Heparan sulfate deficiency leads to Peters anomaly in mice by disturbing neural crest TGF-β2 signaling

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During human embryogenesis, neural crest cells migrate to the anterior chamber of the eye and then differentiate into the inner layers of the cornea, the iridocorneal angle, and the anterior portion of the iris. When proper development does not occur, this causes iridocorneal angle dysgenesis and intraocular pressure (IOP) elevation, which ultimately results in developmental glaucoma. Here, we show that heparan sulfate (HS) deficiency in mouse neural crest cells causes anterior chamber dysgenesis, including corneal endothelium defects, corneal stroma hypoplasia, and iridocorneal angle dysgenesis. These dysfunctions are phenotypes of the human developmental glaucoma, Peters anomaly. In the neural crest cells of mice embryos, disruption of the gene encoding exostosin 1 (Ext1), which is an indispensable enzyme for HS synthesis, resulted in disturbed TGF-β2 signaling. This led to reduced phosphorylation of Smad2 and downregulated expression of forkhead box C1 (Foxc1) and paired-like homeodomain transcription factor 2 (Pitx2), transcription factors that have been identified as the causative genes for developmental glaucoma. Furthermore, impaired interactions between HS and TGF-β2 induced developmental glaucoma, which was manifested as an IOP elevation caused by iridocorneal angle dysgenesis. These findings suggest that HS is necessary for neural crest cells to form the anterior chamber via TGF-β2 signaling. Disturbances of HS synthesis might therefore contribute to the pathology of developmental glaucoma.

Introduction
Developmental glaucoma is a congenital blinding disease associated with elevated intraocular pressure (IOP) because of anomalies of the drainage structure for the aqueous humor in the eye. The major drainage structure for aqueous humor consists of the iridocorneal angle, which is the place in which the iris and cornea meet (1, 2). Developmental studies have revealed that the iridocorneal angle originates from the periocular mesenchyme and mainly consists of neural crest cells (3, 4). During ocular morphogenesis, these neural crest cells migrate into the eye to create the anterior chamber, which is a small space within the anterior ocular segment (3, 4). The migrating neural crest cells cover the anterior chamber and differentiate into the iridocorneal angle, the corneal endothelium, and the anterior portion of the iris (5–9). Several of the transcriptional factor genes, such as forkhead box C1 (Foxc1), paired-like homeodomain transcription factor 2 (Pitx2), and paired box gene 6 (Pax6), have been identified as the causative genes for developmental glaucoma, and they have been demonstrated to be responsible for the differentiation of the neural crest cells (10–18). In addition to the iridocorneal angle, developmental glaucoma is also highly associated with anomalies in the neural crest–derived tissues such as the corneal endothelium defect (Peters anomaly; OMIM #604229), iris coloboma (Axenfeld-Rieger syndrome; OMIM #180500, #601499, and #602482), cleft palate, jaw defect, and ear deformity (15, 17, 19). Thus, failed differentiation of the neural crest cells causes anterior chamber dysgenesis, which ultimately results in developmental glaucoma.

Both the orderly migration and differentiation of the neural crest cells are controlled by cues from various cell guidance factors, morphogens, and extracellular matrices. The TGF-β2 superfamily has been found to be especially associated with mediating the differentiation of the ocular neural crest cells (20–22). The genetic disruption of TGF-β2 causes anterior chamber dysgenesis (20), with TGF-β2 signaling regulating the expression of the causative genes for developmental glaucoma, Foxc1 and Pitx2 (22). On the other hand, extracellular matrix molecules, such as fibronectin, laminin, peanut agglutinin–binding molecules, and chondroitin sulfate proteoglycans, are thought to affect the behavior of the neural crest cells (23–27). However, despite the influence of these molecules in vitro, it has yet to be determined in vivo whether these extracellular matrices are critical factors for neural crest cell development. The drainage structure has been found to have abundant extracellular matrices (28). Both the accumulation and changes in the component in the human trabecular meshwork are thought to affect the aqueous outflow resistance. Abnormal expression of glycosaminoglycans and their glycoproteins, along with proteoglycans in the trabecular meshwork, have been found in human glaucoma (29–31). Heparan sulfate (HS) is a glycosaminoglycan component of the proteoglycans that is expressed in the
extracellular matrix of the iridocorneal angle and on cell surfaces of the trabecular meshwork cells (32, 33). Interestingly, genetic studies in Drosophila showed that the defect of a HS proteoglycan (HSPG) homolog, dally, disturbs signaling mediated by the TGF-β homolog, dpp, suggesting that TGF-β-mediated morphogenesis may be dependent upon HSPGs (34). Moreover, a genetic interaction between the HSPG homolog, UNC-52, and the TGF-β homologs is observed during the development of the Caenorhabditis elegans gonad arm (35). Therefore, we hypothesized that HS/HSPGs may contribute to the development of the ocular neural crest cells. HS synthesis is governed by a series of enzymes. Exostosin 1 (EXT1) is indispensable for HS synthesis, because it has a critical role in the polymerization process of the alternating D-glucuronic acid and N-acetyl-D-glucosamine that ultimately provide the HS sugar chain backbone (36–38). Ext1-disrupted cells exhibit a complete loss of HS (37). In the current study, we found that the disruption of Ext1 in the neural crest cells leads to the Peters anomaly phenotype in the anterior chamber and, thus, affects the TGF-β2-mediated morphogenesis. We also found that gene reduction of Ext1 and Tgfb2 results in developmental glaucoma with an elevated IOP. This suggests that the neural crest cells require HS for TGF-β2-dependent iridocorneal angle development.

Results

Loss of Ext1 and HS in neural crest–derived tissues of the Wnt1-CreExt1<sup>flox</sup>/<sup>fl</sup> mutant. Ext1 mRNA was broadly expressed throughout the embryo. Similar to a previous report, intense signals for Ext1 were especially observed at E10.5 in the head region and branchial arches of the embryo (Figure 1A) (39). Subsequently, we demonstrated that Ext1 was particularly localized in the forebrain, hindbrain, facial region, and limb buds at E12.5 (Figure 1C). In order to conditionally disrupt Ext1 in the neural crest cells during embryogenesis, transgenic mice carrying the loxp-modified Ext1 allele (Ext1<sup>flox</sup> mice) were bred to transgenic mice with Cre recombinase driven by the Wnt1 promoter (Wnt1-Cre mice). The mutant carrying homozygous floxed alleles of Ext1 and an allele of Wnt1-Cre (Wnt1-CreExt1<sup>flox</sup>/<sup>fl</sup>) mice exhibited substantially decreased signals for Ext1 mRNA in the branchial arches and the facial region, including the optic vesicle at E10.5 (Figure 1B). In spite of the intense signal of the control (Figure 1, C and E), loss of the signal for the perilocular mesenchyme of the mutant occurred at E12.5 (Figure 1, D and F). As compared with the control tissue, immunohistochemistry with an anti-HS antibody revealed there was little staining for the mutant perilocular mesenchyme (Figure 1H). The other ocular tissues (including the lens, presumptive ciliary body, and neural retina) in the mutant (Figure 1H) showed similar levels of expression to those in the control (Figure 1G).

HS deficiency causes a Peters-like anomaly. All of the mutants died within the first day of life. Appearance was grossly normal except for severe malformation of the craniofacial tissues such as cleft palate (Figure 2, A–F) and ear deformity (Figure 2, A and B). Eyes of mutants exhibited multiple anomalies. All the eyes of mutants displayed eyelid defects (Figure 2, G–I). Of the 67 mutants at E18.5, 66 (98.5% of the total mutants) had ventral iris coloboma (Figure 2H). The coloboma was consecutively observed in the ciliary body of 39 (58.2%) mutants (Figure 2I). The anterior chamber structure was also affected in the mutant, with an abnormally thin cornea and dysgenesis of the iridocorneal angle (Figure 3, A and B). While a histological study showed that the values of the mutant corneal thickness and anterior chamber depth were significantly smaller than those of the control (Figure 3, A and C), the size of the lens derived from the surface ectoderm was not affected (Figure 3C). Van Gieson staining revealed that collagen was not distributed in the mutant corneal stroma (Figure 3D). Each of the corneal endothelial cells expressed ZO-1, a tight junction protein, in
the cell margins (Figure 3E). Immunostaining for ZO-1 revealed loss of ZO-1-positive cells in the mutant corneal endothelium (Figure 3E). These phenotypes in the anterior chambers are very similar to the ocular phenotypes of Peters anomaly, which is associated with disturbed differentiation of the ocular neural crest cells. Moreover, both a histological study performed between E11.5 and E18.5 (Figure 3, A and B) and a fate-mapping study for the neural crest cells (Figure 3F) demonstrated that neural crest cells of the mutant migrated to the periocular region. Moreover, both a histological study performed (Figure 3F) demonstrated that cell proliferation was decreased in the mutant cornea and iridocorneal angle at E15.5 (Figure 4). However, there were no differences in the frequency of the apoptotic cells observed (data not shown).

To exclude the possibility that decreased numbers of BrdU-positive cells in the mutant corneal stroma were due to limited number of cells in the thinner mutant corneal stroma at E15.5, we counted both the number of BrdU-positive cells and the total corneal cells during the earlier embryonic stage (E13.5). No differences for the corneal thickness or the distribution of neural crest cells (β-gal-positive cells traced by Rosa26R mouse strain) were noted between the mutant and control at E13.5. However, there was a significant (Figure 4J) reduction in the number of BrdU-positive cells in the mutant corneal tissue. These results indicated that reduced mutant neural crest proliferation is associated with poor anterior chamber development.

**HS deficiency is associated with the inactivation of TGF-β2 signaling and downregulation of Foxc1 and Pitx2.** The characteristics of the phenotypes in the anterior chamber were similar to those of the mutant genes for the TGF-β2 signaling molecules (Figure 5, A and B) (20, 22). Binding assays demonstrated that TGF-β2 had an affinity to HS, whereas epidermal growth factor did not bind to HS (Figure 5C). If there is disturbance of the TGF-β2 signaling in the HS-deficient eye, then there should be activation of downstream mediators of TGF-β2. It has been previously reported that TGF-β2 expression in the ocular anterior segment peaks between E13.5 and E15.0 (22). In the current study, while no major changes were noted for TGF-β2 distribution in the mutant eye at E13.5 (Figure 5, D and I), there was specific inhibition, in the periorcular mesenchyme of the mutant, of the phosphorylation of Smad2, which is a downstream molecule of TGF-β2 signaling (22). At E15.5, Pitx2 was observed in the cornea (Figure 5H), and there was localized expression of Foxc1 in the corneal endothelium and primitive trabecular beam (Figure 5G). In the mutant eye, however, a dramatic downregulation of Foxc1 and Pitx2 expression was noted (Figure 5, L and M). This reduced expression of the downstream molecules suggests that the HS deficiency is responsible for the disturbances in the TGF-β2 signaling.

In addition, it has also been shown that Tgfb2-disrupted mutants exhibit cleft palate (40). In the palatal tissue of these HS-deficient embryos, a dramatic downregulation of the immunoreactivity of phosphorylated Smad2 was observed (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38519DS1).

FGFs are known to be HS-binding morphogens, which are essential factors for HS-mediated lens development (41). Thus, it is possible that FGF signaling might be involved with anterior chamber morphogenesis. To further study this, we examined the expression of phosphorylated ERK1/2, which are downstream effectors of the FGF-MAPK pathway (Supplemental Figure 1). However, no differences in the phosphorylated ERK1/2 immunoreactivity in the anterior chamber were found between the mutant and control.

**TGF-β2 signaling in neural crest cells requires cell-autonomous expression of HS.** The transfection of the adenovirus that encodes the Cre recombinase gene showed there was expression of Cre recombinase in the cultured pericoronal neural crest cells with the Ext1flox/flox alleles (Figure 6A). Immunocytochemistry with the anti-HS antibody showed that the transfection of the Cre recombinase gene caused a loss of HS in the neural crest cells (Figure 6A). In primary cultures of pericoronal neural crest cells, TGF-β2-dependent...
HS deficiency causes Peters-like anomaly. (A and B) Thionin staining of the eyes of Wnt1-CreExt1<sup>fl</sup>box and control embryos during embryonic development. At E15.5 and E18.5, Wnt1-CreExt1<sup>fl</sup>box embryos exhibited abnormal thinning of the central cornea (arrows) and dysgenesis of the iridocorneal angle (open arrowheads). While the control eye shows lid closure (black arrowheads in B), the mutant embryo lacked eyelids. Boxed regions in B indicate the areas shown at higher magnification in A. (C) Impaired ocular growth in Wnt1-CreExt1<sup>fl</sup>box embryos. The central cornea thickness and the anterior chamber depth in the mutant embryos were significantly smaller, as compared with the control eyes. However, the lens thickness was not affected in the eyes of mutants. (D) Van Gieson staining revealed collagen accumulation in the control corneal stroma. In contrast, Wnt1-CreExt1<sup>fl</sup>box embryos lacked collagen matrix. (E) Immunohistochemistry using anti-ZO-1 antibody exhibited a defect of the endothelial layer in the mutant cornea. (F) Fate mapping for neural crest cells. Cre-positive neural crest cells had already migrated to the periocular region as early as E11.5, while the neural crest cells remained distributed at E18.5. Data represent mean ± SEM. *P < 0.05, **P < 0.01, Student’s t test (n = 6). c, cornea; ICA, iridocorneal angle; KO, Wnt1-CreExt1<sup>fl</sup>box. Scale bar: 50 μm.

Discussion

In the present study, genetic disruption of Ext1 in neural crest cells caused anterior chamber dysgenesis, similar to that which is observed in Peters anomaly in humans. In addition, the anterior eye segment exhibited reduced phosphorylation of Smad2 and downregulated expression of Pitx2 and Foxc1, which are the downstream molecules of TGF-β2. Moreover, in vitro BrdU assays indicated that the TGF-β2 signaling in neural crest cells was dependent on the HS that is expressed by the cell itself (cell autonomous). When there is a reduced interaction between TGF-β2 and HS, this can lead to an elevated IOP that is associated with decreased cell numbers in the trabecular beam and the loss of Schlemm canal. Overall, the present study demonstrated that in order for morphogenesis to occur in the anterior chamber of the eye, neural crest cells require an interaction between the TGF-β2 and HSPGs that are expressed cell autonomously. When there is impaired interaction, this leads to developmental glaucoma.

In humans, the major phenotypes found for Peters anomaly include defects of the corneal endothelium, Descemet membrane, and posterior corneal stroma (42, 43). Patients with Peters anomaly also have a high association with anomalies in other craniofacial neural crest–derived tissues such as ear deformities and cleft palates (19, 44). This association strongly suggests that the pathogenesis of Peters anomaly depends upon the maldevelopment of the craniofacial neural crest cells. In some pedigrees, the inheritance has been described as being autosomal dominant (11) or autosomal recessive (45, 46), while in others it has been stated as being sporadic (47). This suggests that there are multiple causative genes for Peters anomaly. Although large subsets of Peters anomaly cases occur without any molecular characterization, a mutation of PITX2 is highly associated with Peters anomaly. When there is a loss of PITX2, this leads to a defect of the corneal endothelium and corneal stroma, which indicates an essential role for this gene in corneal development (48). PITX2 promotes collagen synthesis via the activation of the procollagen lysyl hydroxylase (14). In the current study, the HS-deficient mutant manifested ocular phenotypes associated with Peters anomaly, presenting both downregulated expression of Pitx2 and reduced collagen in the corneal stroma. These data provided support for a role for HS in the regulation of the expression of Pitx2 in ocular development that leads to corneal phenotypes. However, in order to regulate the expression of intracellular Pitx2 in the neural crest cells, extracellular HS is required for an interaction with a morphogen, which in this case is the upstream molecule of Pitx2. For anterior chamber development, TGF-β2 is the most critical morphogen (20). In mammals, there are 3 members of the TGF-β family that are known to exist: TGF-β1, TGF-β2, and TGF-β3 (49, 50). Since TGF-β2 predominates in the eye during embryonic development, no anomalies are seen in the eyes of either TGF-β1– or TGF-β3–null mice (20). However, TGF-β2–null embryos exhibit Peters ocular phenotypes (20) and cleft palate (40), similar to the phenotype seen for the HS-deficient embryo. Lyon et al. (51) performed affinity chromatography and demonstrated that there are 2 TGF-βs, TGF-β1 and TGF-β2, that can bind to heparin and highly sulfated HS. The binding assay in the current study confirmed this affinity between HS and recombinant TGF-β2. Moreover, HS-deficient neural crest cells in vitro failed to show TGF-β2-dependent proliferation without the phosphorylation of Smad2, which is a downstream mediator specific to TGF-β.
research article

When taken together, our results indicated that the loss of HS disturbs the TGF-β2 signaling pathway in the ocular neural crest cells and, thus, leads to the Peters phenotypes.

There are 2 modes of action that HS employs to control the TGF-β2 signaling. First, HS modulates the diffusion and the gradient of TGF-β2 within the local environment. For example, the Drosophila TGF-β homolog, Dpp, moves along the cell surface via restricted extracellular diffusion that involves HSPGs (52). When there is a loss of HSPGs, this disturbs Dpp-dependent wing disc development. However, in the present study, we found that the distribution of TGF-β2 was not affected in the mutant periocular mesenchyme, suggesting that there is a smaller contribution of the morphogen gradient for TGF-β2–induced morphogenesis of the anterior chamber. The second mode of action involves promotion of the interaction between TGF-β2 and the receptors by the HSPGs on the cell surface. For example, another morphogen, FGF, requires cell-surface HS as a coreceptor for FGF signaling, which combines to form a 2:2:1 ratio that consists of a FGF/FGF receptor/HS ternary complex (53). Our present data strongly suggested that HS on the cell surface of neural crest cells modulates the ligand-receptor interaction for TGF-β2 (Figure 8).

Because several HS-binding morphogens and growth factors can contribute to the craniofacial development, interactions between morphogens/growth factors other than TGF-β2 and HS may also be impaired in HS-deficient ocular neural crest cells. Previous studies have reported that genetic disruption of Fgf8 and Shh in the neural crest caused facial dysmorphism (54–56). In addition, the loss of Fgf8 has been shown to induce defects of the eyelids and outer ears (55). However, these ocular phenotypes are not similar to Peters anomaly. A variety of ocular abnormalities, including the iridocorneal angle hypoplasia and elevated IOP, have been reported in heterozygous Bmp4 mutants (21). However, reduced corneal stroma or corneal endothelial defects were not found in the Bmp4 mutant (21). We also did not find any significant disturbance of the phosphorylation of Smad1/5/8 (data not shown) or the phosphorylation of ERK1/2.
HS deficiency leads to the inactivation of TGF-β2 signaling and downregulation of the transcription factors Foxc1 and Pitx2. A thin cornea (black arrows in A) and iridocorneal dysgenesis (black arrowheads in B) were observed in the mutant embryos that lacked the gene for TGF-β2 (E18.5). (C) Binding assay indicated that HS had an affinity for TGF-β2 but not for EGF. Immunohistochemical staining of TGF-β2 (D and I), Smad2 (E and J), and phosphorylated Smad2 (F and K) in the iridocorneal angle of the E13.5 embryo, and Foxc1 (G and L) and Pitx2 (H and M) in the anterior eye segment of the E15.5 embryo. A disturbance was noted for the phosphorylation of Smad2 in the periciliar mesenchyme (dotted lines in F and K) in the Wnt1-CreExt1 flox/flox embryos. In the eyes of mutants, the expression of Foxc1 was substantially reduced in the corneal endothelial layer (white arrows) and in the iridocorneal angle (open arrows), while Pitx2 expression was hardly detected in the corneal stroma or endothelium. en, corneal endothelium; epi, corneal epithelium; str, corneal stroma; *, presumptive ciliary body. Scale bar: 20 μm.
is HS deficiency, this leads to severe anterior chamber dysgenesis, which is reminiscent of Peters anomaly in humans. In addition, the impaired interaction between TGF-β2 and HS causes developmental glaucoma. These findings suggest that disturbances in the synthesis of HS might contribute to the pathology of developmental glaucoma.

**Methods**

**Mice.** The mutant mouse strains used in this study have all been reported previously (6, 22, 39, 62). To produce mutant mice with the Ext1-deficient periocular mesenchymal cells during embryogenesis, Wnt1 promoter-driven Cre-transgenic mice (The Jackson Laboratory) were mated with mice carrying the Ext1lox allele. Subsequently, in order to obtain mutants with a Wnt1-Cre Ext1lox allele, the Wnt1-Cre Ext1lox/wild male mice were crossed with female mice that were homozygous for the Ext1lox allele. Littermates carrying Ext1lox/lox or Ext1lox/wild were used as controls. To detect the neural crest cells in the anterior eye segment, Wnt1-Cre transgenic mice were crossed with Rosa26R mice (a gift from H. Okita, National Research Institute for Child Health and Development, Tokyo, Japan, and P. Soriano, Mount Sinai School of Medicine, New York, New York, USA) (6, 22, 39, The Jackson Laboratory), as they express β-gal following Cre-mediated recombination (63). Tgfβ2 transgenic mice (The Jackson Laboratory) were generated via gene targeting, as has been previously reported (40). Tgfβ2 heterozygous mice were mated with Ext1 heterozygous mice. Genotyping of the mice was performed by PCR-based methods that used DNA prepared from tail biopsies. All of the mice strains used in this study were backcrossed with C57BL/6 more than 10 times. All of the procedures involving the mice were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines were approved by the Kumamoto University Committee on the Use and Care of Animals.

**Whole-mount in situ hybridization.** To study the expression pattern of Ext1 mRNA in developing embryos, in situ hybridization with a digoxigenin-conjugated riboprobe for Ext1 was performed, as has been previously reported (39). Briefly, the riboprobe was hybridized at 55°C overnight, fol-
Haploinsufficiency of Ext1 and Tgfb2 leads to aberrant iridocorneal development. (A) There were no major anomalies noted in Schlemm canal (black arrows) or the trabecular beam (white arrowheads) for either the controls (A) or the Ext1 (B) or Tgfb2 (C) haploinsufficient mutants. As compared with the wild-type mice, the double heterozygote mice (D) had a hypoplastic trabecular beam, and they lacked a Schlemm canal. The double heterozygote mice also exhibited a significant reduction of the total cell number for the iridocorneal angle (E), in addition to an elevated IOP (F). Data represent mean ± SEM. *P < 0.01, Student’s t test (n = 9). Scale bar: 50 μm.
12-dUTP was then incorporated at the 3′-OH DNA ends through the use of terminal deoxynucleotidyl transferase recombinant enzyme. Sections were then analyzed by confocal laser microscopy (Olympus).

**Binding assay.** To study the affinity of morphogens for HS, we performed a binding assay that used the ELISA method, as has been previously reported (64). Briefly, TGF-β2 and epidermal growth factor (R&D Systems) were applied to the polystyrene ELISA tray and incubated for 1 hour at 37°C. For the coculture study, transfected cells were cultured in DMEM/F12 containing 0.1% BSA at 37°C, cells were incubated in DMEM/F12 containing 0.1% BSA and BrdU (final concentration of 10 μM) with or without TGF-β2 (5 ng/ml) for 12 hours at 37°C. Cells incorporating BrdU were identified by staining with anti-BrdU monoclonal antibody (Chemicon) and 3,3′-diaminobenzidine. After analysis of each of the 9 samples, a Student’s t test was used to determine the statistical significance.

**JOP measurement.** JOP was measured using the TonoLab rebound tonometer for rodents (M.E. Technica), according to the manufacturer’s recommended procedures. After a few minutes of acclimation, JOPs of conscious mice at P42 were measured between 11:30 AM and 12:30 PM. Statistical significance was determined by Student’s t test.

**Statistics.** For statistical comparison of 2 samples, we used a 2-tailed Student’s t test. P values of less than 0.05 were regarded as statistically significant.

**Protein extraction and Western blot analysis.** Following a 16-hour incubation in DMEM/F12 containing 0.1% BSA at 37°C, cells were treated with 5 ng/ml of TGF-β2 (R&D Systems) for 90 minutes at 37°C, as has been previously described (22). Proteins were extracted from cells with RIPA buffer (Thermo Fisher Scientific) that contained protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific). Proteins in the cell extracts were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (Whatman). The membranes were blocked with 5% normal rabbit serum/Tris-buffered saline containing 0.1% Tween20 (TBST) and 5% skim milk/TBST for the anti-Smad2 (Santa Cruz Biotechnology Inc.) and the anti-phosphorylated Smad2 (Chemicon) antibodies. Immunopositive bands were visualized by chemiluminescence with the ECL Western Blotting Detection Reagents (Amersham Biosciences) and LAS 4000 Mini UV (Fujifilm).

**Cell culture and transfection.** Periocular mesenchymal tissues were removed from E11.5 embryos at E11.5 by microdissection. The mesenchymal cells were cultured in DMEM/F12 medium (Gibco, Invitrogen) containing 10% fetal bovine serum (Sigma-Aldrich) at 37°C in 5% CO2. To disrupt the HS synthesis, an adenovirus, which included the sequence (65) of the Ext1 gene, was added to cultured periocular mesenchymal cells. The virus solution was diluted accordingly and then added to the cultured cells for 1 hour at 37°C. For the coculture study, transfected cells were additionally cultured with HS-positive periocular mesenchymal cells for an additional day. After virus transfection, the cells were cultured in DMEM/F12 with 10% fetal bovine serum for 16 hours. To detect the expressions of HS and Cre recombinase, we performed immunohistochemistry using anti-HS and anti-Cre recombinase antibody.

**Figure 8**

Hypothetical schema of the HS-dependent TGF-β2 signaling on the cell surface of neural crest cells. (A) HS has an affinity for TGF-β2, which enhances the ligand presentation to the TGF-β receptors. Subsequently, transduction of the signal to the nucleus occurs via phosphorylation of the Smads. (B) For the HS defect, there is deterioration of the efficiency of the interaction between TGF-β2 and the receptors, which leads to a disturbance of the Smads phosphorylation. The loss of phosphorylated Smads then inhibits the expression of Foxc1 and Pitx2.
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