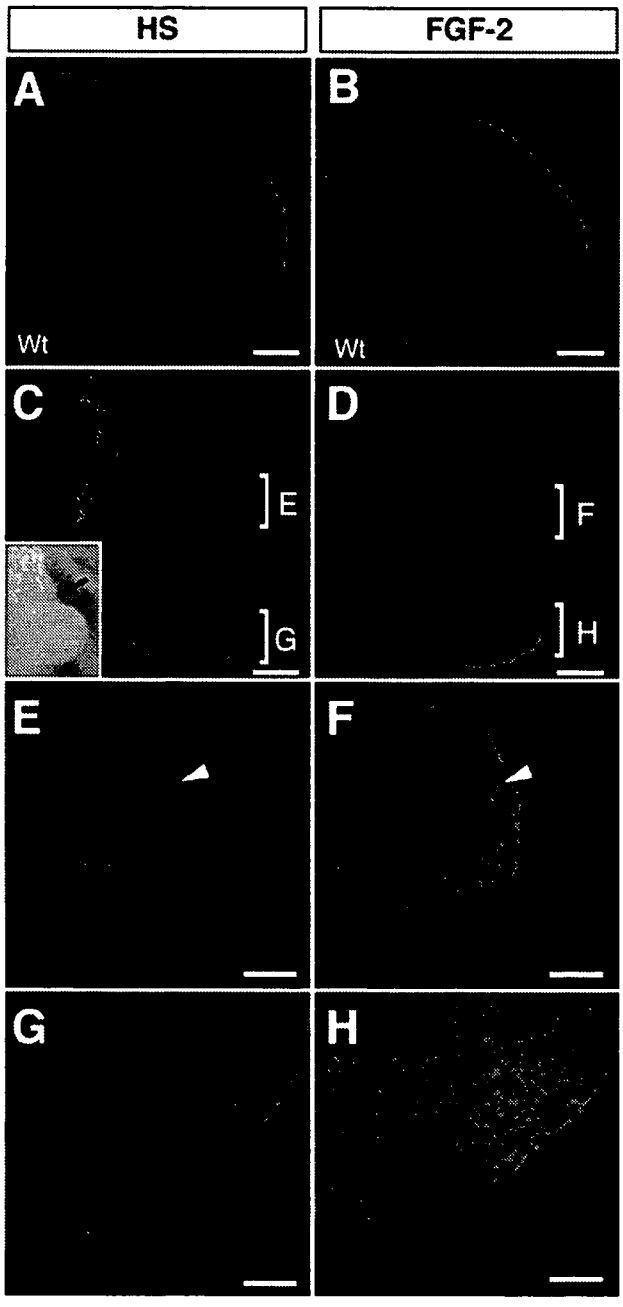

FIGURE 3. **Alteration of 2-*O*-sulfated HS by HS2ST RNAi.** *A* and *C*, HS was detected by 3G10 antibody. *B* and *D*, the 2-*O*-sulfated HS was detected as described under "Experimental Procedures." Longitudinal sections of limb buds (removal of endogenous HBGFs by treatment with 2 M NaCl) were exposed 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.

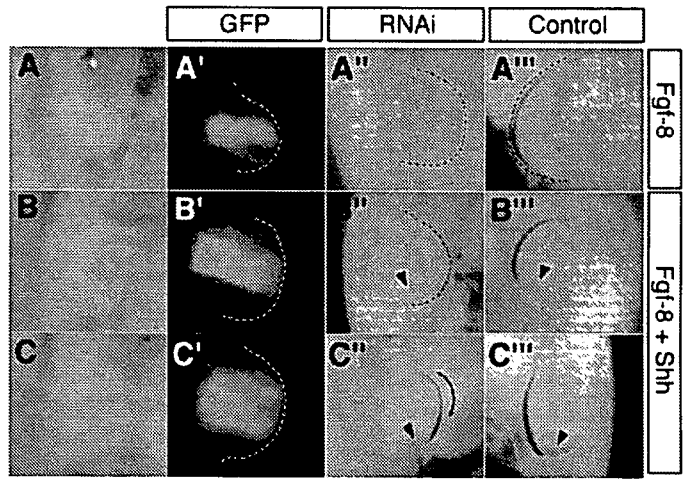

FIGURE 4. **Abnormal limb buds showed lower *Fgf-8* and *Shh* expression.** *A–C*, the expression patterns of *Fgf-8* and *Shh* in stage 23 embryos were detected by whole-mount *in situ* hybridization. *A′–C′*, GFP signals, which were derived from coelectroporated pEGFP-N1, indicated the siRNA-injected 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.

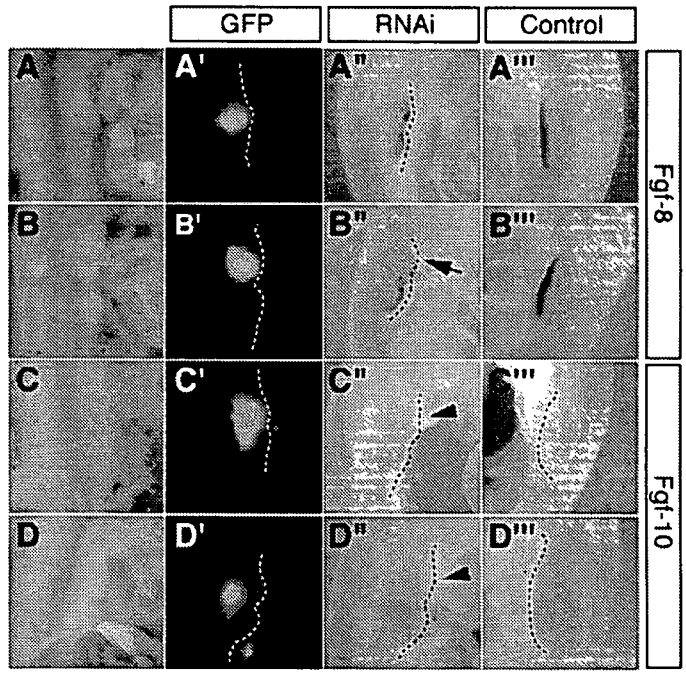

FIGURE 5. **HS2ST RNAi disrupted the FGF signaling loop.** *A–D*, *Fgf-8* and *Fgf-10* expression patterns at stage 18 (*A–C*) or 19 (*D*) were detected by whole-mount *in situ* hybridization (13–15 h after treatment). *A′–D′*, GFP signals, which were derived from coelectroporated pEGFP-N1, indicated 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*).

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. 6*C*). 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.

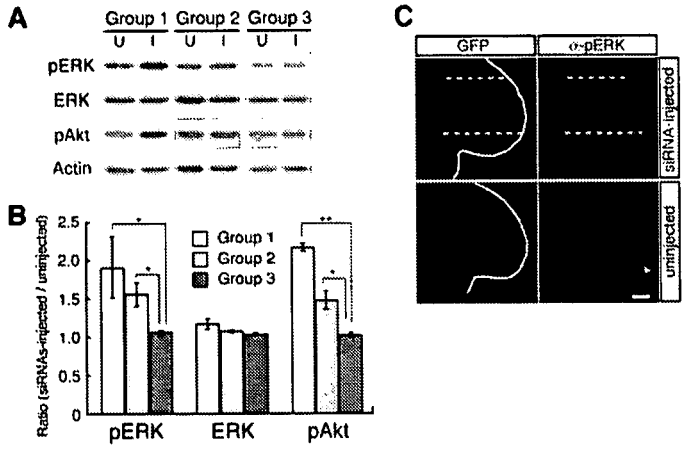

FIGURE 6. **Changes in ERK and Akt phosphorylation by HS2ST RNAi.** Phosphorylation of ERK and Akt was determined by Western blot analysis. *Group 1*, HS2ST esiRNA-injected and truncated limb buds (450 ng/μl). *Group 2*, HS2ST esiRNA-injected and almost normally developed limb buds (450 ng/μl). *Group 3*, luciferase esiRNA-injected and almost normally developed limb buds (300 ng/μl). *A*, Western blot analysis. Proteins were extracted from injected right buds (*I*) and the corresponding untreated left buds (*U*) of embryos at stage 23. *B*, the ratio of esiRNA-injected right buds to uninjected left buds. Each ratio of pERK, ERK, or pAkt is normalized to the respective actin expression. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$ (Student's *t* test). *C*, localization of pERK. HS2ST siRNA-injected right bud and uninjected left bud at stage 23 were longitudinally sectioned and immunostained with anti-pERK antibody. A higher level of pERK was observed in AER (*arrowheads*) and ectoderm as reported previously (45). A relatively high level of pERK was observed in the HS2ST siRNA-injected region indicated by GFP signal (area between *dotted lines*), whereas a lower level of pERK was observed in the mesenchyme of uninjected control buds. *Scale bar*, 200 μm.

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. 7*A*). No binding was detected without FGFs or with the treatment with a mixture of heparitinases (Fig. 7*A*). In the HS2ST RNAi-affected region, the binding of FGF-8 and FGFR2c was significantly weak (Fig. 7*B*). 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. 7*A*). In the RNAi-affected region, interestingly the binding of FGF-10 and FGFR2b was increased (Fig. 7*B*). The binding was particularly increased in the mesenchyme under the ectoderm (Fig. 7*B*). 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* expressions observed in the RNAi-treated limb bud, these changes might affect the chick limb bud development.

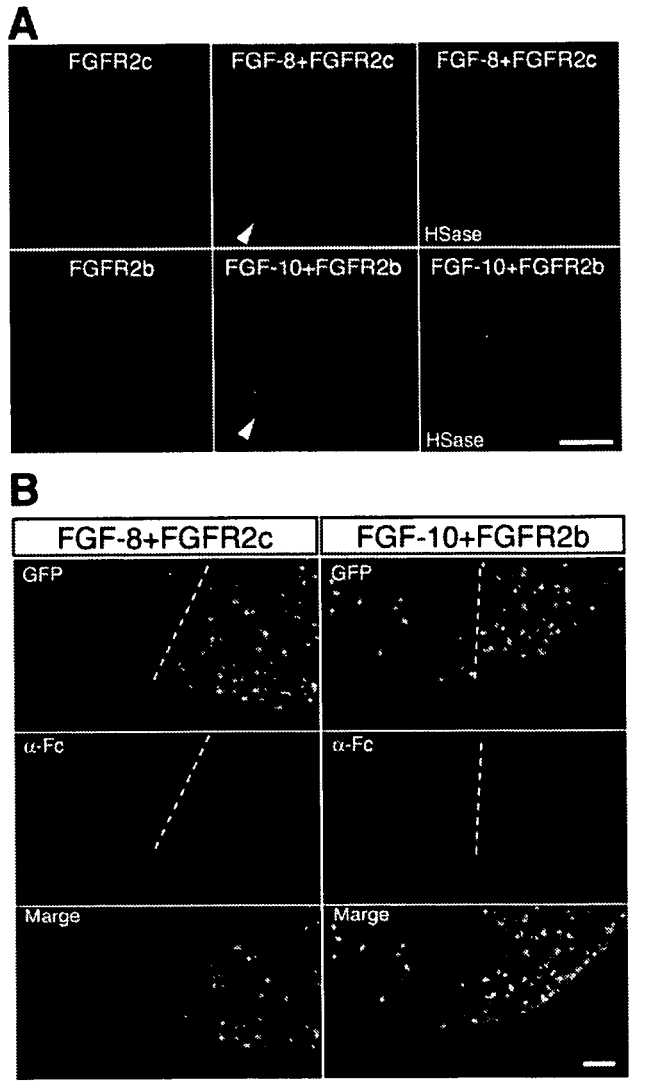

FIGURE 7. **Alteration of FGF and FGFR binding by HS2ST RNAi.** Interaction of FGF-8·FGFR2c and FGF-10·FGFR2b with HS was examined using the ligand and carbohydrate engagement assay. *A*, bindings of FGF-8·FGFR2c and FGF-10·FGFR2b on normal limb bud sections. Transverse sections of stage 23 limb buds were exposed to exogenous FGF-8 or FGF-10 and FGFR2c-Fc or FGFR2b-Fc and then probed with anti-Fc antibody. The binding of FGF-8·FGFR2c or FGF-10·FGFR2b was observed, whereas the binding of only FGFR2b or FGFR2c was not observed. When sections were pretreated with heparitinase (*HSase*), the bindings of FGF·FGFR were significantly reduced. Strong signals in the AER (*arrowheads*) were not detected either for the binding of FGF-8·FGFR2c or for that of FGF-10·FGFR2c. *Scale bar*, 50 μm. *B*, bindings of FGF-8·FGFR2c and FGF-10·FGFR2b in the longitudinal sections of limb bud after HS2ST RNAi treatment. GFP signals show RNAi-affected region. HS2ST siRNAs were injected into the area to the *right* of the *dotted lines*. The binding of FGF-10·FGFR2b is significantly increased in the RNAi-affected region, whereas the binding of FGF-8·FGFR2c is reduced in the RNAi-affected region. The nonspecific binding of FGF-8·FGFR2c was observed in the AER possibly due to the folding section. *Scale bar*, 50 μm.

DISCUSSION

Limb bud development is regulated by various growth factors, especially FGF-8 and FGF-10. HS is known to be involved in their signaling by its binding activity with specific structures (23). We therefore expected that the interaction between HS and growth factors requires a specific *O*-sulfation pattern. The expression patterns of HS *O*-sulfotransferases (HS2ST and HS6ST) are spatiotemporally regulated during embryogenesis and in adult tissues (34, 47, 48) and appear to coordinate with

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 FGF·FGFRs to HS may be different from bindings of FGFs to HS. In fact, interaction of FGF-8b and FGFR3c with HS was not affected in *Hs2st*-deficient mice, although interactions between FGF-8b and FGFR2c 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·FGFR2c 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. 7*B*). 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*-sulafted 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·FGFR2c was shown to decrease in the mesenchyme after RNAi, other FGF or FGFR signaling such as FGF-4 or FGFR1 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. 6*C*).

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 mutants resemble *Fgf-10* mutants, which have dysfunctional pectoral fins, suggesting that EXT2 and EXTL3 are required for FGF-10 signaling during fin bud development (59). Although the EXT family of genes are glycosyltransferases, which are responsible for HS chain synthesis, a similar fin bud defect also resulted from HS6ST knockdown in zebrafish (31). These reports indicate that fine structures of HS are essential for pectoral fin bud development in zebrafish. On the other hand, EXT1- or EXT2-deficient mice failed in gastrulation (60, 61). However, the hypomorphic mutation of EXT1 in mice resulted in reduced skeletal size, fusions of the elbow and knee joints, and occasionally syndactyly of digits (62). Similarly HS2ST or C-5 epimerase knock-out mice have a high frequency of polydactyly (28, 63). Considering these different effects depending upon the animal species, the fine structures of HS are more important being definitively involved in chick limb and fish fin development than in mammalian limb development. In fact, HS6ST expressions in developing limb reveal different patterns in these animals. For example, HS6ST-1 and -2 are expressed in AER of developing mouse limb bud, whereas significant expressions of HS6STs in AER are not observed in chick limb and zebrafish fin (34, 48, 64). Thus, the different fine structures of HS may play different roles in the regulation of various growth factors and morphogen signaling, the extent of which appears to vary depending on different vertebrates.

In accordance with different expression patterns of HS modification enzymes, the expression of some Wnt family proteins in developing limb buds appears to be different between chicks and mice (65, 66). At the initiation stage of chick limb buds, Wnt2b is expressed in intermediate mesoderm and LPM in the prospective forelimb region, and Wnt8c is expressed in LPM at the prospective hind limb (67). Both signals induce and maintain the expression of *Fgf-10* and are capable of inducing ectopic limbs in the embryonic flank. However, similar expression patterns of Wnt2b and Wnt8c at the limb initiation stage were not detected in mouse embryos (68). FGF-10 induces Wnt3a expression in the AER of chick limb buds, leading to the induction of *Fgf-8* expression (69). In contrast, Wnt3, but not Wnt3a, is expressed ubiquitously in the mouse limb ectoderm (70). A conditional Wnt3 mutant mouse exhibits a lack of *Fgf-8* expression in the limb buds (71). Several recent studies show that heparan sulfate proteoglycans regulate Wnt/Wg signaling during vertebrate and invertebrate development (10, 72). Although it has not been shown whether specific HS structures are required for the interaction of Wnt family proteins, knockdown of zebrafish HS6ST caused the up-regulation of Wnt and hedgehog signaling (31). Similar up-regulation of Wnt signaling was demonstrated by the action of HS-specific 6-*O*-endosulfatases (73, 74). The up-regulation of this signaling is considered a result of the release of growth factors from HS by the reduction of 6-*O*-sulfation. It is likely that HS2ST RNAi affects specific Wnt signaling by a similar action. However, further studies are required to show such possibilities.

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