The interactions of heparan sulfate (HS) with heparin-binding growth factors, such as fibroblast growth factors (FGFs), depend greatly on the chain structures. O-Sulfations at various positions on the chain are major factors determining HS structure; therefore, O-sulfation patterns may play a crucial role in controlling the developmental and morphogenetic processes of various tissues and organs by spatiotemporally regulating the activities of heparin-binding growth factors. In a previous study, we found that HS-2-O-sulfotransferase is strongly expressed throughout the mesoderm of chick limb buds during the early stages of development. Here we show that inhibition of HS-2-O-sulfotransferase in the prospective limb region by small inhibitory RNA reduced Fgf-8 expression in the apical ectodermal ridge. The treatment also reduced Fgf-10 expression in the mesenchyme. Moreover 2-O-sulfated HS, normally abundant in the basement membranes and mesoderm under ectoderm in limb buds, was significantly reduced in the treated buds. Phosphorylation levels of ERK and Akt were up-regulated in such truncated buds. Thus, we have shown for the first time that 2-O-sulfation of HS is essential for the FGF signaling required for limb bud development and outgrowth.

Chick limb bud is a useful model of limb patterning, which involves various growth factors and morphogens such as fibroblast growth factor (FGF), hedgehog, wingless/Wnt, and bone morphogenetic proteins (1–4). Limb bud initiation requires reciprocal interactions between a specialized ectodermal structure called the apical ectodermal ridge (AER) and the underlying mesoderm (5, 6). At the time of limb initiation, in stages 13–15 of the chick embryo, Fgf-8 in the intermediate mesoderm induces Fgf-10 expression in the lateral plate mesoderm (LPM) of the prospective limb region. FGF-10 produced in the prospective limb mesoderm then induces Fgf-8 expression in the overlying surface ectoderm (AER). FGF-8 secreted from AER maintains Fgf-10 expression in the underlying mesoderm. This FGF signaling loop establishes the maintenance of AER and outgrowth of limb buds. FGF-8 also contributes to Shh induction, which in turn induces Fgf-4 expression in the AER and maintains AER function in the posterior underlying mesoderm called the zone of polarizing activity (7, 8).

Although growth factors and morphogens are required for limb bud development and patterning, it is unclear how their signaling and distribution are regulated. Heparan sulfate proteoglycans, which are ubiquitous on the cell surface and in the extracellular matrix including basement membranes, are important regulators of such growth factor signaling and distribution (9–11). For example, chicken syndecan-3 is expressed throughout the distal mesenchymal cells and plays a role in limb bud outgrowth by controlling FGF signaling between the AER and mesoderm (12). Heparan sulfate proteoglycans play such roles by interacting with these growth factors (13–15); however, it is unclear what role HS chains play in these processes, although HS chains are known to interact with a variety of heparin-binding growth factors (HBGFs) such as FGF.

In FGF signaling, the ternary complex composed of HS chains, FGF family molecules, and FGF receptors (FGFRs) is formed on the cell surface (16–18). Specificities of interactions between heparan sulfate proteoglycan and ligand molecules are thought to reside in the fine structures of HS chains with specific sequences consisting of highly sulfated monosaccharides (N-2-O-, and/or 6-O-sulfated) (9, 19, 20). In fact, the different O-sulfation patterns of HS chains are involved in interactions with FGF family molecules (21–23). These fine structures are generated by the following modification reactions with specific enzymes after the backbone chain of HS is synthesized by the addition of alternating D-glucuronic acid and N-acetylated-S-glucosamine (GlcNAc) residues from the respective UDP sugars by EXT family proteins (24): GlcNAc N-deacetylation and N-sulfation by N-deacetylase/N-sulfotransferase, C5 epimerization of D-glucuronic acid residues to iduronic acid by C5 epimerase, and O-sulfations at various places that take place first at C2 of IdOA and D-glucuronic acid by heparan sulfate 2-O-sulfotransferase (HS2ST), subsequently at C6 of GlcNAc and N-sulfate-
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D-glucosamine units by heparan sulfate 6-O-sulfotransferases (HS6STs), and lastly at C-3 of N-sulfate-D-glucosamine residues by heparan sulfate 3-O-sulfotransferases (25–27).

O-Sulfation residues and patterns have been shown to be critical to HS function in several developmental processes. For example, gene trap mutation of HS2ST in mice causes renal agenesis, eye and skeleton defects, and neonatal lethality (28). HS6ST-1 null mice die between embryonic day 15.5 and the perinatal stage and show a reduction in the number of fetal microvessels in the labyrinthine zone of the placenta (29). In addition, a recent report on mutant HS2ST and HS6ST-1 mice showed defects of specific axon guidance at the optic chiasm (30). Morpholino-mediated knockdown of HS6ST in zebrafish results in disruption of somitic muscle development and defects in the branching morphogenesis of the caudal vein (31, 32). Inhibition of Drosophila HS6ST expression by RNA interference (RNAi) reduces FGF signaling activity and disrupts the primary branching of the tracheal system (33).

Previously we showed distinctive expression patterns of HS O-sulfotransferases in developing chick limb buds (34). HS6ST-1 and HS6ST-2 transcripts are preferentially localized in the anterior and posterior proximal regions of the limb bud, respectively, whereas HS2ST transcripts are distributed rather uniformly throughout the bud. HS sulfation patterns in chick limb buds correspond exactly with these expression patterns (34). Thus, specific HS sulfation patterns may regulate the development and patterning of limb buds by binding to growth factors and morphogens. In this study, we examined the role of 2-O-sulfation in the morphogenesis of the chicken limb. Electroporation of RNA duplexes (RNAi technique) is effective at suppressing the expression of target genes in chick embryos (35). Disruption of the HS2ST gene by small inhibitory RNA (siRNA) reduced 2-O-sulfation levels and led to the abnormal development of limb buds, which showed less expression of Fgf-8 and Fgf-10 accompanied by the up-regulation of both Erk and Akt. Taken together, these results suggest that reducing 2-O-sulfate residues affect limb bud formation and outgrowth.

EXPERIMENTAL PROCEDURES

Preparation of Chicken Embryos—Fertile White Leghorn chicken eggs were obtained from Takeuchi Farm (Nara, Japan), incubated in humidified air at 38 °C, and staged according to Hamburger and Hamilton (36).

RNAi—Double-stranded RNA corresponding to the HS2ST open reading frame sequence was prepared and then digested with RNase III. Chicken HS2ST open reading frame was subcloned into pBluescriptII KS(−) (Stratagene, La Jolla, CA) before transcribing sense and antisense single-stranded RNA from T3 and T7 RNA polymerase promoters. RNA was incubated with DNase I to degrade the template DNA. Sense and antisense RNA were annealed into duplexes by combining equal amounts of each type of RNA, then denatured at 95 °C for 5 min, and incubated at 37 °C overnight. Annealed duplexes were incubated with RNase I (Ambion, Austin, TX) at 37 °C for 30 min and then treated with phenol–chloroform. The purified duplexes were resuspended in distilled water. To generate endonuclease-digested small inhibitory RNA (esiRNA), double-stranded RNA was incubated with RNase III (New England Biolabs, Beverly, MA) at 37 °C for 30 min. The reaction products were purified with siRNA Purification Units (Ambion), then precipitated with ethanol, and resuspended in distilled water. As controls for the siRNA treatment, siRNA mixtures for luciferase, the gene of which is derived from pGL3-Basic (Promega, Madison, WI), were prepared by the same procedure.

In Utero Electroporation—For electroporation experiments, eggs were opened after 2 days of incubation at 38 °C, corresponding to stage 13–14, and a solution of India ink diluted in Tyrode’s solution was applied below the blastoderm to enhance contrast. The vitellin membrane overlying the presumptive forelimb region was also carefully removed using a fine tungsten needle. RNA solutions containing 0–700 ng/μl esiRNA, 0.1% fast green, and 2 μg/μl pEGFP-N1 (Clontech) were injected into the LPM of the presumptive right forelimb region of embryos, and several drops of Tyrode’s solution were added after injection. Electrodes were placed above (cathode) and below (anode) the forelimb region containing the injected RNA, and electroporation (three 15-V, 25-ms pulses) was performed with ElectrosquarePorator T820 (BTX; Inovio Biomedical Corp., San Diego, CA). Experimental embryos were observed and/or harvested 16–48 h after electroporation. For cryosections, embryos were fixed in 4% paraformaldehyde, PBS at 4 °C overnight and then incubated in 30% sucrose, PBS at 4 °C for 2–12 h. Embryos were embedded in OCT compound (Sakura, Tokyo, Japan), and 10-mm-thick cryosections were made.

In Situ Hybridization—Whole-mount and section in situ hybridization was performed as described previously with slight modifications (34, 37–39). For whole-mount in situ hybridization, embryos were fixed in 4% paraformaldehyde, PBS overnight and then digested with 1 μg/ml proteinase K in PBS containing 0.1% Tween 20 at 20 °C for 15 min. Hybridization was performed at 65 °C in 5× SSC, 50% (v/v) formamide, 1% (w/v) SDS, 50 μg/ml heparin, and 50 μg/ml yeast tRNA using digoxigenin-labeled RNA as a probe.

In Situ FGF-2 Binding Assay and Immunohistochemistry—For in situ detection of 2-O-sulfated HS, we used an exogenous FGF-2 binding assay (40). Cryosections were air-dried and then washed with PBS. To remove endogenous HS-bound molecules, sections were incubated with 2 M NaCl, PBS at room temperature for 10 min and then washed three times with PBS. When necessary, sections were treated with a mixture of heparitinases (Seikagaku Corp., Tokyo, Japan) at 37 °C for 2 h. Sections were incubated with 5–30 nm recombinant human FGF-2 (Roche Applied Science) at 4 °C overnight followed by blocking for 1 h at room temperature with PBS containing 10% bovine serum albumin (Sigma). After five washes with PBS, FGF-2 binding was analyzed using an anti-FGF-2 monoclonal antibody (05-118; Upstate Biotechnology, Lake Placid, NY) and Alexa-conjugated secondary antibody (Molecular Probes, Eugene, OR). HS was detected with 3G10 antibody (Seikagaku Corp.) and secondary antibody.

Western Blot Analysis—For Western blot analysis, tissue lysates of stage 22–23 limb buds were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blots were incubated with
10% skim milk in Tris-buffered saline/Tween 20 (TBST; Tris-buffered saline containing 0.1% Tween 20) and probed with anti-phosphorylated ERK (pERK) antibody (catalog number 9106; Cell Signaling Technology, Beverly, MA), anti-ERK antibody (catalog number 9102; Cell Signaling Technology), anti-phosphorylated Akt (pAkt) antibody (catalog number 9275; Cell Signaling Technology), or anti-actin antibody (catalog number A2066; Sigma) diluted with 1% skim milk in TBST. The signal was visualized using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Western Lighting Plus; PerkinElmer Life Sciences) according to the manufacturer’s instructions.

Ligand and Carbohydrate Engagement Assays—In situ binding of the FGF-FGFR complex to heparan sulfate in the assay enables us to detect HS structure alterations (41, 42). The frozen sections were incubated in 2 M NaCl, PBS for 10 min followed by treatment with 10% BSA, PBS to block nonspecific binding. Slides were then incubated with 10–30 nM FGF-8 or FGF-10 (Peprotech Inc., Rocky Hill, NJ) together with 10–20 nM FGFR2b-Fc or FGFR2c-Fc (R&D Systems, Minneapolis, MN), respectively, which were fused with the human IgG Fc domain, at 4 °C overnight. After five washes with PBS, the bound FGFR-Fc was detected using a Cy3-conjugated anti-human Fc IgG secondary antibody (Chemicon International Inc., Temecula, CA).

RESULTS

Expression Patterns of HS2ST—HS2ST is highly expressed in the developing limb buds of chick embryos (34). Whole-mount in situ hybridization showed that HS2ST transcripts were localized uniformly throughout both wing and leg buds at stage 23 (Fig. 1A). In a transverse section of the wing bud region, HS2ST expression was strong in the mesenchyme and weak (but significant) in the overlying ectoderm including the AER (Fig. 1, B and C). This characteristic expression pattern suggests that HS2ST is deeply involved in limb bud development.

Abnormal Limb Bud Development and Outgrowth by HS2ST RNAi—To investigate the role of 2-O-sulfated HS in growth factor signaling in limb bud development, siRNA mixtures were transfected into the LPM of the prospective right forelimb region in stage 13-14 embryos via electroporation. HS2ST esiRNA-injected limbs caused abnormal development 2 days after the operation (Fig. 2, B–E), whereas PBS-injected limbs showed less abnormality (Fig. 2A). The abnormal limb buds were truncated and reduced in size (Fig. 2, B–D) or occasionally indented (Fig. 2E). When higher concentrations of esiRNA were injected, severe abnormal phenotypes were observed in the limb buds with higher frequency (Table 1). Green fluorescent protein (GFP) signals derived from coelectroporated pEGFP-N1, indicated the siRNA-injected region. A, PBS-injected control samples had almost normal limb buds. B, mildly truncated limb; C, moderately truncated limb; D, severely truncated limb; and E, indented limb. F–I, Alcian blue staining of skeletal patterns in HS2ST esiRNA-injected limbs revealed their abnormalities. G, severely affected right forelimb showing an abnormal skeletal pattern compared with untreated left forelimb (F). I, mildly affected right forelimb showing a defective digit (arrowhead) and size reduction compared with untreated left forelimb (H).

Abnormal limb bud development by HS2ST RNAi. A–E, HS2ST esiRNA- or PBS-injected stage 22-23 limb bud. HS2ST siRNA mixtures (150 ng/μl) or PBS was injected, via electroporation, into the lateral plate mesoderm of the prospective right wing bud region of stage 13-14 embryos. A′–E′, GFP signals, derived from coelectroporated pEGFP-N1, indicated the siRNA-injected region. A, PBS-injected control samples had almost normal limb buds. B–E, HS2ST esiRNA-injected samples showed truncated limb buds. B, mildly truncated limb; C, moderately truncated limb; D, severely truncated limb; and E, indented limb. F–I, Alcian blue staining of skeletal patterns in HS2ST esiRNA-injected limbs revealed their abnormalities. G, severely affected right forelimb showing an abnormal skeletal pattern compared with untreated left forelimb (F). I, mildly affected right forelimb showing a defective digit (arrowhead) and size reduction compared with untreated left forelimb (H).

Expression pattern of chicken HS2ST mRNA. A, whole-mount in situ hybridization of stage 23 embryo. A high level of HS2ST transcripts was detected in wing (W) and leg (L) buds and in the branchial arches (b). B and C, in situ hybridization of transverse sections of stage 22 wing buds. HS2ST is expressed weakly in the AER and ectoderm (C). B–F, in situ hybridization of transverse sections of stage 22 wing buds. HS2ST siRNA was injected into the prospective region of the right wing bud of stage 13 embryos (D). Treated embryos were fixed at stage 23-24 (45 h after treatment). The right bud (F) showed lower levels of HS2ST mRNA compared with the untreated left bud (E). Scale bars in B, D, and F, 200 μm; scale bar in C, 50 μm.
unlikely that the truncation is due to such effects. Furthermore the frequency of severe phenotypes in these controls was significantly less than that in HS2ST esiRNA-injected buds (Table 1). These results suggest that the injection of HS2ST esiRNA specifically caused truncation of the limb buds. In addition, HS2ST transcripts were reduced in HS2ST RNAi limb buds compared specifically caused truncation of the limb buds. In addition, HS2ST mRNA was observed in control limb buds (data not shown). These results indicate that siRNA mixtures effectively reduce HS2ST transcripts.

*Alteration of HS Structures by HS2ST RNAi*—We then examined whether the abnormal morphology of limb buds resulted from the loss of 2-O-sulfate residues in HS. Because no suitable antibodies for the detection of 2-O-sulfate residues in HS are available, we took advantage of the following binding properties of exogenous FGF-2 to HS to determine the distribution of 2-O-sulfated HS in tissues (40). We have shown that 2-O-sulfated HS is sufficient to bind FGF-2 (21). We pretreated the limb bud sections with 2 M NaCl to remove endogenous HBGFs, including FGF-2, bound to HS and then stained the sections with exogenous FGF-2 in combination with anti-FGF-2 antibody (Fig. 3 and supplemental Fig. 1). In the normal limb bud at stage 23, exogenous FGF-2 was strongly bound to the basement membranes and mesenchyme underneath the ectoderm (Fig. 3B and supplemental Fig. 1). These staining patterns coincided with HS distribution in the limb detected by 3G10 antibody (Fig. 3A and supplemental Fig. 1). When sections were treated with heparitinases before applying FGF-2, the FGF-2 binding region was markedly reduced, suggesting that exogenous FGF-2 binds specifically to HS (supplemental Fig. 1). These results indicate that 2-O-sulfated HS is abundant in the basement membranes and mesenchyme underneath the ectoderm in the developing limb bud. We then tested FGF-2 binding activity in siRNA-injected limb buds at stage 23 (Fig. 3). In RNAi-treated limb buds, exogenous FGF-2 bound only weakly to the truncated region compared with the unaffected region, whereas HS distributions themselves were not affected (Fig. 3, E–H). We could not see such a decrease of exogenous FGF-2 binding in luciferase RNAi-treated limb buds (data not shown). These results show that HS2ST RNAi also reduced 2-O-sulfation and FGF-2 binding. Interestingly 2-O-sulfation was significantly reduced in the basement membranes (Fig. 3, E and F, arrowheads). The decrease of 2-O-sulfation in the basement membranes could affect the movement of HBGFs between the mesoderm and ectoderm.

### Table 1

| Injection | ng/ml | No. | Total | Mild | Moderate | Severe | Indentation |
|-----------|-------|-----|-------|------|----------|--------|-------------|
| HS2ST esiRNA |       |     |       |      |          |        |             |
| 700       | 27 (49.1%) | 56 | 12 (14.8%) | 5 (18.5%) | 4 (44.4%) | 6 (22.2%) |
| 450       | 12 (32.4%) | 37 | 2 (33.3%) | 4 (16.7%) | 2 (16.7%) | 4 (33.3%) |
| 250       | 16 (25.8%) | 62 | 7 (56.3%) | 9 (43.7%) | 0 (0%)   | 0 (0%)   |
| 150       | 20 (25.64%)| 78 | 7 (50.0%) | 10 (35.0%)| 1 (5.0%) | 2 (10.0%) |
| Luciferase esiRNA | |     |       |      |          |        |             |
| 700       | 4 (22.2%) | 18 | 3 (25.0%) | 1 (75.0%) | 0 (0%)   | 0 (0%)   |
| 300       | 3 (13.0%) | 23 | 2 (33.3%) | 1 (66.7%) | 0 (0%)   | 0 (0%)   |
| Buffer (PBS) |       |     |       |      |          |        |             |
| 0         | 84 (11.13%)| 11 | 8 (27.3%) | 3 (72.7%) | 0 (0%)   | 0 (0%)   |

* Typical examples are shown in Fig. 2, B–F.

### Effects on FGF Signaling Loop

To investigate whether HS2ST RNAi disrupted FGF signaling during limb bud development, we examined Fgf-8 expression using whole-mount in situ hybridization of stage 22-23 limb buds (Fig. 4). A marked reduction of Fgf-8 transcripts was observed in truncated buds by siRNA injection, whereas Fgf-8 is expressed in the AER of untreated buds (Fig. 4, A and B). Shh, which is induced by FGF-8 signaling, was also reduced in limbs that showed decreased Fgf-8 expression (Fig. 4B, arrowhead). In some cases (4 of 13 samples), the reduction of Fgf-8 expression was also observed even in HS2ST esiRNA-injected but almost normally developed limb buds (Fig. 4C); however, no such reduction was observed in luciferase esiRNA-injected limb buds (11 samples).

We also examined Fgf expression in early developmental stages. Chick wing buds develop and grow at stages 18 and 19 at which point Fgf-8 expression is detectable in the ectoderm (43). In the present study, Fgf-8 expression was significantly reduced in the ectoderm of RNAi-treated limb buds compared with the untreated opposite limb bud (Fig. 5, A and B). In a few cases, Fgf-8 expression was almost undetectable at this stage (Fig. 5B, arrow). As Fgf-8 expression is induced by Fgf-10 derived from the mesoderm and Fgf-10 expression is maintained by Fgf-8 derived from the AER (5, 6), we examined Fgf-10 expression in RNAi-treated limb buds at similar stages. Similar to Fgf-8, Fgf-10 expression in the mesoderm was also reduced in siRNA-injected buds (Fig. 5, C and D). Taken together, these results suggest that a reduction of 2-O-sulfated HS down-regulates both Fgf-8 and Fgf-10 expression in the developing limb bud.

### Up-regulation of ERK and Akt Pathway

The intracellular response to FGFs is mediated by several signal transduction pathways including the MAPK/ERK and phosphatidylinositol 3-OH-kinase/Akt pathways. Recently both pathways have been shown to be essential for limb bud development and patterning (44–46). We examined the activation of ERK and Akt, which are activated in the MAPK and phosphatidylinositol 3-OH-kinase pathways, respectively. We dissected stage 23 esiRNA-injected right limb buds and untreated opposite left limb buds and probed for pERK, ERK, and pAkt by Western blot analysis. The truncated limb buds among those treated with HS2ST esiRNA showed approximately 2 times higher pERK levels than those of the untreated buds, although total ERK levels were not altered between the RNAi-treated and untreated buds (Fig. 6, A and B, Group 1). Similarly pAkt was also increased about 2-fold in the HS2ST RNAi limb buds (Fig. 6, A and B). No up-regulation of the phosphorylation of ERK and Akt was observed in
luciferase esiRNA-injected buds (Fig. 6, A and B, Group 3). Interestingly in HS2ST esiRNA-injected but almost normally developed buds, increases of pERK and pAkt were also observed but not to the extent as those in Group 1 (about 1.5-fold) (Fig. 6, A and B, Group 2). When limb buds were sectioned and stained with anti-pERK antibody, a higher level of pERK was observed around the HS2ST esiRNA-injected region (Fig. 6C). These results suggest that phosphorylation of ERK and Akt was up-regulated by HS2ST RNAi, and truncation may be caused by up-regulation of these signaling molecules.

**FIGURE 3.** Alteration of 2-O-sulfated HS by HS2ST RNAi. A and C, HS was detected by 3G10 antibody. B and D, the 2-O-sulfated HS was detected as described under “Experimental Procedures.” Longitudinal sections of limb buds (removal of endogenous HBGFs by treatment with 2 M NaCl) were exposed to exogenous FGF-2 and then probed with anti-FGF-2 antibody. The top of each figure shows the anterior region, and the bottom shows the posterior. A and B, normal limb buds at stage 23. C and D, HS2ST siRNA-injected limb bud (450 ng/μl) at stage 23 (45 h after treatment). The anterior half of the bud was truncated by RNAi treatment (arrow in C, inset). E–H, magnified images of C and D. FGF-2 binding activity was reduced in the siRNA-injected region (F) compared with unaffected regions (H), whereas HS distributions were not different between injected (E) and untreated regions (G). The basement membranes also showed a marked reduction in FGF-2 binding (compare arrowheads in E and F). Scale bars in A–D, 200 μm; scale bars in E–H, 20 μm. Wt, wild type.

**FIGURE 4.** Abnormal limb buds showed lower Fgf-8 and Shh expression. A–C, the expression patterns of Fgf-8 and Shh in stage 23 embryos were detected by whole-mount in situ hybridization. A”–C”, GFP signals, which were derived from coelectroporated pEGFP-N1, indicated the siRNA-injected regions. A”–C”, right wing buds injected with 150 ng/μl esiRNA. A”–C”, the corresponding untreated left buds. A, moderately truncated and size-reduced limb bud. B, moderately truncated limb bud. C, almost normally developed limb bud. Fgf-8 expression is partially reduced in the anterior region of AER (arch). Arrowheads in B and C indicate Shh expression.

**FIGURE 5.** HS2ST RNAi disrupted the FGF signaling loop. A–D, Fgf-8 and Fgf-10 expression patterns at stage 18 (A–C) or 19 (D) were detected by whole-mount in situ hybridization (13–15 h after treatment). A”–D”, GFP signals, which were derived from coelectroporated pEGFP-N1, indicated the siRNA-injected region. A”–D”, right wing buds injected with 450 ng/μl esiRNA. A”–D”, the corresponding untreated left buds. Fgf-8 expression in the ectoderm was significantly reduced by HS2ST RNAi treatment (A and B) and disappeared in the most affected regions (arrow in B). Similar to Fgf-8 expression, Fgf-10 expression in the mesoderm was also reduced in affected limbs (arrowheads in C and D).
Alteration of HS Structures for FGF-FGFR Binding by HS2ST RNAi—FGF signaling was up-regulated by HS2ST RNAi. Thus, the complex formation of HS, FGF, and FGFR would be inevitably affected due to the alteration of the HS structure. To test this possibility, we subjected limb buds with or without the HS2ST RNAi treatment to ligand and carbohydrate engagement assays using FGF-8, FGF-10, and their specific receptors FGFR2c and FGFR2b. Limb bud sections were incubated with a mixture of FGF-8 and FGFR2c or FGF-10 and FGFR2b. We observed significantly strong binding of FGF-8 and FGFR2c to limb mesenchyme and weak binding to basement membranes and ectoderm including the AER (Fig. 7A). No binding was detected without FGFRs or with the treatment with a mixture of heparitinases (Fig. 7A).

In the HS2ST RNAi-affected region, the binding of FGF-8 and FGFR2c was significantly weak (Fig. 7B). On the other hand, we observed strong binding of FGF-10 and FGFR2b in the limb mesenchyme and basement membranes and weak binding in the ectoderm including the AER (Fig. 7A). In the RNAi-affected region, interestingly the binding of FGF-10 and FGFR2b was increased (Fig. 7B). The binding was particularly increased in the mesenchyme under the ectoderm (Fig. 7B). These results suggest that the reduction of 2-O-sulfate by RNAi changes HS structures in the limb bud in that FGF-10-FGFR2b had a tendency to bind more and FGF-8-FGFR2c had a tendency to bind less to HS in the mesenchyme. Considering effects on the Fgf-8 and Fgf-10 expression observed in the RNAi-treated limb bud, these changes might affect the chick limb bud development.
changes in the activity of growth factors. To test this hypothesis, we examined the function of HS2ST in chick limb bud development. Inhibition of HS2ST (using siRNA mixtures) led to abnormal limb development and a disruption of AER formation and maintenance. This study provides the first evidence that HS2ST is essential for limb bud development.

Abnormal Limb Development by HS2ST RNAi—The knockdown of HS2ST in chick limb buds led to limb truncation, and the AER of these truncated limb buds did not function properly due to reduced Fgf-8 expression (Figs. 4 and 5). Fgf-8 expression in the prospective wing bud ectoderm begins in stage 16, and wing buds start to develop and grow in stage 17 (43). The activation of genes expressed in the AER, including Fgf-8, requires the signaling of FGF-10 derived from underlying mesenchymal cells. In the prospective forelimb region, Fgf-10 is expressed in the LPM at stage 14-15 (5). In this study, we injected HS2ST siRNA mixtures into the LPM of the prospective forelimb region at stage 13-14. Although it is unknown how long it takes for RNAi-affected cells to completely replace normal HS with low 2-O-sulfated HS, the induction of Fgf-10 expression in the LPM remains for several hours after RNAi treatment because it takes time to silence target genes and replace normal HS with de novo synthesized abnormal HS. Considering these factors, limb bud truncation by HS2ST knockdown occurs after AER induction by FGF-10. In fact, Fgf-8 expression in the AER did not completely disappear after RNAi treatment (Figs. 4 and 5). Therefore, truncation by HS2ST RNAi is likely caused by the disruption of AER maintenance, which involves the FGF-8 and FGF-10 signaling loop and other factors such as Shh.

Role of 2-O-Sulfation in FGF Signaling Loop during Limb Development—Previous in vitro studies revealed that both 2-O-sulfation and 6-O-sulfation are necessary for FGF-8 to bind to HS, whereas only 6-O-sulfation is required for FGF-10 to bind to HS (23). Thus, a loss of 2-O-sulfation should not affect the binding of FGF-10 but should affect the binding of FGF-8. Bindings of FGFs to HS may be different from bindings of FGFs to HS. In fact, interaction of FGF-8b and FGF3 with HS was not affected in Hs2st-deficient mice, although interactions between FGF-8b and FGF2c were not detected in the deficient mice (41). In the developing limb bud, FGFR2b and FGFR2c, which are specific to FGF-10 and -8, respectively, are expressed in the ectoderm and mesoderm, respectively (5, 6). Therefore, the suppression of 2-O-sulfation may interrupt FGF-8 signaling via FGFR2c in the mesoderm. Consistent with this estimation, a significant decrease in the FGF-8/FGF2c binding was observed at the HS2ST RNAi-affected region (Fig. 7). The reason why the binding was still observed at the RNAi-affected region may be attributed to the remaining 2-O-sulfate residues because of incomplete loss of 2-O-sulfate residues by RNAi. In contrast, interaction of FGF-10/FGFR2b was significantly enhanced in the HS2ST RNAi-treated limb mesenchyme (Fig. 7B). Because FGFR2b is not expressed in the mesenchyme (5, 6), this result may explain the disruption of FGF-10 signaling as follows. FGF-10 is expressed in the limb mesenchyme and acts as a ligand in the AER, which expresses FGFR2b. Therefore, FGF-10 must diffuse to the AER from mesenchyme for signaling. HS in the HS2ST RNAi-treated mesenchyme would have a high affinity for FGF-10, and so FGF-10 in the treated mesenchyme might be difficult to diffuse to the AER and tend to remain in the mesenchyme. Furthermore in HS2ST mutant mice or HS2ST mutant Drosophila, a compensating increase in 6-O-sulfation has been observed (49, 50). If this is also the case with HS2ST knockdown limb buds, such highly 6-O-sulfated HS would bind more strongly to FGF-10 than would normal HS, resulting in the disruption of FGF-10 diffusion through the extracellular matrix.

Up-regulation of ERK and Akt Phosphorylation in HS2ST RNAi-treated Limb Buds—As the affected limb buds by HS2ST RNAi reduced Fgf-8 and -10 expressions and also possibly showed the interruption of FGF-8 and FGF-10 signaling as discussed above, it is likely that the signal transduction of FGFs may also be decreased. The reduction of O-sulfation is expected to decrease the phosphorylation of ERK under FGF signal transduction. For example, RNAi of Drosophila Hs6ST reduces ERK phosphorylation in the tracheal system (33). However, we observed the up-regulation of ERK and Akt phosphorylation by HS2ST RNAi in developing limb buds (Fig. 6). This up-regulation of ERK and Akt phosphorylation could be caused by abnormal FGF signaling and other signaling through any tyrosine kinase receptors, although the respective mechanisms are estimated to be different. A more probable possibility is that the reduction of 2-O-sulfate affected the distribution or translocation of those HBGFs. The reduction of HS often causes the broad diffusion of HBGFs (9–11). Such abnormal distributions of HBGFs would widely activate their signals compared with localized HBGFs. In addition, fibroblast cells from HS2ST-deficient mice have a normal response for FGF-2 signaling although they have 2-O-sulfate-deficient HS that does not have binding activity with FGF-2 (49). Thus, the reduction of 2-O-sulfate in chick limb buds may affect the localization of HBGFs but not their signaling, although their signal levels and locations are probably imprecise, resulting in the up-regulation of pERK and pAkt.

Alternatively 2-O-sulfate of HS may negatively regulate the FGF signaling in chick limb bud development. In some reports, heavily 2-O-sulfated regions of HS are negative regulators of FGF signaling (51–54). If the 2-O-sulfation level in the limb bud is high enough to inhibit FGF signaling, HS2ST RNAi would reduce the inhibitory HS domain, resulting in the acceleration of FGF signaling. Because the interaction of FGF-8/FGF2c was shown to decrease in the mesenchyme after RNAi, other FGF or FGF signaling such as FGF-4 or FGFRI may be up-regulated.

In chick limb bud development, both the MAPK/ERK and phosphatidylinositol 3-OH-kinase/Akt pathways have been shown to be essential for limb bud development and patterning (44–46). The ERK phosphorylation level in the limb bud is regulated by MAPK phosphatase 3, which antagonizes ERK phosphorylation and is induced through the phosphatidylinositol 3-OH-kinase/Akt pathway (45, 46). Recent reports have demonstrated that the MAPK/ERK pathway itself also induces MAPK phosphatase 3 expression and negatively regulates its phosphorylation level (pERK) in chick limb buds (46, 55). Indeed the levels of pERK and pAkt seemed to differ depending upon the different phenotypes or injections (Fig. 6C). Application of FGF-8 or other FGF in the developing limb bud causes truncation, although such FGFs induce ectopic limb
buds in the flank of chick embryos (56, 57). In addition, it has been shown that ERK activation induces apoptosis in the limb mesenchyme (45). However, there has been no report on roles of Akt signaling in apoptosis during limb bud development, and this signaling is rather considered to have an antiapoptotic function (58). As it is possible that pERK and pAkt were up-regulated in different cells, abnormal apoptosis might happen to the cells with up-regulated pERK. Thus, knockdown of HS2ST by RNAi in the chick limb buds would cause abnormal apoptosis by up-regulation of ERK phosphorylation due to abnormal HS structures, resulting in truncation in severely affected limbs.

The Function of HS in Limb Development—We have shown that HS2ST is essential for wing bud development in chicken embryos. Interestingly the role of HS in limb bud development is different among tetrapods. In zebrafish, EXT2 and EXTL3 are expressed ubiquitously in the mouse limb ectoderm (70). A specific Wnt signaling by a similar action. However, further studies are required to show such possibilities.

Acknowledgments—We thank N. Nagai, S. Ashikari-Hada, and K. Kamimura for technical support and helpful discussions.

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