Glycosaminoglycan-dependent restriction of FGF diffusion is necessary for lacrimal gland development

Xiuxia Qu¹, Yi Pan², Christian Carbe¹*, Andrea Powers¹, Kay Grobe³ and Xin Zhang¹,‡

SUMMARY

Glycosaminoglycans (GAGs) play a central role in embryonic development by regulating the movement and signaling of morphogens. We have previously demonstrated that GAGs are the co-receptors for Fgf10 signaling in the lacrimal gland epithelium, but their function in the Fgf10-producing periocular mesenchyme is still poorly understood. In this study, we have generated a mesenchymal ablation of UDP-glucose dehydrogenase (Ugdh), an essential biosynthetic enzyme for GAGs. Although Fgf10 RNA is expressed normally in the periocular mesenchyme, Ugdh mutation leads to excessive dispersion of Fgf10 protein, which fails to elicit an FGF signaling response or budding morphogenesis in the presumptive lacrimal gland epithelium. This is supported by genetic rescue experiments in which the Ugdh lacrimal gland defect is ameliorated by constitutive Ras activation in the epithelium but not in the mesenchyme. We further show that lacrimal gland development requires the mesenchymal expression of the heparan sulfate N-sulfation genes Ndst1 and Ndst2 but not the 6-O and 2-O-sulfation genes Hs6st1, Hs6st2 and Hs2st. Taken together, these results demonstrate that mesenchymal GAG controls lacrimal gland induction by restricting the diffusion of Fgf10.

KEY WORDS: FGF, Heparan sulfate, Glycosaminoglycan, Lacrimal gland, Branching morphogenesis, Morphogen gradient, Mouse

INTRODUCTION

Glycosaminoglycans (GAGs) are the major constituents of the extracellular matrix that control the transport and signaling of numerous growth factors (Hacker et al., 2005). Consisting of 50-400 repeats of disaccharide units, GAGs can be divided by their composition, sulfation and epimerization into chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS)/heparin, and keratan sulfate (KS). A common precursor of all GAGs is UDP-glucose acid, which is synthesized from UDP-glucose by a single mammalian enzyme, UDP-glucose dehydrogenase (Ugdh). Together with an amino sugar such as N-acetylgalactosamine for HS and N-acetylgalactosamine for CS, these monosaccharides are incorporated by polymerization enzymes into the backbone of the polysaccharide and are further modified by a series of sulfotransferase enzymes (Ndst, Hs6st, Hs2st). Taken together, these results demonstrate that the sulfation modification of HS is crucial for its function as a requisite partner of FGFR on the cell surface to detect FGF signals.

Previous studies have also suggested that GAGs might regulate the diffusion of FGFs in the extracellular matrix. It has been observed that GAG family proteins often can bind to highly sulfated GAG sequences, including HS, CS and DS (Jemth et al., 2002; Bao et al., 2004; Kreuger et al., 2005; Taylor et al., 2005). On the one hand, through rapid and reversible binding, cell surface GAGs might protect FGF from proteolytic degradation and control the movement of FGF within the extracellular matrix, thus maintaining the position-dependent gradient of FGF (Beer et al., 1997; Dowd et al., 1999). On the other hand, enzymatic cleavage of GAGs or their attached core protein has been suggested to turn FGF into a long-range signaling molecule by releasing the FGF-bound GAGs. Indeed, it was shown in Xenopus embryos that secreted serine protease xHtrA1 stimulated FGF signaling by cleaving GAGs.

Note

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containing proteoglycans, thus promoting the movement of FGF. Consistent with this, injection of HS and DS in Xenopus embryos induced a similar posteriorization effect as that by xHtrA1 and FGF signals (Hou et al., 2007).

The role of GAGs in FGF diffusion during branching morphogenesis is poorly understood. In the vertebrate lung, signals (Hou et al., 2007) induced a similar posteriorization effect as that by xHtrA1 and FGF.

In the ductal system of the lacrimal gland, the secretory epithelial cells are surrounded by a basement membrane rich in HS. It has been shown that even heterozygous mutations in HS produce subtle but significant defects in gland development (Yan and Lin, 2007). These results thus raised questions about the role of HS in the FGF-producing mesenchyme.

The lacrimal gland also develops through branching morphogenesis regulated by FGF signaling. At mouse embryonic day (E) 12.5, the conjunctival epithelium at the temporal side of the eye invades the Fgf10-expressing mesenchyme to form the initial lacrimal gland bud. The bud elongates posteriorly until E15.5 when secondary branching begins to establish the complex tubuloalveolar structure. This allows a continuous flow of nutrient and secretory products to the distal tubules, which are undergoing branching morphogenesis, and in whole-lung culture addition of over-sulfated heparin actually suppressed branching morphogenesis (Izvolsky et al., 2003). However, HS digestion by heparanase in submammalian gland culture was shown to be critical for the release of Fgf10 from the basement membrane and subsequent MAPK signaling (Patel et al., 2007). By contrast, genetic analysis of Drosophila demonstrated that, although tracheoblast cells required HS to respond to FGF signaling, ablation of HS in the FGF-producing or surrounding cells did not affect trachea development (Yan and Lin, 2007). These results thus raised questions about the role of HS in the FGF-producing mesenchyme.

The lacrimal gland development was studied in an in vitro culture model. The lacrimal gland was dissected from E15.5 embryos and cultured in conditioned medium supplemented with FGF. The gland was allowed to develop for 3 days, and the resulting glands were analyzed for their morphology and gene expression. The results showed that HS is crucial for mesenchymal differentiation and FGF diffusion. HS-depleted media inhibited gland development, while HS supplementation restored normal gland morphology and gene expression.

MATERIALS AND METHODS

Mice

The Ugdhlox targeting vector was constructed using the recombineering method from a 12.7 kb genomic fragment (Liu et al., 2003; Carbe et al., 2012), which was retrieved from a C57BL/6 bacterial artificial chromosome (BAC) clone (RP23-477N9, BACPAC Resources Center at Children’s Hospital Oakland Research Institute, CA, USA). It contains a neomycin (Neo) selection cassette surrounded by two fRT sites and exon 6 of the Ugdh gene flanked by two loxP sites (Fig. 1A). The linearized targeting construct was electroporated into 129S6/SvEvTac embryonic stem (ES) cells and recombinant clones were screened by Southern blot analysis with 5’ (SacI) and 3’ (BamHI) external probes before being injected into C57BL/6 blastocysts. Chimeras were further bred with C57BL/6 mice for germline transmission, which was confirmed by PCR genotyping using the following primers: Ugdhlox^F: 5’-TTCTGAGG-CTGTATTTCACTTCC-3’; Ugdhlox^R: 5’-AGGCAAGGGCACAGTT-AGGA-3’. The amplification bands of 214 bp and 314 bp corresponded to the wild-type and flox alleles, respectively. After crossing with an F1 transgenic line (stock number 009086, Jackson Laboratory, Bar Harbor, ME, USA), the fRT-flanked Neo cassette was removed in the Ugdhlox mice.

Ndst1^fl/fl and Hs6st2^fl/fl mice were described previously (Grobe et al., 2005; Qu et al., 2011b). Hs6st2^fl/fl is a kind gift from Dr Wellington V. Cardoso (Boston University School of Medicine, Boston, MA, USA) (Izvolsky et al., 2008). Hs6st2^fl/fl is a kind gift from Dr Jeffrey D. Esko (University of California San Diego, La Jolla, CA, USA) (Stanford et al., 2010). Tg-HrasG12D^fl/fl is a kind gift from Dr Paul A. Overbeek (Baylor College of Medicine, Houston, TX, USA) (Burgess et al., 2010). Ndst2^fl/fl is a kind gift from Dr Lena Kjellén (University of Uppsala, Uppsala, Sweden) (Forberg et al., 1999). The P0.5 lacZ reporter transgenic mice were kindly provided by Drs Paul A. Overbeek and Richard Lang (Children’s Hospital Research Foundation, Cincinnati, OH, USA) (Makarenkova et al., 2000). LSL-Kras^G12D^mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute (Tuveson et al., 2004). Wnt1-Cre mice were isolated from Jackson Laboratory (stock number 009107) (Danielian et al., 1998). The Wnt1-Cre; Ugdhlox^fl/fl embryos were generated by crossing Wnt1-Cre; Ugdhlox^fl/fl mice with Ugdhlox^fl/fl mice, and the Wnt1-Cre; Ugdhlox^fl/fl embryos in the same litter were used as wild-type controls. The animals were maintained in mixed genetic backgrounds. All experiments were performed in accordance with institutional guidelines.

Histology and immunohistochemistry

Hematoxylin and Eosin (H&E) histology, carcino staining and immunohistochemistry were performed as previously described (Pan et al., 2008). For HS detection, sections were treated with 50 μg/ml heparinase I (Seikagaku, Tokyo, Japan) for 2 hours at 37°C to expose the terminal disaturated hexuronic residues at the non-reducing ends of HS, which are recognizable by 3G10 antibody (David et al., 1992). By contrast, 10E4 antibody binds HS epitopes that are N-sulfated and N-acetylated rather than O-sulfated (David et al., 1992; van den Born et al., 2005). For phospho-ERK staining, the Tyramide Signal Amplification kit (TSA Plus System, PerkinElmer, Waltham, MA, USA) was used to amplify the signal. The following antibodies were used: anti-phospho-ERK1/2 (#9107, Cell Signaling Technology, Beverly, MA, USA), anti-Pax6 (PRB-278P, Covance, USA), anti-E-cadherin (U3254, Sigma), anti-Fgf10 (#sc-7917, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-ERK1/2 (#9102, Cell Signaling Technology, Beverly, MA, USA), anti-Fgf10 (R&D Systems, Minneapolis, MN, USA). 3G10 (#H1890-75, United States Biological, Swampscott, MA, USA), anti-chondroitin sulfate (#A28035, Sigma, St Louis, MO, USA), anti-E-cadherin (U3254, Sigma), anti-Fgf10 (#sc-7917, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Pax6 (PRB-278P, Covance, Berkeley, CA, USA). At least three embryos of each genotype were analyzed.

RNA in situ hybridization

RNA in situ hybridization on cryosections was performed according to a standard protocol (Pan et al., 2008). Briefly, the digoxigenin-labeled probe was hybridized on cryosections overnight at 65°C, followed by stringent washing. After equilibration with maleic acid buffer, the sections were incubated with an AP-conjugated anti-digoxigenin antibody (Roche, Indianapolis, IN, USA). The amplification bands of 214 bp and 314 bp corresponded to the wild-type and flox alleles, respectively. After crossing with an F1 transgenic line (stock number 009086, Jackson Laboratory, Bar Harbor, ME, USA), the fRT-flanked Neo cassette was removed in the Ugdhlox mice.

DEVELOPMENT

CTGTATTTCACTTCC-3’; Ugdhlox^F: 5’-TTCTGAGG-
**RESULTS**

**Mesenchymal Ugdh is required for lacrimal gland development**

Ugdh is a biosynthetic enzyme for UDP-glucose, a substrate required for all GAG synthesis. To investigate the general function of GAG in embryonic development, we constructed a conditional allele of Ugdh by inserting twoloxP sites flanking the Ugdh exon 6, which encodes its catalytic domain (Fig. 1A) (Campbell et al., 2000; Sommer et al., 2004). Homologous recombination in ES cells was identified by Southern blots using both the 5’ and 3’ probes and germline transmission was confirmed by PCR using primers that surround the 5’loxP site (Fig. 1B,C). Ugdhlox/lox mice were born healthy at the normal Mendelian ratio and were fertile as adults, indicating that the conditional allele had no overt hypomorphic effects.

We have previously shown that Wnt1-Cre mediated ablation of Ndst1, an HS biosynthetic enzyme, disrupted the sulfation pattern of HS in the periorcular mesenchyme (Pan et al., 2008). Interestingly, no lacrimal gland phenotype was observed in this animal model, suggesting that lacrimal gland development did not require mesenchymal modification of HS by Ndst1. To investigate further...
the role of GAGs in lacrimal gland development, we crossed Wnt1-Cre mice with the Ugdhlox allele to deplete all GAGs in the periocular mesenchyme. The Wnt1-Cre; Ugdhloxflx (hereafter referred to as UgdhCKO) mice were born with a normal body size but died at birth with craniofacial defects including cleft palate (data not shown). In contrast to the Wnt1-Cre; Ndst1lox/lox mutants, carmine staining further revealed a complete absence of the lacrimal gland in the UgdhCKO pups (Fig. 1D,G, arrows). The lacrimal gland develops at mid-gestation through budding morphogenesis from the conjunctival epithelium, which can be identified by Pax6 expression (Fig. 1E,F, arrows). In the E14.5 UgdhCKO embryos, however, no such buds were ever observed (Fig. 1H,I, arrows; see Fig. 6F for statistics). Therefore, lacrimal gland development requires Ugdh function in the periocular mesenchyme.

Ugdh ablation disrupted the biosynthesis of GAGs

To determine the mechanism of Ugdh function, we next examined the biosynthesis of GAGs in the UgdhCKO mutants. Using an antibody specific to CS, we observed that at E10.5 this major family of GAGs was expressed in the control periocular mesenchyme but lost in the UgdhCKO mutants (Fig. 2A,E). Another major GAG, HS, can be detected by the antibody 3G10, which recognizes a common stub motif present in all HS after heparitinase I digestion (Pan et al., 2006). We showed that 3G10 staining was also lost in the Ugdh mutant mesenchyme but preserved in the epithelium (Fig. 2B,F, arrows and arrowheads). Consistent with this, both control and mutant embryos at E10.5 expressed N-sulfated HS recognized by the 10E4 antibody in the basement membrane of the epithelium but not in the mesenchyme (Fig. 2C,G, arrowheads) (Pan et al., 2006). Finally, we have previously shown that the assembly of the high affinity Fgf10/Fgfr2b complex on the cell surface required HS with selective secondary modifications, which could be detected by the FGF ligand and carbohydrate engagement assay (LACE) (Qu et al., 2011b). In E10.5 UgdhCKO mutants, the Fgf10/Fgfr2b LACE signal was again lost specifically in the periocular mesenchyme (Fig. 1D,H, arrows), consistent with the HS defects seen in early UgdhCKO embryos.

These results were further confirmed at E14.5 when the lacrimal gland bud had already extended from the fornix of the conjunctival epithelium in control embryos. In contrast to strong expression of GAGs in the control mesenchyme that surrounded the lacrimal gland bud, the UgdhCKO mutants lost CS and HS staining in the periocular mesenchyme underneath the conjunctival epithelium (Fig. 2I-P, arrows). Taken together, these results showed that the Wnt1-Cre mediated ablation of Ugdh indeed disrupted the expression of CS and HS in the periocular mesenchyme, confirming the essential role of Ugdh in GAG synthesis.

Ugdh mutants preserved Fgf10 expression but lost FGF signaling in lacrimal gland development

The profound loss of GAGs in the UgdhCKO mutants prompted us to ask whether development of the periocular mesenchyme was also affected. At E10.5 when GAGs were already depleted in the mutant periocular mesenchyme, we still detected the Fgf10 expression but lost the periocular mesenchyme (Fig. 2A-F, arrows). At E12.5, UgdhCKO mutants not only preserved a similar specific expression of Crabp1, FoxC1 and Pitx2, all markers of ocular anterior segment development (Fig. 3A-F, arrows). At E12.5, UgdhCKO mutants did not only preserve a similar expression of Crabp1, FoxC1 and Pitx2 as the control, but also displayed correct expression of Fgf10 in the periocular mesenchyme underneath the invaginating conjunctival epithelium (Fig. 3G-J, arrows; data not shown). Moreover, we failed to detect any statistically significant difference in cell proliferation as indicated by BrdU incorporation and Ki67 staining between the control and mutants (data not shown). Therefore, the UgdhCKO mutant mesenchyme appeared to be properly developed at the time of lacrimal gland induction.
Despite the apparently normal expression of Fgf10 in the Ugdh mutant mesenchyme, we observed a complete loss of FGF signaling during lacrimal gland development. In E13.5 control embryos, lacrimal gland progenitors first appeared as a patch of thickening cells at the fornix of the conjunctival epithelium, which exhibited elevated phosphorylation of ERK, a downstream mediator of FGF signaling, and increased expression of Erm, an FGF signaling downstream response gene (Fig. 3K,M, arrows). In the UgdhCKO mutants, however, both ERK phosphorylation and Erm expression were abolished in the presumptive lacrimal gland progenitor cells (Fig. 3L,N, arrows). Similarly, strong activation of FGF signaling was evident in the E14.5 control lacrimal gland bud by robust expression of phospho-ERK and Dusp6, another FGF signaling inducible gene. By contrast, the UgdhCKO mutants preserved Fgf10 expression in mesenchyme but failed to display any phospho-ERK and Dusp6 staining in the conjunctival epithelium (Fig. 3O-T, arrows). These results suggest that the Ugdh lacrimal gland defects might be caused by the loss of FGF signaling.

Excessive Fgf10 diffusion disrupted lacrimal gland budding in Ugdh mutants

GAGs in the extracellular matrix might promote cell signaling by protecting growth factors from degradation or by restricting their dispersion. To test these ideas, we first collected periocular mesenchyme from E13.5 embryos and performed western blots. As shown in Fig. 4A,B, both control and UgdhCKO mutants exhibited comparable amounts of Fgf10 protein expression in the periocular mesenchyme, suggesting that GAGs were not required for the stability of Fgf10 protein in lacrimal gland development. We next performed an FGF diffusion assay to determine whether the extracellular GAG regulated Fgf10 dispersion. As the endogenous expression of Fgf10 protein was below the detection limit of immunohistochemistry (data not shown), we decided to implant E10.5 head explants with beads soaked with either BSA or Fgf10 in the periocular mesenchyme. After culturing for 3 hours, a ring of Fgf10 immunostaining could be detected in the control explants closely encircling the Fgf10 beads but not the BSA beads (Fig. 4C,F). By contrast, the UgdhCKO mutants exhibited a much wider span of Fgf10 immunostaining surrounding the Fgf10 beads in the periocular mesenchyme (Fig. 4D,G). However, both the control and UgdhCKO mutants displayed limited Fgf10 diffusion in the limb mesenchyme (Fig. 4E,H,I), which does not contain Wnt1-Cre derived neural crest (data not shown). These results suggested that Ugdh mutation led to an expanded diffusion of Fgf10 in the periocular mesenchyme.

We reasoned that excessive dispersion of Fgf10 in Ugdh mutant mesenchyme would probably lower its local concentration, effectively reducing the inductive strength of Fgf10 in promoting lacrimal gland budding. We thus performed a lacrimal gland induction assay to assess the potency of Fgf10 signaling in ex vivo explant culture. To visualize the budding of the lacrimal gland, we crossed our animals with P6.5 lacZ transgenic mice, which express the lacZ reporter under the control of a Pax6 promoter in the lacrimal gland (Makarenkova et al., 2000). In control explants of E13.5-E14.5 embryos, endogenous lacrimal glands stained by X-gal could be observed to grow spontaneously from the eye rudiments, unperturbed by BSA-soaked beads in the periocular
mesenchyme (Fig. 5A, arrow). By contrast, lacrimal gland budding was disrupted in the UgdhCKO mutant explants (Fig. 5D). As we and others have shown previously, Fgf10-soaked beads were able to efficiently induce ectopic lacrimal gland buds from the control eye rudiments (Fig. 5B, C, arrows) (Makarenkova et al., 2000; Pan et al., 2008). In the UgdhCKO mutant explants, however, the efficiency of ectopic lacrimal gland budding was significantly reduced (Fig. 5E-G, P<0.001). Therefore, the loss of GAGs in periocular mesenchyme disrupted Fgf10-induced lacrimal gland budding in the UgdhCKO mutants.

Fig. 5. Fgf10-induced lacrimal gland budding was abolished in the Ugdh mutants. (A-F) In E13.5 control explant culture, BSA-containing beads did not perturb budding of the endogenous lacrimal gland (A, arrow), whereas Fgf10-containing beads induced ectopic lacrimal gland buds (B, enlarged in C, arrowhead). By contrast, this ectopic induction of lacrimal gland buds was disrupted in UgdhCKO mutant explants (D-F). Beads are marked by asterisks. Endogenous and ectopic buds are marked by arrows and arrowhead, respectively. (G) The efficiency of lacrimal gland budding in explant cultures.

Epithelial Ras signaling rescued the lacrimal gland budding defects in the mesenchymal Ugdh mutants

The above results suggest that GAGs restrict the diffusion of Fgf10 in the periocular mesenchyme, which is necessary for budding of the lacrimal gland from conjunctival epithelium. This non-cell-autonomous model predicts that lacrimal gland defects caused by GAG deficiency in the mesenchyme could be rescued by the restoration of FGF downstream signaling in the epithelium. To test this model, we crossed the UgdhCKO mutant with two transgenic lines that expressed the constitutively active forms of Ras, which is a major downstream mediator of FGF signaling. Driven by the same Pax6 promoter used in the P6 5.0 lacZ transgenic mice, the Tg-HrasG12V line expressed the activated Hras (G12V) specifically in the lens and conjunctival epithelium (Fig. 6A) (Burgess et al., 2010). In the LSL-KrasG12D mouse, however, oncogenic Kras expression was controlled by its endogenous promoter but blocked by a floxed transcription stop cassette (Fig. 6A) (Tuveson et al., 2004). When crossed with the UgdhCKO (Wnt1-Cre; Ugdhflox/flox) mice, this stop cassette was expected to be cleaved by the Wnt1-Cre deleter, which also disrupted the Ugdhflox allele. This would result in a simultaneous ablation of GAG and activation of Kras signaling in the periocular mesenchyme. We have previously demonstrated that this strategy could compensate for the loss of FGF signaling in retinal, lens and lacrimal gland development (Cai et al., 2010; Pan et al., 2010; Qu et al., 2011a). Nevertheless, no
development requires Ndst1, a major N-sulfation enzyme for HS, only in the conjunctival epithelium but not in the periorcular mesenchyme (Pan et al., 2008). Indeed, we confirmed that Wnt1-Cre mediated ablation of Ndst1 (Wnt1-Cre; Ndst1flox/flox or Ndst1<sup>ΔKO</sup>) disrupted both 10E4 staining of HS and the LACE signal of Fgf10/Fgfr2b binding in the mesenchyme, but this conditional knockout of Ndst1 did not affect lacrimal gland budding (Fig. 7A-H, arrows; n=5). To further investigate the role of HS in lacrimal gland development, we generated a combined deletion of both Ndst1 and Ndst2 (Wnt1-Cre; Ndst1flox/flox; Ndst2<sup>ΔKO</sup> or Ndst1Δ<sup>ΔKO</sup>/Ndst2<sup>ΔKO</sup>). Although the staining of the pan-HS marker 3G10 was unaffected, lacrimal gland budding was completely abolished in Ndst1/2<sup>ΔKO</sup> mutants (Fig. 7I-L, arrows; n=6), demonstrating that the N-sulfated HS in the mesenchyme were indeed required for lacrimal gland development. We have also previously shown that lacrimal gland Fgf10 signaling requires 6-O and 2-O sulfated HS in the conjunctival epithelium (Qu et al., 2011b). Interestingly, a Wnt1-Cre-mediated ablation of two HS 6- O sulfotransferases (Hs6st<sup>1</sup> and Hs6st<sup>2</sup>) and 2-O sulfotransferase (Hs2st<sup>Δ</sup>) did not affect HS 3G10 and 10E4 staining, but abolished Fgf10/Fgfr2b binding in the periorcular mesenchyme (Fig. 7M-O, arrows). Nevertheless, lacrimal gland budding was preserved in this triple mutant (Wnt1-Cre; Hs6st<sup>1</sup>flox/flox; Hs6st<sup>2</sup>flox/flox or Hs6st<sup>1-2</sup>flox/flox; Hs2st<sup>Δ</sup>flox/flox) (Fig. 7P, arrows; n=7). Consistent with this, there was no statistically significant difference in the range of Fgf10 diffusion between the control and Hs6st<sup>1-2</sup>flox/flox; Hs2st<sup>Δ</sup>flox/flox mutants (supplementary material Fig. S1). Taken together, these results support that Fgf10 signaling in lacrimal gland development requires distinctive sets of HS modifications in the epithelium and mesenchyme.

**DISCUSSION**

In this study, we have used lacrimal gland development as a model to study the role of GAGs in epithelium-mesenchyme interaction. We showed that mesenchymal ablation of Ugdh eliminated the biosynthesis of HS and CS, two major constituents of GAGs, but it did not affect the differentiation of the periorcular mesenchyme. Importantly, the expression of Fgf10 transcripts and protein was preserved in the Ugdh mutant mesenchyme, but the Fgf10 signaling response was lost in the lacrimal gland mesenchyme. Ex vivo explant experiments suggested that the loss of GAGs leads to unrestricted dispersion of Fgf10, which became too diluted in the mesenchyme to induce significant FGF signaling and budding response in the epithelium. Indeed, we showed that Ugdh lacrimal budding defects could be rescued by activated Ras signaling in the epithelium but not in the mesenchyme, demonstrating a non-cell-autonomous function of GAGs in the periorcular mesenchyme to promote lacrimal gland development. Finally, we showed that the Ugdh lacrimal defect was phenocopied by the simultaneous ablation of HS modification enzymes, Ndst1 and Ndst2. This shows that HS is probably the most crucial GAG regulating Fgf10 dispersion in the periorcular mesenchyme.

Our study thus shows that a complete elimination of GAGs, including HS, in the ligand-producing mesenchyme abolished FGF signaling in the receptor-expressing epithelium. At first glance, this conclusion might appear contradictory to previous studies that showed gain-of-function phenotypes in Fgf mutants with reduced affinity to HS (Harada et al., 2009; Makarenkova et al., 2009). Here, we would like to argue that both observations were parts of the continuum of the FGF signaling response as a result of the perturbation in FGF-GAG interactions. As shown in Fig. 8, we propose that the strong affinity of wild-type FGF to extracellular
GAGs significantly restrict the dispersion of FGFs, resulting in a steep concentration gradient away from the signal source. By contrast, Fgf mutants with reduced GAG affinity exhibit a shallower gradient profile, effectively expanding the range of the FGF signaling response. Elimination of all GAGs, however, leads to a free diffusion of FGFs, which are dispersed too widely to maintain a concentration high enough for lacrimal gland induction. Notably, in vivo imaging in zebrafish showed that the movement of some, but not all, Fgf8 molecules were restricted by HS in the extracellular matrix, and enzymatic degradation of HS indeed extended the range of Fgf8 signaling (Yu et al., 2009). Our study is also consistent with studies in chick and mouse, which demonstrate an essential role of HS in regulating retention versus dispersion of FGFs in embryonic development (Chen et al., 2009; Shimokawa et al., 2011). We would also like to point out that Shimokawa and colleagues recently reported that proteolytic cleavage of HS core proteins was necessary for the spread of FGFs in the extra-embryonic ectoderm (Shimokawa et al., 2011). However, it is not clear how widely applicable this mechanism of FGF transport is beyond early gastrulation, as we did not observe any visible dispersion of HS in our conditional knockouts. Although further studies are required to resolve these issues, it is clear that manipulations of FGF-HS interactions could produce both gain- and loss-of-function responses in FGF signaling.

Finally, our studies also revealed the unique sulfation requirement of mesenchymal HS in lacrimal gland development. Although the mesenchymal knockout of Ndst1 completely abolished the 10E4 staining of HS, only the combined deletion of

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**Fig. 7. Mesenchymal ablation of Ndst1/2, but not Hs6st/Hs2st, disrupted lacrimal gland budding.** (A-H) Mesenchymal deletion of Ndst1 (Wnt1-Cre; Ndst1^lox/lox^ or Ndst1^CKO^) disrupted both HS 10E4 staining and the Fgf10/Fgfr2b LACE signal, but lacrimal gland budding was unaffected. (I-L) Combined mesenchymal deletion of both Ndst1 and Ndst2 (Wnt1-Cre; Ndst1^lox/lox^, Ndst2^CKO^ or Ndst1/2^CKO^) did not affect HS 3G10 staining but abolished lacrimal gland budding. (M-P) Combined deletion of Hs6st1, Hs6st2 and Hs2st genes (Wnt1-Cre; Hs6st1^lox/lox^, Hs6st2^CKO^, Hs2st^CKO^ or Hs6st1^CKO^, Hs2st^CKO^) failed to disrupt HS 3G10 and 10E4 staining, but it did abrogate the Fgf10/Fgfr2b LACE signal. However, lacrimal gland budding was not affected.

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**Fig. 8. Model of a biphasic regulation of FGF signaling by mesenchymal GAGs.** Wild-type GAGs restrict the diffusion of FGF to a sharp concentration gradient (green line), whereas Fgf mutations that weaken the interaction between Fgf and GAGs produced a relatively shallow gradient (yellow line), which expanded the Fgf signaling range. By contrast, ablation of GAGs leads to a free diffusion of Fgf in the mesenchyme (red line), the concentration of which was reduced too rapidly to sustain the FGF signaling response in the epithelium. WT, wild type.
both Ndst1 and Ndst2 would disrupt lacrimal gland development. The 10E4 antibody is widely used to probe tissue-specific expression of HS, but our results suggest that the HS epitope recognized by this antibody is dispensable in the mesenchyme for lacrimal gland development. This is in contrast to the lacrimal gland epithelium, where we showed earlier that ablation of Ndst1 was sufficient to abrogate lacrimal gland FGF signaling (Pan et al., 2008). The distinct sulfation requirement of HS between the epithelium and mesenchyme is also clear from analysis of HS 2-O and 6-O sulfations. Ashikari-Hada and colleagues have previously reported that Fgf10 requires 6-O but not 2-O sulfate groups for interaction with octasaccharides in vitro (Ashikari-Hada et al., 2004). However, our genetic analysis showed that lacrimal gland epithelium requires both 2-O and 6-O sulfated HS for Fgf10-induced budding (Qu et al., 2011b). In this study, we further showed that deletion of both Hs6st and Hs2st genes in the mesenchyme abolished the Fgf10/Fgfr2b LACE staining, but it did not cause any lacrimal gland budding defects. We would therefore like to propose that the function of mesenchymal HS in lacrimal gland development is regulated by its overall sulfation level but not by its specific sulfation pattern. In this view, the loss of HS 2-O and 6-O sulfation in the mesenchyme failed to disrupt lacrimal gland budding, because as we demonstrated earlier in Hs6st; Hs2st knockout MEF cells, the overall sulfation level of HS was maintained by the compensatory increase in N-sulfation (Qu et al., 2011b). By contrast, genetic deletion of both Ndst1 and Ndst2 has been shown to not only abolish HS N-sulfation, but also result in pleiotropic reduction in both HS 2-O and 6-O sulfation (Holmborn et al., 2004). Thus, for mesenchymal HS, it is the overall sulfation that is critical for lacrimal gland development. The requirement of a sulfation level for HS in the mesenchyme is also consistent with its role in the restriction of Fgf10 diffusion during lacrimal gland development. It is likely that the electrostatic interaction between Fgf10 and the negatively charged HS dictated by its sulfation level is sufficient to confine mesenchymal Fgf10 to the vicinity of the lacrimal gland epithelium. Yet the epithelial HS are expected to participate in a trimeric complex with FGF/FGFR as a co-receptor, which might place more stringent requirements for the sulfation pattern of HS. Therefore, there is a distinctive requirement for HS fine structures in the lacrimal gland epithelium versus the mesenchyme. Differential patterns of HS modifications have also been widely observed in both normal tissues and tumors.

We would like to suggest that these dynamic expressions of HS reflect their distinct functional requirement in epithelial-mesenchymal interactions, which might be important for understanding not only embryonic development but also tumorigenesis.

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Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl/doi:10.1242dev.079236/-/DC1

References
GAG controls FGF diffusion


