1 M NaOH. Next, we added 8 μl of acetic acid. The cell lysate was added with Pronase (2 mg/ml; Sigma–Aldrich) and digested overnight at 37 °C. After inactivation at 95 °C for 10 min, the released glycosaminoglycan was enriched by being passed through a Q-column, desalted, and then digested with heparin lyases I, II, and III (Sigma–Aldrich) overnight at 37 °C. We collected the resultant HS disaccharides by filtering the digested glycosaminoglycan though a 3,000 molecular-weight-cutoff (MWCO) filter (Thermo Fisher), labeling with 2-aminoazobenzene, and then carrying out separation with an Agilent 1260 HPLC system with a Propac PA1 (4 × 250 mm; DIONEX) column connected with a Propargyl PA1 Guard column (4 × 10 mm; DIONEX). The separated disaccharides were detected by a fluorescence detector with excitation at 348 nm and emission at 440 nm.

PAGE analysis of intact HS extracted from cell samples. Cell samples were first treated with 100 μl of protein–extraction reagent under sonication for 20 min10. To remove chondroitin sulfate and hyaluronic acid, we added recombiant heparitinase lyase and hyaluronidase (100 μl each) and 500 μl of digestion buffer (50 mM ammonium acetate, 2 mM calcium chloride) to the reaction buffer and incubated the samples under 37 °C for 2 h. Next, 0.1 ml of actinase E solution (20 mg/ml) was mixed with each sample for proteolyis at 55 °C for 24 h. HS was then recovered and purified with a Dynabead MINI Q spin column. We desalted the obtained product solution by passing it through 3 kDa MWCO spin columns, lyophilizing it, and finally redispersing it in 10 μl of deionized water for further use. An analytical PAGE gel with 5 ml of 15% total acrylamide monomer resolving solution was allowed to polymerize for 30 min with 5 ml of TEMED and 30 μl of 10% (w/v) ammonium persulfate. Above the polymerized resolving gel, 2 ml of 5% total acrylamide monomer stacking gel was cast. An aliquot of 5 ml of purified HS was loaded in a solution of 10 μg/ml (w/v) phenol red and 25% (w/v) sucrose. Electrophoresis was conducted for 30 min at a constant voltage of 200 V.

Statistical analysis. For statistical analysis we used two-sided Student's t-test, with P < 0.05 considered statistically significant.

Resource availability. All cell lines are available on request under a standard material transfer agreement with UGA for academic research purposes.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Experimental design

1. Sample size
   - Describe how sample size was determined.
   - No sample size calculation was performed since this is a cell line study.

2. Data exclusions
   - Describe any data exclusions.
   - One transcript data of a triple repeat was excluded due to obvious abnormal value (less than mean of the triple - 3xSD).

3. Replication
   - Describe the measures taken to verify the reproducibility of the experimental findings.
   - The reproducibility of our experimental finding were confirmed by at least three independent repeat experiments.

4. Randomization
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - No randomization was performed as the study are based on cells in different genotype.

5. Blinding
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - The heparan sulfate disaccharide, gene transcript and FGF2 signaling analyses were blind to the investigators. However, the cell surface staining for CD31, VEGFR2, anti-heparan sulfate phage display antibodies and genotyping were not blind to the investigators, because these information were needed essentially to generate the heparan sulfate mutant cell library.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
   - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided
   - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - Test values indicating whether an effect is present
   - Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.
Software

Describe the software used to analyze the data in this study.

Microsoft Excel 2017, Flowjo v8.7 for mac, DSDecodedM (http://skl.scau.edu.cn/dsdecode/), CRISPR-ID (http://crispid.gbiomed.kuleuven.be/), CRISPR gRNA design tool (http://tools.genome-engineering.org)

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

The unique heparan sulfate mouse lung endothelial cell lines were generated in our own laboratory and have been available to researchers in scientific field. To dates, we have shared the cell library with several laboratories world wide.

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The commercial antibodies were widely used and validated in many publications. The anti-heparan sulfate phase display antibodies have been used widely in the heparan sulfate field and validated in many publications too. In our study, we also validated the antibodies are true anti-heparan sulfate antibody using our heparan sulfate-deficient cells.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

All the used mouse lung endothelial cell lines were generated in our own laboratory.

b. Describe the method of cell line authentication used.

The mouse lung endothelial cell lines were authenticated by observing cell surface expression of CD31 and VEGFR2 in flowcytomertry (flow cytometry BD LSRII).

c. Report whether the cell lines were tested for mycoplasma contamination.

All our cell lines have not tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by iCLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Provide all relevant details on animals and/or animal-derived materials used in the study.

All mice in C57BL6 background at 8-10 weeks-old were used including genetically engineered Ext1/f/f, Ndst1/f/f, Ndst2/-/-, Hs2st/f/f, Hs6st1/f/f, Hs6st2-/-, Sulf1/f/f, 2f/f mice.
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.