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ABSTRACT

Heparan sulfate proteoglycans (HS-PGs) regulate several developmental processes, but their possible roles in mandibular and TMJ formation are largely unclear. To uncover such roles, we generated mice lacking Golgi-associated N-sulfotransferase 1 (*Ndst1*) that catalyzes sulfation of HS-PG glycosaminoglycan chains. *Ndst1*-null mouse embryos exhibited different degrees of phenotypic penetrance. Severely affected mutants lacked the temporomandibular joint and condyle, but had a mandibular remnant that displayed abnormal tooth germs, substandard angiogenesis, and enhanced apoptosis. In mildly affected mutants, the condylar growth plate was dysfunctional and exhibited thicker superficial and polymorphic cell zones, a much wider distribution of Indian hedgehog signaling activity, and ectopic ossification along its lateral border. Interestingly, mildly affected mutants also exhibited facial asymmetry resembling that seen in individuals with hemifacial microsomia. Our findings indicate that *Ndst1*-dependent HS sulfation is critical for mandibular and TMJ development and allows HS-PGs to exert their roles *via* regulation of *Ihh* signaling topography and action.

KEY WORDS: *Ndst1*, TMJ, hemifacial microsomia, heparan sulfate, hedgehog signaling.

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Sulfotransferase *Ndst1* is Needed for Mandibular and TMJ Development

INTRODUCTION

Heparan sulfate proteoglycans (HS-PGs) are cell-surface and extracellular matrix macromolecules that regulate numerous developmental processes (Hacker *et al.*, 2005). They consist of a core protein to which one or several glycosaminoglycan chains are covalently attached (Bulow and Hobert, 2006; Bishop *et al.*, 2007). The HS chains are composed of repeating D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) residues, are synthesized in the Golgi complex by Ext1 and Ext2 glycosyltransferases, and are modified by deacetylase/N-sulfotransferases (*Ndsts*) that replace acetyl groups from subsets of GlcNAc residues with sulfate. In addition, C-5 epimerase modifies GlcA to iduronic acid (IdoA), and further O-sulfation of IdoA, GlcA, and GlcN can occur as well. These additional modifications are dependent on prior N-sulfation of GlcN residues by *Ndsts* (Esko and Selleck, 2002), signifying that the latter enzymes have a key role in establishing the overall structural characteristics of HS chains.

The overall degree and patterns of sulfation are critical for HS-PG function (Grobe *et al.*, 2005). Indeed, a major mechanism by which HS-PGs exert their roles is by HS-mediated interactions with powerful signaling factors, including members of the hedgehog and bone morphogenetic protein families. Genetic studies showed that mice lacking *Ndst1* display malformations of several developing organs, and their HS-PGs contain lower levels of N- and O-sulfated residues and iduronic acid (Grobe *et al.*, 2005). A recent study on mice carrying a hypomorphic mutation in *Ext1* showed that the long bones were short and their growth plates contained lower amounts of HS, but a much wider distribution of Indian hedgehog (*Ihh*) (Koziel *et al.*, 2004). Analysis of these data reaffirmed the notion that HS-PGs and their HS chains are critical to the establishment and determination of the ranges of hedgehog protein diffusion, distribution, and action on target cells (Pacifici *et al.*, 2005; Koyama *et al.*, 2007).

Upon reaching their targets, *Ihh* and other family members act by interaction with the cell-surface receptor Patched and modulation of signaling activity of Smoothened in cooperation with primary cilia and Gli transcription factors (Corbit *et al.*, 2005; Haycraft *et al.*, 2005; Koyama *et al.*, 2007). Recently, we studied the pre- and post-natal development of the mandibular condyle and temporomandibular joint (TMJ) in conditional *Ihh*-deficient

Figure 1. Craniofacial skeletal development is abnormal in *Ndst1*-null embryos. Skulls from E18.5 wild-type (A-B) and mutant (C-F) littermates were analyzed by micro-computed tomography (μ CT) that revealed a spectrum of mild (C-D) to severe (E-F) defects. The images are shown by frontal view (A, C, E) and parasagittal view (B, D, F). In the mildly affected mutant (C-D), the right side of the neurocranium is flat (C, arrowhead), the frontal fontanella is wider (C, double arrow), the mandibular condylar and coronoid processes are missing (D, arrowhead), and the distal mandibular edge is defective (D, double arrowhead). In the severely affected mutant (E-F), the mandibular symphysis is fused (E, arrowhead), and the proximal part of mandibular bone and an entire part of the ramus, including condylar, coronoid, and angular processes, are missing (F). co, coronoid process; cp, condylar process.

mice (Shibukawa *et al.*, 2007; Ochiai *et al.*, 2010). Normally, the condylar epiphyseal end contains chondroprogenitor cells that proliferate, differentiate into chondrocytes, and are apposed onto the sub-adjacent cartilaginous growth plate (Kantomaa *et al.*, 1994). The chondrocytes then undergo maturation into pre-hypertrophic and hypertrophic chondrocytes and are replaced by endochondral bone (Sarnat, 1966). In *Ihh*-deficient condyles, several of those growth plate developmental steps were defective, and, interestingly, the articular disc and flanking joint cavities were deranged as well. Analysis of these data, together, strongly indicated that *Ihh* signaling is essential for condylar and TMJ development where it regulates chondroprogenitor cell proliferation, expression of parathyroid-hormone-related protein (*PTHrP*), condylar growth, and joint and disc formation. Given that hedgehog proteins require HS-PGs for function, we asked whether mandibular and TMJ development are deranged in *Ndst1*-null mice and whether the defects may be due to aberrant *Ihh* distribution and action.

MATERIALS & METHODS

Generation of *Ndst1*-null Mice

The generation of *Ndst1*- and *Ihh*-null mice has been described previously (Grobe *et al.*, 2005; Young *et al.*, 2006). Fragments of embryonic tails were genotyped by PCR with the following primers: for *Ndst1*, 5'-CCCAGATGGCGAGACT-3' and 5'-CATCCTCTGAGG TGACCGC-3' yield a 350-bp product corresponding to deletion of exon2; for *Ihh*, 5'-AGGAG GCAGGGACATGGATAGGGTG 3' and 5'-TACCGGTGGAT GTGAATGTG TGCG-3' generating a 300-bp product. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Micro-computed Tomography

Embryos were scanned by micro-computed tomography (μ CT) with a μ CT40 SCANCO system with a 36-mm holder at 45 kV of energy, 12 μ m scanning thickness, and medium resolution. We used two-dimensional slice images to generate three-dimensional reconstructions with the following parameters: filter width sigma = 0.8, support level = 1.0, and threshold = 173. Three-dimensional images were rotated at specific angles to generate frontal, lateral, and superior views of the craniofacial skeleton.

Histology, Immunohistochemistry, Apoptosis Assay, and Gene Expression Analysis

Embryos were fixed with 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. For immunohistochemical detection of endothelial cells, sections were incubated with a 1:100 dilution of rat antibody CD31 (BD Pharmingen™, San Diego, CA, USA), followed by detection with goat anti-rat HRP-conjugated antibody (Zymed, San Francisco, CA, USA). For control, sections were incubated with rabbit IgG. Apoptosis detection was performed on paraffin sections with a TUNEL Assay Kit (Roche, Mannheim, Germany). For *in situ* hybridization, sections were hybridized with antisense or sense 35 S-labeled riboprobes (Nagayama *et al.*, 2008).

RESULTS

Abnormal Growth and Craniofacial Skeletal Development in *Ndst1*-null Mice

Ndst1-null embryos developed to term, but died perinatally. Anatomical inspection and soft x-ray and μ CT analyses showed that the mutant embryos displayed developmental defects ranging from mild to severe (Fig. 1 and Appendix Fig. 1) (Grobe *et al.*, 2005; Pallerla *et al.*, 2007). In controls, the neurocranium displayed a typical round morphology, and the osteogenic front of calvarial bones reached the midline (Fig. 1A). The cranial base displayed a typical elongated morphology along the antero-posterior axis (Appendix Fig. 1B), and the mandibular bones were well-developed, with clearly defined condylar and coronoid processes (Fig. 1B, arrows). In mildly affected E18.5 mutants, the neurocranium was slightly shorter along the antero-posterior axis, and the post-cranial skeleton was fairly normal (Appendix Fig. 1C). The skull was often malformed and asymmetric, with a wider anterior fontanella (Fig. 1C, double arrow). One side of the skull was flat and hypoplastic (Fig. 1C, arrowhead) and lacked the temporal bone, zygomatic arch (Appendix Fig. 1D, arrowhead), and condylar and coronoid processes

(Fig. 1D, arrowhead). The ethmoid and other midfacial structures were underdeveloped (Appendix Fig. 1D), the mandibular ramus was shorter, and the distal mandibular edge covering the developing incisor was rough and defective (Fig. 1D, double arrowhead). The contralateral side of the skull was less affected and contained most skeletal structures, including a condylar process and a TMJ (see below). In severely affected mutants, the growth deficiency affected the appendicular skeleton, viscerocranium, neurocranium, and overall body (Appendix Fig. 1E). Indeed, the viscerocranial and neurocranial bones were largely missing (Figs. 1E, 1F), the mandible was hypomorphic, and its symphysis was fused (Fig. 1E, arrowhead).

Analysis of Mandibular and Maxillary Defects

Mandibular and maxillary developmental defects were particularly evident in severely affected embryos, and thus we focused on these specimens to investigate possible underlying mechanisms. In control E18.5 embryos, the maxillary and mandibular bones and the oral cavity were already well-formed (Fig. 2A). Incisor and molar tooth germs were at the bell stage and displayed typical structures within the dental organ and mesenchyme, including polarized pre-ameloblasts, stratum intermedium, stellate reticulum, and pre-odontoblasts (Fig. 2C and inset). In contrast, mutant maxillary and mandibular bones were severely defective, and the tongue was missing (Fig. 2D). Incisor and molar tooth germs were hypoplastic and composed of poorly differentiated odontogenic cells, and both upper and lower first molars displayed abnormal organization (Fig. 2F and inset). Immunohistochemistry with antibodies to endothelial cell marker CD31 showed that there were fewer arterial and venous vessels in mutant mandibular tissues (Fig. 2E) than in controls (Fig. 2B), and TUNEL staining showed that there were more dying cells in mutant (Fig. 2E, inset) than in wild-type mandibles (Fig. 2B, inset).

Figure 2. Mandibular and maxillary bones and structures are defective in *Ndst1*-null embryos. Coronal sections from E18.5 wild-type embryo (A–C) and severely affected mutant (D–F) were processed for Safranin-O/fast green staining (A, C, D, F), CD31 immunohistochemical staining (B, E), and TUNEL staining (insets in B, E). In wild-type embryos, note the presence of well-defined mandibular first molar (mo), incisor (in), and tongue (to) and the absence of Meckel's cartilage at this portion of the mandible (A). CD31-positive endothelial cells depict the developing arteries and/or veins (B, arrowheads and arrow, respectively), and few TUNEL-positive ectomesenchymal cells are present near the developing mandibular bone (inset in B). In the *Ndst1*-null mandible, two upper first molars and one lower molar resemble each other and are much smaller (D) and contain substandard dental epithelium and dental mesenchyme (inset in F). Note also the presence of Meckel's cartilage (mc), several TUNEL-positive apoptotic ectomesenchymal cells (inset in E), and the absence of CD31-positive blood vessels (E). mo, molar; in, incisor; to, tongue; mc, Meckel's cartilage; eo, enamel organ; dm, dental mesenchyme; pab, pre-ameloblast; si, stratum intermedium; and sr, stellate reticulum. Bars in A for A and D, 300 μ m; in B for B and E, 65 μ m; in inset for B and E, 35 μ m; in C for C, 300 μ m; in F for F, 250 μ m; and inset in F for the insets in C and F, 60 μ m.

Altered Cell Proliferation and Bone Formation in *Ndst1*-null Condyles

Next, we analyzed the condyle and TMJ present in mildly affected mutants. Control E18.5 condyles displayed growth plate-like zones, including the superficial (sf) layer, polymorphic (pm) precursor cell layer, flattened chondrocyte (fc) zone, and hypertrophic chondrocyte (hc) zone (Appendix Fig. 2A)

Figure 3. Polymorphic chondroprecursor cell layers are thicker and more proliferative in *Ndst1*-null condyles. Parasagittal serial sections of E18.5 wild-type (A-C) and mutant (D-F) condyles were processed for *in situ* hybridization analysis of indicated genes (A-F). In controls, note that the polymorphic cell layer displays abundant *Aggrecan* transcripts (red in A) and very low *type II collagen* transcripts (blue in A) and contains *H4C*-positive proliferating cells (B). In mutants, the polymorphic layer is much thicker (D, double vertical yellow arrow), contains more proliferating cells (E, double vertical yellow arrow), and displays much higher levels of *Ptc-1* transcripts (F) compared with controls (C). *sf*, superficial layer; *pm*, polymorphic precursor cell layer. Bar in D for A-F, 180 μ m.

(Luder *et al.*, 1988). The superficial and polymorphic layers displayed strong expression of *aggrecan*, while the flat chondrocyte layer displayed strong expression of *type II collagen* (Fig. 3A). Interestingly, mutant condyles displayed thicker superficial and polymorphic layers (Appendix Fig. 2B, vertical yellow double arrow) and a proportionally expanded *aggrecan* expression area (Fig. 3D, yellow double arrow) compared with controls (Fig. 3A). The thicker apical condylar layers contained also a larger number of *histone 4C* (*H4C*)-positive proliferating cells (Fig. 3E) than controls (Fig. 3B). When we analyzed *Ihh* expression, we found that it was similar in controls and mutants (not shown). However, transcripts encoding *Ptc-1*, a direct transcriptional target of hedgehog signaling, were more abundant and more widely distributed in mutant superficial and polymorphic layers (Fig. 3F) than controls (Fig. 3C), indicating that *Ihh* signaling topography was far wider in mutants. To corroborate the above findings, we examined *Ihh*-null E18.5 embryos and found that, in this case, the polymorphic layer was in fact thinner and proliferation was

Figure 4. Analysis of intramembranous bone formation and working model of HS via regulation of *Ihh* function. Serial parasagittal sections from E18.5 control (A-B) and *Ndst1*-null (C-D) condyles were processed for *in situ* hybridization analysis. In controls (A, B), early osteogenic markers such as *Osterix* (*Osx*) and *collagen I* (*Col I*) are abundant in differentiating osteoblasts below the perichondrium-periosteum border (arrowheads) and are less prominent in chondroprogenitors and differentiating chondrocytes at the condylar apical end. In mutants, strong expression of *Osterix* and *collagen I* is evident far closer to the apical condylar end (C and D, arrows), indicating an expansion of intramembranous bone formation. Bar in B for A-D, 250 μ m. Our current working model (E). Under normal circumstances, *Ihh* produced in pre-hypertrophic and hypertrophying chondrocytes would diffuse laterally and apically and create shallow gradients that reach the perichondrium, periosteum, and polymorphic and superficial layers. These gradients would be limited and restricted by HS-PGs and would sustain normal chondroprecursor proliferation, *Osx* and *Col I* expression, and bone collar formation. In mildly affected *Ndst1*-mutant embryos, HS-PGs would be partially under-sulfated and would still sustain *Ihh* diffusion, but *Ihh* would actually diffuse more broadly and trigger excessive condylar cell proliferation and bone collar formation. Note that this model may not apply to severely affected embryos in which HS-PGs would be severely under-sulfated; *Ihh* may not be able to diffuse from its site of synthesis, and condylar as well as long bone development would be severely abnormal.

lower (Appendix Figs. 2C, 2D, double arrow) than in controls (Fig. 3B and Appendix Fig. 2A).

Ihh also regulates formation of intramembranous bone at the periphery of the growth plate (Koyama *et al.*, 1996; St-Jacques *et al.*, 1999; Maeda *et al.*, 2007) and does so in a direct manner (Nakamura *et al.*, 1997). Thus, we asked whether the broader distribution of Ihh signaling activity in mildly affected *Ndst1*-mutant condyles was accompanied by enhanced ossification along its border. In control condyles, low levels of *Osterix* and *collagen I* transcripts were detectable in differentiating osteogenic cells and chondrocytes in apical portions (Figs. 4A, 4B), and more abundant levels were observed at the transition between perichondrial and periosteal/bone along the lateral border (Figs. 4A, 4B, arrowheads). In *Ndst1* mutants, the *Osterix* and *collagen I* transcripts were present near the apical end (Figs. 4C, 4D, arrows), indicative of precocious and expanded ossification along the lateral condyle border.

DISCUSSION

Analysis of our data demonstrated that *Ndst1* is necessary for development of the mandible, mandibular condyle, TMJ, and tooth germs. Phenotypic penetrance in *Ndst1*-null mutants varies from embryo to embryo, as also previously reported (Grobe *et al.*, 2005). The reason for this intriguing variation remains to be clarified, but may involve epigenetic mechanisms or variable onset of compensating activities such as those provided by other *Ndst* proteins. Whatever the mechanism, analysis of our data showed that, in the mildly affected embryos, the craniofacial skeleton is particularly affected by the lack of *Ndst1* function compared with the appendicular skeleton. In severely affected embryos, the skull is almost absent, and the mandibular remnant is one of the few skeletal elements still able to form. Clearly, *Ndst1* has major and encompassing roles in craniofacial development, and its essential function reflects the fundamental importance that *Ndst1*-dependent sulfation patterns and sulfation levels of heparan sulfate chains have for HS-PG function and action in development (Bishop *et al.*, 2007).

The mandibular condyle is usually present in mildly affected embryos, but is absent in severely affected embryos. In the latter, we observed a severe reduction in blood vessels. Analysis of these data suggests that the lack of local blood vessels may have had a major negative role in the onset of condylar development, leading to its absence. This interpretation is in line with the fact that the condylar primordium forms from the mandibular periosteum at early stages of embryogenesis (Shibata *et al.*, 2006), and that lack of local blood vessels could have fully impaired this early morphogenetic step. Angiogenic factors, including vascular endothelial growth factor (VEGF) and FGFs, interact with, and require, HS-PGs (Dreyfuss *et al.*, 2009) and are expressed during TMJ development (Molteni *et al.*, 1999; Aoyama *et al.*, 2004). Thus, lack of *Ndst1* expression may have impaired HS-PG function and vessel formation. Though present in mildly affected embryos, the mandibular condyle still exhibits defects, including increased proliferation in superficial and polymorphic layers and increased thickness. These changes are accompanied by increases in Ihh signaling distribution and

intensity over the same topographical areas, strongly suggesting that these events are correlated in a causal manner. This interpretation fits well with previous studies, from this and other laboratories, indicating that Ihh is a major regulator of proliferation in growth plates and that HS-PGs are critical for regulating hedgehog signaling topography and action (Kozziel *et al.*, 2004; Koyama *et al.*, 2007; Shibukawa *et al.*, 2007). Thus, the defective HS-PGs in *Ndst1*-null condyles would not bind Ihh well (Grobe *et al.*, 2005) and would allow it to diffuse more broadly from its site of synthesis in the pre-hypertrophic zone and elicit broader and even ectopic effects on condylar cell proliferation and ossification, as analysis of our data indicated. It should be noted that this interpretation differs from the conclusion reached in a previous study on *Ndst1*-null long bone growth plates, in which Ihh diffusion and signaling to the periosteum were actually found to be reduced compared with wild-types (Pallerla *et al.*, 2007). One possible explanation for these differing observations is that, though related structurally and functionally, condylar and long bone growth plates and adjacent tissues may obey different mechanisms. A second and more likely possibility is that the phenotype of mildly affected condyles may be the result of compensation by other *Ndst* isoenzymes; hence, the HS-PGs in these embryos would be partially sulfated and would still be able to sustain Ihh diffusion and signaling, albeit too broadly. An analogous situation would occur in *Ext1* hypomorphic mutant mice containing low levels of HS chains (Kozziel *et al.*, 2004). In contrast, in severely affected embryos, the HS-PGs would be severely under-sulfated, and Ihh would not be able to diffuse from its site of synthesis, leading to severe defects in craniofacial structures and long bones. Clearly, side-by-side comparisons of condylar and long bone growth plates from mild and severe *Ndst1*-null embryos will be needed to sort out these interesting possibilities.

In closing, we should point out that the mildly affected *Ndst1* mutants exhibit phenotypic traits reminiscent of those in persons with hemifacial microsomia (HFM) and Treacher-Collins syndrome (TCS). HFM is characterized by various degrees of asymmetric underdevelopment of structures originating from multiple pharyngeal arches (Grayson *et al.*, 1983). The *Ndst1* mutants exhibit various degrees of tongue agenesis and dysmorphogenesis that have only recently been recognized as part of the HFM spectrum (Chen *et al.*, 2009). HFM has been thought to be due to embryonic hemorrhages (Poswillo, 1974), but a more recent model hypothesized a genetic basis (Cousley *et al.*, 2002; Dabir and Morrison, 2006). TCS is an autosomal-dominant disorder of craniofacial development caused by loss-of-function mutation in *TCOF1* that encodes a nucleolar phosphoprotein involved in ribosomal biosynthesis (Trainor *et al.*, 2009), and, notably, the *Ndst1* gene is located in the flanking region of the *TCOF1* locus (Dixon *et al.*, 1995). It is thus interesting to speculate whether skeletal defects in both syndromes may involve some changes in HS-PG function and/or hedgehog signaling. A small proportion of persons with facial microsomia, including TCS and HFM, present with unexplained TMJ ankylosis, and it is conceivable that such joint fusion could result from dysregulated hedgehog function and ectopic osteogenesis due to up-regulated *Osterix* and *collagen I* expression, as we observed here.

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