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Review

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Editor's Choice

The significant role of glycosaminoglycans in tooth development

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This review delves into the roles of glycosaminoglycans (GAGs), integral components of proteoglycans, in tooth development. Proteoglycans consist of a core protein linked to GAG chains, comprised of repeating disaccharide units. GAGs are classified into several types, such as hyaluronic acid, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate. Functioning as critical macromolecular components within the dental basement membrane, these GAGs facilitate cell adhesion and aggregation, and play key roles in regulating cell proliferation and differentiation, thereby significantly influencing tooth morphogenesis. Notably, our recent research has identified the hyaluronan-degrading enzyme Transmembrane protein 2 (Tmem2) and we have conducted functional analyses using mouse models. These studies have unveiled the essential role of Tmem2-mediated hyaluronan degradation and its involvement in hyaluronan-mediated cell adhesion during tooth formation. This review provides a comprehensive summary of the current understanding of GAG functions in tooth development, integrating insights from recent research, and discusses future directions in this field.

Key words: glycosaminoglycan; proteoglycan; hyaluronic acid; tooth development; extracellular matrix.

Overview of glycosaminoglycan

Proteoglycans are composed of a core protein with covalently attached glycosaminoglycan (GAG) chains. The predominant components of proteoglycans, GAGs are composed of repeating disaccharide units. The biosynthesis of these negatively charged polysaccharides begins in the cell cytoplasm and is transferred to the Golgi apparatus for further modification and sulfation (Esko et al. 2009). However, hyaluronic acid (HA), a non-sulfated GAG, is synthesized in the plasma membrane rather than the Golgi apparatus. The synthesis of GAGs is regulated by the complex interplay of several tissue-specific enzymes (Esko et al. 2009).

GAGs, which are ubiquitously found on the cell surface and within the extracellular matrix (ECM), play pivotal roles in numerous biological processes. These include cell signaling, cell adhesion, growth and proliferation, ECM assembly, tissue repair, and coagulation. GAGs can be classified into four groups depending on the variance of their repeating disaccharide units: HA (non-sulfated), heparan sulfate/heparin (HS, sulfated), chondroitin sulfate (CS, sulfated), dermatan sulfate (DS, sulfated), and keratan sulfate (KS, sulfated).

In the dental context, GAGs act as major macromolecular components of the dental basement membrane, stimulating cell adhesion and aggregation, controlling cell proliferation and differentiation, and influencing tooth morphogenesis (Lau and Ruch 1983). This review will focus specifically on the roles of different types of glycosaminoglycans during tooth development, particularly in the differentiation of ameloblasts and odontoblasts.

Tooth development

Teeth are ectodermal organs and an essential part of the digestive system in humans and animals. While the dentition may vary between different species, teeth serve consistent and multiple functions that are vital for various aspects of our daily lives. One of the primary functions of teeth is mastication, by breaking down food into smaller pieces, which helps in the digestion and absorption of nutrients (van der Bilt et al. 2006). Teeth also play a significant role in speech production and maintaining the patent airway, ensuring that air passage remains open during breathing. Teeth also serve as the foundation for the vertical dimensions of the face. Teeth provide support to the lips, cheeks, and surrounding facial tissues, influencing the aesthetics of the smile and the overall facial profile (Faure et al. 2002).

Tooth development, or odontogenesis, is a complex biological process that requires the precise spatiotemporal coordination of several cellular and molecular events. This process begins in the early embryonic stages and continues through to adult life, with the replacement of deciduous teeth by permanent dentition. The process can be categorized into distinctive stages: the bud stage, cap stage, bell stage, and finally, the secretory and maturation stage. Each stage of tooth

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development is characterized by morphological changes that occur in the dental organ and the differentiation of specific cell types, namely ameloblasts and odontoblasts (Yu and Klein 2020).

During the cap stage of tooth development, the tooth bud morphologically resembles a cap, and the dental epithelial cells differentiate into ameloblasts that eventually produce enamel, the hardest tissue in the body. Concomitantly, dental papilla cells differentiate into odontoblasts, which are responsible for dentin formation. Subsequently, in the bell stage, ameloblasts and odontoblasts undergo further differentiation and start producing enamel and dentin, respectively. Ameloblasts elongate to form the outer layer of the enamel organ, whereas odontoblasts initiate the synthesis of the dentin matrix. This sequence of events leads to initial mineralization and hard tissue formation in the tooth.

The complex interplay between these two cell types and the different stages of tooth development underscores the tightly regulated nature of odontogenesis (Thesleff 2006; Jussila and Thesleff 2012). Any disruption to this delicate balance can lead to dental anomalies and diseases, emphasizing the importance of understanding these processes in greater detail.

Chondroitin Sulfate in tooth development

CS is known to be an abundant sulfated GAGs of extracellular matrix, predominantly composed of glucuronic acid and N-acetylgalactosamine incorporated with one or more sulfate attached 4- or 6-carbons of galactosamine moiety (Nieminen et al. 1993; Liu et al. 2017). Stage-specific regulation of ECM is crucial for epithelial-mesenchymal interactions (Vainio et al. 1989b). Odontogenesis is an ideal model for sequential and reciprocal epithelial-mesenchymal interactions. The spatial–temporal alterations in the structural properties of the extracellular matrix modulate cellular events and distinctive gene expression. Thus, the expression of CS proteoglycan, a prevalent component of the extracellular matrix, varied according to the degree of sulfation and the position of the sulfate ester groups (Mark et al. 1989). The expression patterns of CS and CS proteoglycan have been extensively examined (Fig. 1 and Table 1). *Mark et al.* reported monoclonal antibody (mab) *2B6* marker for chondroitin-4-sulfate was detected on the dental lamina at the earliest developmental stage to the bud stage and was restricted during the initiation of cusp morphogenesis (Mark et al. 1990). Strong expression of chondroitin-4-sulfate was observed in the occlusal region of the dental papilla after the onset of cusp formation. Prior to the bud stage, mab *3B3* and *MC21C* epitope chondroitin-6-sulfate accumulated in the dental mesenchyme and consequently remained in the corresponding early mesenchymal cell condensation and disappear from the dental papilla of the cusp and anterior region of the incisor. However, the monoclonal antibody (mab) *7C5* epitope labelled for chondroitin-6-sulfate showed robust reactivity in the dental papilla, especially during cusp formation at the bell stage, and reduced expression was also observed in the developing incisor from the posterior and lingual to the anterior and buccal part of the papilla (Nieminen et al. 1993). Contrarily, during the bud to early bell stages, chondroitin-6-sulfate and chondroitin-4-sulfate expressions were missing in the dental mesenchyme. In the odontoblastic layer, while chondroitin-6-sulfate was detected during outset of differentiation in between pre-odontoblasts, and chondroitin-4-sulfate was profoundly expressed in pre-dentin. However, *NIEMINEN et al.* revealed the existence of chondroitin-6-sulfate in the pre-dentin and pulp of pigs, sheep, rats, and human molars. While mab *3b3* reactivity was absent in the sub-odontoblastic layer, intense expression of the *7C5* epitope was found in the sub-odontoblastic layer (Nieminen et al. 1993). In addition to the dental mesenchyme, both isomers of CS are found in the stellate reticulum (Mark et al. 1990). The abundant enamel matrix protein amelogenin plays a critical role in enamel matrix formation, and non-amelogenin proteins also regulate ameloblast differentiation and enamel formation. *Thieberg et al.* declared the temporal–spatial pattern participation of the GAGs epitope during ameloblast differentiation (Thieberg et al. 1999). They exhibit localization of chondroitin-4-sulfate in the secretory ameloblasts and adjacent stratum intermedium, suggesting that CS proteoglycans modulate ameloblast cyto-differentiation and enamel matrix formation.

The large chondroitin-6-sulfate proteoglycan, versican is commonly found in the dental mesenchyme. A temporal–spatial pattern study elucidated versican mRNA is expressed in the thickened dental epithelium at E12, persisting in the enamel organ until the bell stage. By E15, versican mRNA expression occurs in the dental papilla of the cap stage, continuing as late as the secretory stage. Concurrently, from the bud to the late bell stage, versican immunolocalization is observed surrounding the stellate reticulum areas, dental papilla, and dental follicle. This suggests that the dental epithelium is responsible for the synthesis of large CS proteoglycan versican during odontogenesis (Jiang et al. 2010).

The chimeric protein dentin sialophosphoprotein (*Dspp*) constitutes the chief proteins of tooth dentin and is proteolytically cleaved into Dsp (dentin sialoprotein), Dgp (dentin glycoprotein) and Dpp (dentin phosphoprotein). As a member of the secretory calcium-binding phosphoprotein (SCPP) gene family, *Dspp* participates in bone, enamel, and dentin mineralization (Kawasaki 2009; Yamakoshi et al. 2011). Studies have demonstrated that porcine Dsp has two GAG attachments, chondroitin-6-sulfate and chondroitin-4-sulfate. Moreover, during primary dentinogenesis, a strong expression of Dspp is observed in differentiating odontoblasts (Hou et al. 2012). The dentin matrix composed of 90% organic matrix where type I collagen is the major structural protein and 10% inorganic compound. Apparently, the interaction of non-collagenous component and type I collagen was critical for dentinogenesis (Veis 1993). Interestingly, more than half of the non-collagenous part is formed by Dspp-derived proteins. Alterations in DSPP genes can cause inherited dentin defects, such as dentinogenesis imperfecta type II and III, and dentin dysplasia type II (Table 2. These reports suggest that the interaction between Dsp GAG chains and collagen during dentin matrix formation may be regulated by chondroitinase activity (Yamakoshi et al. 2005; Yamakoshi et al. 2011). DSPP is also known as a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family that is transiently expressed in the pre-secretory stage of differentiating ameloblasts (Bègue-Kirn et al. 1998; Fisher et al. 2001; Fisher and Fedarko 2003). Another study has demonstrated DSPP expression during the differentiation of secretory ameloblasts. They detect DSPP expression at the developmental ages E16.5–E18.5, and postnatal day (P10). These reports on DSPP expression provide insight into how ameloblast differentiation and cell physiology might be modulated by DSPP (Verdelis et al. 2016). Furthermore, *Dspp* mutant mice exhibit reduced enamel mineral densities,

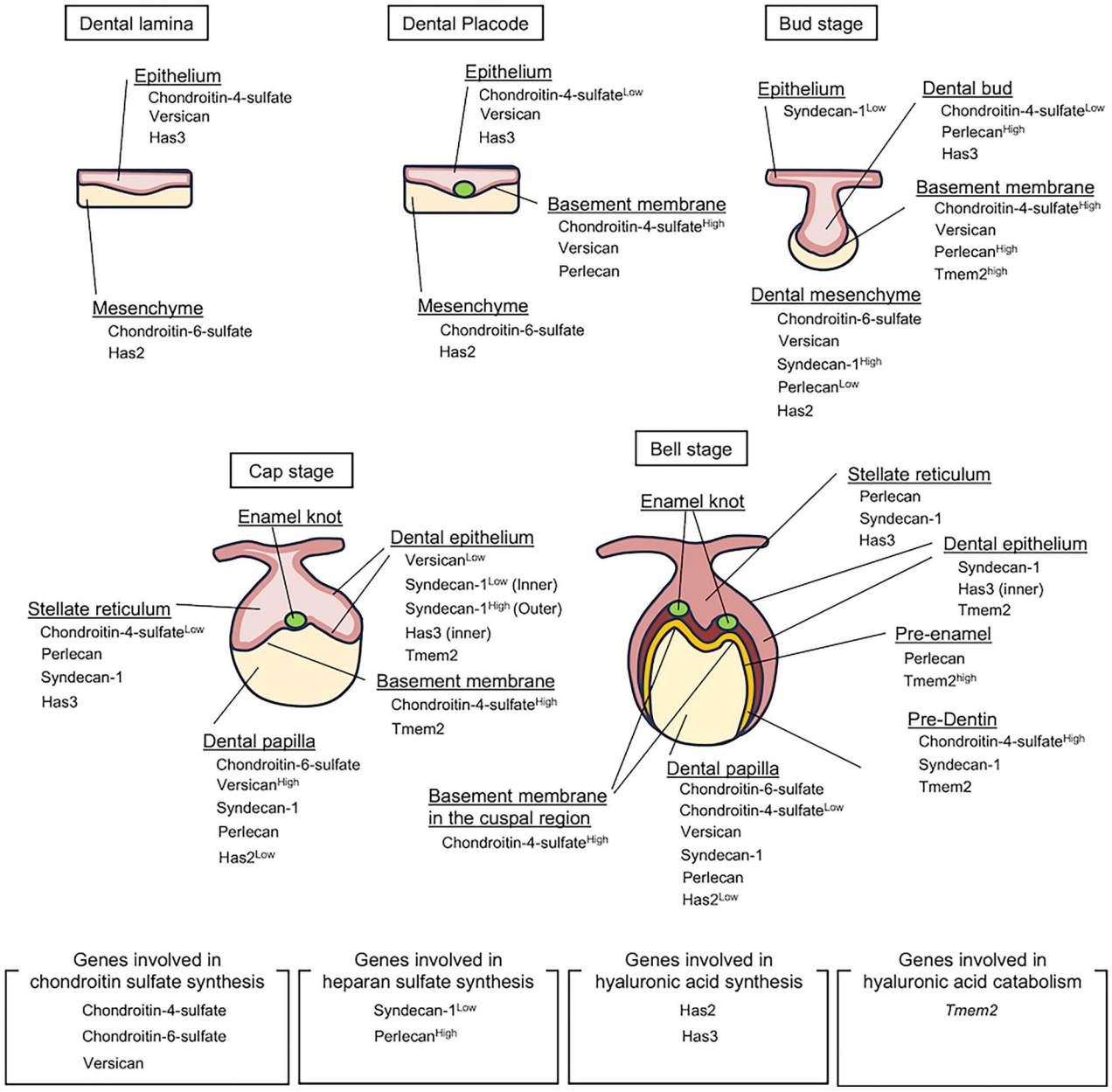

Fig. 1. Expression patterns of GAG related molecule during tooth development. This figure illustrates the complex interplay between the oral epithelium and the underlying mesenchyme, which is crucial for tooth development.

delayed enamel maturation, and accelerated enamel attrition after tooth eruption, resulting in malformed enamel (Liang et al. 2021).

Additional reports have clarified the role of CS in dentinogenesis. Tooth germ organs cultured with 4-methylumbelliferyl-b-D-xyloside (Xyl-MU), which inhibits CS incorporation into CS proteoglycans, influenced odontoblast differentiation. This led to decreased secretion of type 1 collagen, DMP1, and DSPP, and altered tooth morphogenesis and dentinogenesis (Table 2) (Liu et al. 2017). However, it is critical to consider that Xyl-MU might also affect the synthesis of HS, potentially contributing to the observed biological effects not exclusively due to changes in CS. Employing specific antibodies to distinguish the effects on CS and HS synthesis influenced by Xyl-MU could provide deeper insights into how Xyl-MU impacts the synthesis of these GAGs, thereby clarifying its role in tooth development.

Hou et al. observed strong co-localization of versican and DSPP expression in the sub-odontoblastic layer of the coronal pulp until crown formation at P5 (Hou et al. 2012). Afterwards, CS localization was found in the apical papilla region of developed molars, suggesting that CS supports odontoblast differentiation and is involved in dental pulp cell proliferation, pro-angiogenic action in the early stages of development, and the healing process of dental pulp (Table 2) (Ida-Yonemochi et al. 2022).

Heparan Sulfate in tooth development

The production of HS is a complex, multistage procedure carried out by a series of enzymes, which are organized into physical clusters. Key contributors in this synthesis process are exostosin glycosyltransferases (EXTs) and bifunctional deacetylase-N-sulfotransferases (NDSTs) (Lind et al. 1998;

Table 1. Expression of GAG related molecules.

Molecule	Expression	Detection method	Reference
Chondroitin-4-sulfate	Dental lamina, bud stage, occlusal region of dental papilla	Immunostaining with *2B6*	Mark et al. 1990
Chondroitin-4-sulfate	Pre-dentin, stellate reticulum, secretory ameloblast, stratum intermedium	Immunostaining with *2B6*	Mark et al. 1990 Thieberg et al. 1999
Chondroitin-6-sulfate	Dental mesenchyme, dental papilla (bell stage), Pre-odontoblast	Immunostaining with *3B3, MC21C, 7C5*	Mark et al. 1990 Nieminen et al. 1993
Chondroitin-6-sulfate	Pre-odontoblast, pre-dentin, pulp, stellate reticulum	Immunostaining with *3B3, MC21C, 7C5*	Mark et al. 1990 Nieminen et al. 1993
Versican	Thickened dental epithelium, enamel organ, bell stage	In situ hybridization Immunostaining	Jiang et al. 2010
Versican	Dental mesenchyme, dental papilla, stellate reticulum, dental follicle	In situ hybridization Immunostaining	Jiang et al. 2010
DSPP	Differentiating odontoblast, Pre-ameloblast, secretory ameloblast, sub-odontoblastic layer of the coronal pulp	In situ hybridization Immunohistochemistry	Hou et al. 2012, Fisher et al. 2001, Fisher and Fedarko 2003, Bègue-Kirn et al. 1998, Verdelis et al. 2016 Liang et al. 2021
Syndecan-1	Developing human and rodent molar and incisors, differentiating mesenchymal cells	Immunohistochemistry	Vainio et al. 1989a, 1991; Bai et al. 1994; Mitsiadis et al. 1995, Dias et al. 2005; Muto et al. 2007, Kero et al. 2014
Perlecan	Epithelial tooth bud, basement membrane, stellate reticulum, dental mesenchyme, pre-ameloblast	In situ hybridization Immunohistochemistry	Ida-Yonemochi et al. 2005
Heparanase	Dental epithelial cells, cervical loops, dental lamina, stratum intermedium, HERS cells	In situ hybridization	
HAS2	Epithelial tooth bud and condensed mesenchyme, dental papilla	In situ hybridization Immunohistochemistry	Yang et al. 2016 Morita et al. 2016
HAS3	Dental placode, epithelial tooth bud, stellate reticulum, stratum intermedium, inner enamel epithelium (IEE)	In situ hybridization Immunohistochemistry	Yang et al. 2016 Morita et al. 2016
TMEM2	Odontoblasts, stratum intermedium, Pre-ameloblast, secretory ameloblast, mature ameloblast	In situ hybridization Immunohistochemistry	Nag et al. 2023

McCormick et al. 1998; Esko and Lindahl 2001). EXTs extends the HS chain by the adding repeated disaccharide segments, whereas NDSTs induce sulfation at specific points within the HS structure. The specific sulfation configurations created by NDSTs are essential for all subsequent modefications of HS, encompassing the post-biosynthesis cleavage of the glycosidic bond by the endoglucuronidase heparanase 1 (Hpse1/HPSE1) (Poon et al. 2014). These modifications thus determine the binding properties of HS, which affect the availability of HS proteoglycans to interact with ECM constituents and form gradients of HS-binding factors (Yasuda et al. 2010).

HS is a highly sulfated GAG involved in dentinogenesis (Bishop et al. 2007). Among HS proteoglycans, syndecan-1 has been extensively studied in developing tooth germs. The expression domains of syndecan-1 and the 10E4 antibody (which binds to specific motifs on intact HS side chains) appear almost as mirror images of each other (Bai et al. 1994). At the initial bud stage, the 10E4 epitope is diffusely expressed throughout the jaw mesenchyme. As development progresses to late bud stage, staining becomes more concentrated in the dental mesenchyme. From the cap stage onwards, 10E4 staining is primarily localized to the dental follicle and surrounding mesenchyme, with the dental papilla remaining unstained. Notably, the staining pattern of the 10E4 epitope exhibits asymmetry, being more intense on the posterior side, particularly in the outer layers of the sac. By the late bell stage, differentiating odontoblasts within the dental papilla begin to express the 10E4 epitope on their apical surface and in the presumed pre-dentin. The supporting tissue and the alveolar bone continue to express the 10E4 epitope. Syndecan-1 is present on the oral epithelium and temporarily on the epithelial cells of the budding tooth, yet remains 10E4-positive until the late bell stage. The other enamel organ structures, such as the stratum intermedium and the stellate reticulum, exhibit weak or moderate staining for syndecan-1. In the dental mesenchyme, syndecan-1 expression is more restricted and less persistent than the 10E4 epitope. In the early cap stage, syndecan-1 expression is mainly limited to certain area of the dental mesenchyme, and the staining lasts in the dental papilla only up to the cap stage, later disappearing from the dental follicle, whhile the 10E4 epitope continues to be expressed in these areas. Consistently, various studies document the presence of syndecan-1 in the

Table 2. Possible function of GAGs.

GAGs	Model	Phenotype	Reference
Chondroitin sulfate	In vitro	Decrease secretion of type 1 collagen, DMP1, DSPP and altered tooth morphogenesis and dentinogenesis	Liu et al. 2017
Chondroitin sulfate	*T1 knockout mice*	Decrease cell proliferation in the dental pulp and collagen I-positive reparative dentin formation during healing process and dentin hypomineralization.	Ida-Yonemochi et al. 2022
Heparan sulfate	*Ndst1* knockout mice	hypodontia of incisor and molar, abnormal organization and differentiation of odontoblasts	Yasuda et al. 2010
Heparan sulfate	*Sulf1/Sulf2* double null mice	Thin dentin matrix and short roots, reduced *Dspp* expression	Hayano et al. 2012
Sulfated GAGs	*Slc26a2 knockout mice*	Small upper incisor, hypoplasia of upper molars, hypoplasia of dentin matrix and shorten tooth root	Yoshida et al. 2023
Hyaluronic acid	Ex vivo	HA synthesis inhibited, Increased in the proliferation and size of developing molars and inhibiting the molars sequential formation	Sánchez et al. 2020
Hyaluronic acid	*Tmem2* knockout mice	Enamel hypoplasia and soft enamel	Nag et al. 2023

developmental stages of rodent molars and incisors (Vainio et al. 1989a; Vainio et al. 1991; Mitsiadis et al. 1995; Dias et al. 2005; Muto et al. 2007). Additionally, Kero et al. have expanded our understanding by identifying syndecan-1 expression in the developmental phases of human dentition (Kero et al. 2014). According to the expression patter during tooth development, syndecan-1 plays a key role in directing mesenchymal cells towards dental or non-dental fates, crucial for proper tooth development, and controls tooth morphogenesis by influencing differentiation events. Considering its distribution, syndecan-1 is essential for normal tooth morphogenesis, highlighting its role in facilitating communication between epithelial and mesenchymal tissues during odontogenesis (Vainio et al. 1989a).

Perlecan expression is most enhanced in the stellate reticulum cells and is also detected in the central area of the epithelial tooth germ, the basement membrane, and the dental mesenchyme (Ida-Yonemochi et al. 2005; Yonemochi and Saku 2006). As the enamel organ matures, perlecan accumulates in the intercellular spaces of the stellate reticulum, leading to the emergence of its characteristic myxoid appearance. Perlecan is likewise found in the interstitial spaces and at the distal end of pre-ameloblasts prior to the enamel matrix deposition. It has been suggested that perlecan plays a contributory role in the differentiation of ameloblasts, facilitating their production of enamel matrices.

Transgenic mouse models have been utilized to perform functional analysis of HS and HS proteoglycans in tooth development (Table 2). Ndst1-deficient mice exhibited hypodontia of incisors and molars, and showed the abnormal odontoblasts differentiation and organization (Yasuda et al. 2010). In contrast, mice with syndecan-1 gene deletion appear healthy and show no evident abnormalities in their organs, with the sole exceptions being the mammary glands and the re-epithelialization of the cornea and skin (Stepp et al. 2002; Liu et al. 2004). Given their shared evolutionary background and substantial sequence similarity, it is possible that other syndecan proteoglycan family proteins may functionally compensate during development.

Interestingly, Hayano et al. reported that the extracellular glucosamine-6-sulfatases, Sulf1 and Sulf2, play a critical role in odontoblast differentiation and dentin matrix synthesis by removing sulfate groups from the 6-O-position on N-acetylglucosamine during dentinogenesis. Specifically, *Sulf1/Sulf2* double-null mice displayed substantially thinner dentin matrices, shortened roots, and a decrease in Dspp mRNA expression (Hayano et al. 2012). These findings suggest that sulfation and desulfation of HS proteoglycans and N-acetylglucosamine are important processes for proper tooth development, highlighting the critical roles of sulfation and desulfation in the regulation of odontoblast differentiation and function. Importantly, these observations are corroborated by documented cases of dental formation abnormalities in Multiple Sulfatase Deficiency (Adam et al. 1993).

Similarly, the involvement of the HS catabolic enzyme, heparanase, which is recognized as the sole active endo-β-glucuronidase in mammals, in tooth morphogenesis is proposed due to its expression during tooth development in mice and humans (Ida-Yonemochi et al. 2010; Kero et al. 2018). Beginning at E11.5, heparanase was localized within dental epithelial cells, with HS notably aligning linearly along the basement membrane and diffusely within the underlying mesenchyme. By E17.5, a marked increase in heparanase expression is observed in the cervical loops and dental lamina, areas critical for the ongoing formation and growth of the dental structures. The expression of heparanase intensifies by E19, particularly within cells of the stratum intermedium, suggesting a function in enamel maturation processes. Since intense expression of heparanase is detected in Hertwig's epithelial root sheath (HERS) cells with the progression of root formation, heparanase may contribute to tooth root formation (Hirata and Nakamura 2006). However, mice with a knockout of the heparanase coding gene, Hpse1, exhibit no discernible phenotype, suggesting the existence

of potential compensatory mechanisms that complicate the direct interpretation of biological roles of heparanase during tooth development.

Hyaluronic acid in tooth development

HA is one of the most well-known and extensively studied GAGs. Unlike other GAGs, HA is unique due to its non-sulfated nature. It is an integral component of the ECM and is present in various tissues, including epithelial, connective, and neural tissues. HA has a simple linear polymer structure, consisting of repeating disaccharide units that alternate between glucuronic acid and N-acetylglucosamine. The absence of sulfate groups in its structure contributes to its unique properties and functions. One of the most critical roles of HA in the ECM is to provide a structural network through its interactions with other ECM components, such as proteoglycans and collagens. HA serves as a backbone for these proteoglycans, enabling them to form complexes and contribute to the physical properties of the ECM. In addition to its structural role in the ECM, HA is also involved in various biological processes, including wound healing, tissue regeneration, embryonic development, and carcinogenesis (Chen and Abatangelo 1999; Spicer and Tien 2004; Jiang et al. 2007; Contreras et al. 2009; Preston and Sherman 2011).

The biosynthesis of HA occurs through the activity of a group of integral membrane proteins, collectively referred to as hyaluronan synthases (HAS). Three distinct isoforms of hyaluronan synthase, namely HAS1, HAS2, and HAS3, have been identified. Each isoform is encoded by a separate gene and exhibits a unique expression and regulation profile (Itano and Kimata 2002). The gene encoding hyaluronan synthase 1 (Has1) is predominantly expressed in cells of mesenchymal origin and is responsible for the synthesis of low molecular weight HA, in contrast to the Has2 gene, which exhibits broad expression across various cellular lineages and is responsible for the synthesis of high molecular weight HA. The Has3 gene shows widespread expression and can generate low molecular weight variants of HA (Jiang et al. 2011; Fouladi-Nashta et al. 2017).

Earlier research has investigated the distribution of HA throughout the process of tooth development. HA has been found to be prominently present in the stellate reticulum, dental basement membrane, and the apical region of presecretory ameloblasts (Felszeghy et al. 2000; Shibata et al. 2002). However, it is not expressed in the ameloblasts and odontoblasts of both mouse and human molars. (Fig. 1 and Table 1) (Felszeghy et al. 2000; Yang et al. 2016). The aforementioned HA plays a crucial role in facilitating signal transmission between the inner dental epithelium and the ectomesenchymal cells located on the periphery of the dental papilla, pivotal for odontoblast differentiation. As the tooth germ develops into the bell stage, HA is predominantly found in the stellate reticulum, stratum intermedium, and the dental papilla. The stellate reticulum, a layer of cells situated between the inner enamel epithelium and the outer dental papilla, along with the intercellular space between the cells, contains a special network of extracellular matrix molecules, including HA, that plays a critical role in maintaining normal tooth shape development (Fig. 1 and Table 1). Furthermore, it has been found that fetal enamel contains HA throughout its entirety, with the concentration of HA increasing during the fetal period of tooth development. Investigation also revealed robust immunoreactivity of HA in the dental papilla, where mesenchymal cells derived from neural crest cells migrate, proliferate, and differentiate into odontoblasts and pulp cells.

Hyaluronic acid anabolism

The expression of HASs during the development of mouse incisor and molar tooth germ has been extensively studied (Fig. 1 and Table 1) (Spicer and Tien 2004; Tien and Spicer 2005; Morita et al. 2016; Yang et al. 2016). These studies offer insights into the role of HA in tooth development. Spicer et al., demonstrated that Has3 mRNA is first expressed at E10.5 by tooth-forming cephalic neural crest cells. At E11.5 during molar development, a thickened oral epithelium forms a dental placode, with weak and scattered Has2 expression detected in the underlying mesenchyme (Tien and Spicer 2005; Morita et al. 2016). Has3 mRNA is expressed in the dental placode (Tien and Spicer 2005; Morita et al. 2016). As development progressed to E12-E13, the dental placode expanded into a tooth bud, and the mesenchyme begins to condense beneath the enamel organ of the tooth germ. At E13, weak expression of Has2 mRNA is detected in the epithelial tooth bud and condensed mesenchyme, while Has3 is expressed in the epithelial tooth bud (Tien and Spicer 2005; Morita et al. 2016). As the incisor tooth germ advances to the cap stage at E14, the enamel organ, consisting of the inner enamel epithelium, stellate reticulum, and outer enamel epithelium, forms, while the condensed mesenchyme differentiates into the dental follicle and dental papilla (Morita et al. 2016). At this stage, Has2 mRNA is weakly detected in the dental papilla, while Has3 is strongly expressed in the inner dental epithelium adjacent to the enamel knot (Tien and Spicer 2005; Morita et al. 2016). By E16, the tooth germ extends in length, forming pre-dentin. Has2 mRNA is slightly expressed in the dental papilla adjacent to the labial apical bud, while Has3 mRNA is intensely expressed in the labial apical bud, stratum intermedium, and invaginated inner enamel epithelium, with no visible Has2 expression (Morita et al. 2016). Enamel and dentine formation are observed at postnatal day (P7), with the inner enamel epithelium and odontoblasts differentiating at various stages (Morita et al. 2016). The stellate reticulum is found in the apical bud but gradually disappears towards the oral cavity, leading to the formation of the papillary layer. Has3 mRNA is expressed in the labial apical bud and the papillary layer adjacent to mature enamel (Morita et al. 2016). These investigations suggest that hyaluronan is a primary regulatory factor for both prenatal and postnatal HA synthesis, as well as for tooth germ and embryonic mouse molar morphogenesis and cytodifferentiation, regulated by HAS isoforms. Additionally, an ex vivo study indicates that inhibiting HA synthesis significantly increases the proliferation and size of developing molars (Sánchez et al. 2020). However, inhibiting the sequential formation of molars resulted in the disruption of cellular orientation (Table 2) (Sánchez et al. 2020).

Hyaluronic acid catabolism

The physiological process of HA turnover is observed to be quite stable and notably rapid in humans. It is postulated that approximately one-third of the entire body's HA content, estimated at around 15 g in a 70 kg individual, undergoes turnover daily (Fraser and Laurent 1989). This turnover rate is vital in maintaining the homeostasis of HA in the body, which plays significant roles in various physiological processes, including tissue repair, inflammation, and hydration.

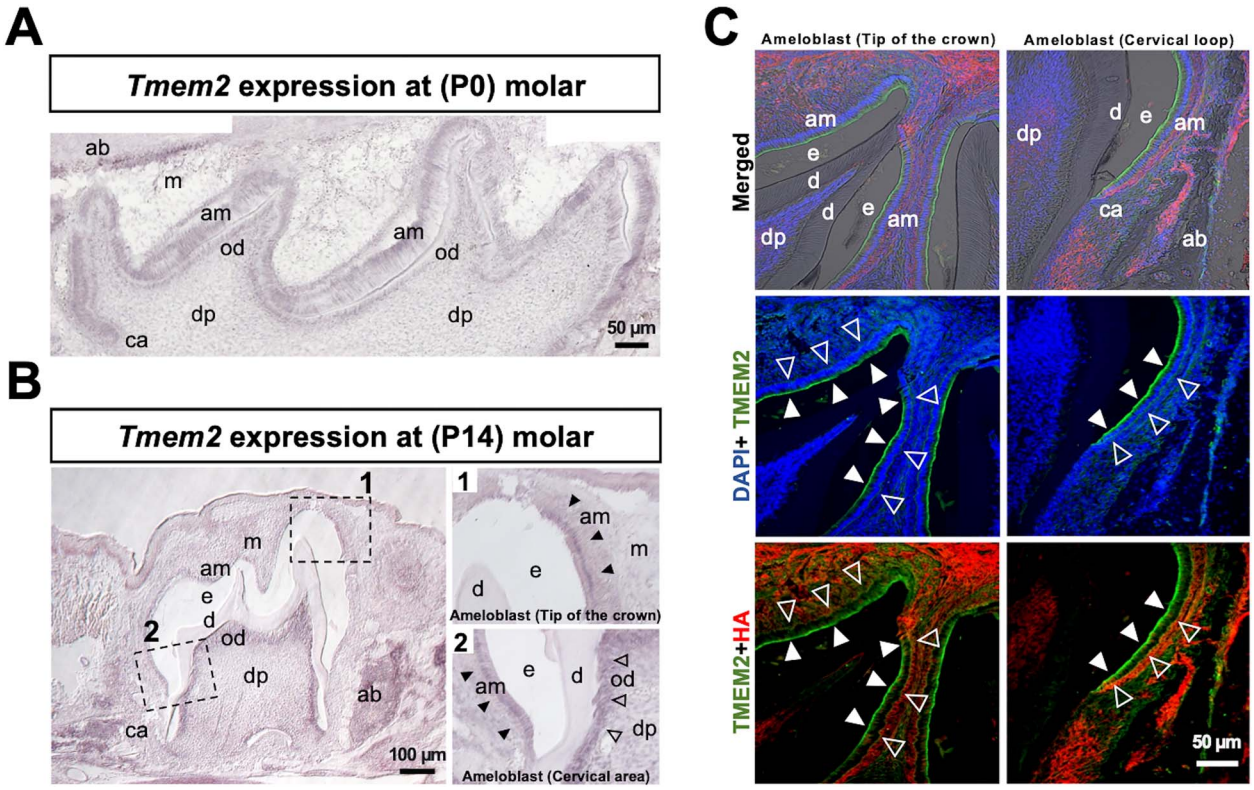

Fig. 2. The expression of Tmem2 in mouse molar during tooth development. In situ hybridization on transverse paraffin sections at P0 (A) and P14 (B) shows distinct and robust expression at developing molar ameloblasts (white arrowheads) around stratum intermedium, and odontoblasts (open arrowheads). Panel (1) and (2) are enlarged images of boxed areas in (B). (C) To detect HA and Tmem2 protein localization, sagittal sections of mouse molar at P14 were double labeled with an anti-Flag antibody and biotinylated hyaluronic acid binding protein (HABP), with nuclear counterstaining was performed with DAPI. The sagittal section of a P14-aged Tmem2-Flag-knock-in mouse revealed strong expression of Tmem2-FLAG protein (green) on the apical side of ameloblasts, and its expression at the basal end of ameloblasts. In contrast HA (Red) immunoreactivity was detected in the stratum intermedium cells and the mesenchymal tissue surrounding the ameloblasts. For detailed information on the materials and methods used, please refer to Nag et al. 2023. ab, alveolar bone; am, ameloblasts; ca, cervical area; d, dentin; dp, dental pulp; e, enamel; m, mesenchyme; od, odontoblasts.

HA is degraded by an enzyme called hyaluronidase (HYAL). There are six distinct isoforms of HYAL, each with unique functions and tissue distributions. HYAL1 is the primary and only form of hyaluronidase found in human plasma, and its mRNA is present in various organs, including the heart, kidney, liver, lung, skeletal muscle, and placenta. The acid-active HYAL2 is crucial for non-enzymatic functions and is also expressed in various tissues. HYAL3 is found in the mammalian testis, bone marrow, and neonatal kidney, while HYAL4 is expressed in the placenta and skeletal muscle but is suspected of having no HA-degrading activity. PH-20/SPAM1, also known as testicular HYAL, is important during egg fertilization by sperm. Finally, HYALP1 is a human pseudogene that results from premature codon termination (Csoka et al. 2001; Stridh et al. 2012; Nagaoka et al. 2015; Fouladi-Nashta et al. 2017; Yamaguchi et al. 2019). Beyond their role in HA degradation, members of the HYAL family, such as HYAL1 and HYAL4, extend their enzymatic activities to the breakdown of other GAGs, including CS and DS (Stern and Jedrzejas 2006). Specifically, HYAL1 has been identified to possess potent chondroitinase activity (Yamada 2015), and HYAL4 is also functionally characterized as a chondroitinase (Stern and Jedrzejas 2006), highlighting the multifaceted enzymatic capabilities of the HYAL family in extracellular matrix remodeling.

In a groundbreaking study published in 2017, researchers discovered that Tmem2 (Transmembrane protein 2) functions as a novel cell surface hyaluronidase (Yamamoto et al. 2017). Specifically, this enzyme is responsible for cleaving extracellular high molecular weight HA into intermediate-sized fragments directly on the cell surface before their internalization. These intermediate HA fragments are subsequently fully degraded in the lysosome. Unlike HYAL family proteins, Tmem2 does not cleave CS or DS, highlighting its specificity on HA (Yamamoto et al. 2017). This specificity underlines unique role of Tmem2 in modulating HA dynamics within the extracellular matrix. A recent study by Inubushi et al. reported detection of *Tmem2* mRNA in several regions during the mid-gestation period (E8.5-10.5), including the forebrain, midbrain, hindbrain, trigeminal ganglion, branchial arches, heart, and dorsal root ganglia (Inubushi et al. 2022). The study further revealed that Tmem2 plays a critical role in neural crest development, as the conditional ablation of *Tmem2* through Wnt1-Cre-mediated deletion resulted in severe craniofacial defects. In addition, they reported that a lack of *Tmem2* results in an endocardial cushion defect (Inubushi et al. 2022). Nag *et al.* conducted a study examining the expression of *Tmem2* mRNA during embryonic and postnatal tooth development (Nag et al. 2023). Their findings demonstrated that in mouse incisor at P14, the Tmem2 protein is localized in odontoblasts, around the stratum intermedium, and at the pre-secretory (PAB), secretory (SAB), and maturation (MAB) stages of ameloblasts. In the mouse molar

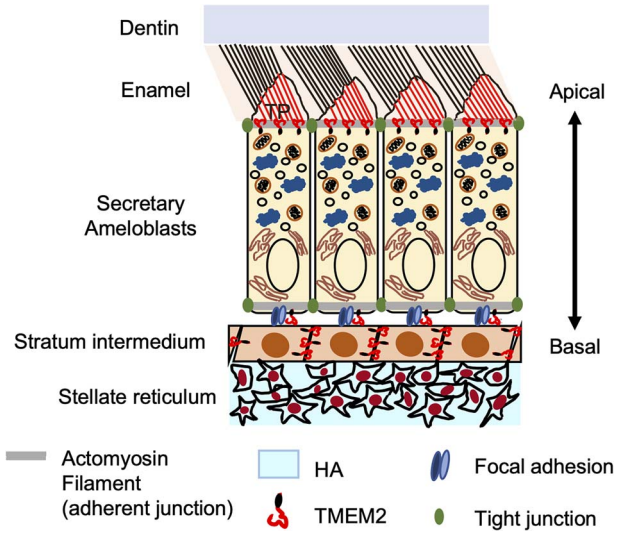

Fig. 3. The potential roles of Tmem2 in enamel formation. Tmem2 protein localization at the apical and basal end of secretory ameloblasts and in the stratum intermedium. Tmem2 plays border function at the basal end of ameloblasts by inhibiting HA accumulation in ameloblasts. This figure was adapted from Nag et al. 2023, journal of dental research. Description of the figure and any modifications made. Reprinted with permission.

at P0 and P14, distinct and robust expression of Tmem2 is observed in developing molar ameloblasts, around the stratum intermedium, and in odontoblasts (Fig. 1, Fig. 2A and B and Table 1). The Tmem2 protein localizes on both the apical and basal sides of the ameloblasts (Fig. 2C). This expression pattern contrasts with that of HA, which is localized in the mesenchymal tissue (Fig. 2C). Notably, in tissues where Tmem2 is expressed, HA localization is not observed, indicating a mutually exclusive distribution. Conditional deletion of *Tmem2* in mouse dental epithelium resulted in severe enamel hypoplasia, highlighting the importance of TMEM2 in enamel formation (Table 2). These observations clearly indicate that TMEM2 is crucial for adhesion to the HA-rich extracellular matrix, ameloblast differentiation, and enamel matrix mineralization (Fig. 3). However, the role of TMEM2 in non-epithelial tissues, including odontoblasts, has not yet been elucidated.

Other GAGs in tooth development

Previous studies have identified *fibromodulin*, a proteoglycan harboring KS chains, expression in the outer enamel epithelium at E14.5 (Wilda et al. 2000) and in the stratum intermedium alone at P0 (Goldberg et al. 2006). Chen et al., have also reported that *fibromodulin* is expressed in the dental epithelium and mesenchyme at E 11.5 and E13.5, but *lumican* is expressed markedly higher than *fibromodulin* (Chen et al. 2023). In contrast, Randilini et al., reported that the expressions of *fibromodulin* and *lumican* are not identified in the tooth germs through the developmental stage from E13.5 to P7 (Randilini et al. 2020). Collectively, these findings suggest that KS proteoglycan could play a significant regulatory role in the dental epithelium throughout the process of tooth development. Yet, to date, relatively few studies have explored its function in this context.

The CS backbone is composed of glucronic acid (GlcA) and N-Acetyl-D-galactosamine (GalNAc), while DS is a stereoisomer of CS, featuring iduronic acid (IdoA) instead of GlcA. It has been reported that DS proteoglycan and KS proteoglycan, present in pre-dentin, inhibit the calcification of collagen fibrils within the uncalcified matrix and are not found at the calcification front (Moriguchi et al. 2004). Nishikawa et al. have demonstrated that rat dental pulp cells synthesize CS and DS, with an enhanced production following TGF-β stimulation (Nishikawa et al. 2000). More importantly, there are indications of a link between DS defects and tooth malformation in Musculocontractural Ehlers-Danlos Syndrome (mcEDS) (Minatogawa et al. 2022). This condition is caused by pathogenic variants in the CHST14/D4ST1 (carbohydrate sulfotransferase 14/dermatan-4 sulfotransferase-1) or DSE (dermatan sulfate epimerase) genes, which are involved in DS biosynthesis (Kosho et al. 2019). However, further investigation is necessary to elucidate the role of DS in tooth development.

Sulfate metabolism

The sulfation of GAGs imparts a negative charge at various sites on proteoglycans, instigating a diversity of biological functions attributable to structural microheterogeneity. In mammals, the SLC26 gene family is composed of 11 genes (SLC26A1-A11) that encode anion transporters. This gene family facilitates the transportation of a wide variety of anionic substrates, such as sulfate, bicarbonate (HCO^{3-}), chloride (Cl^{-}), oxalate, iodide (I^{-}), and formate. It has been reported that Slc26a2 is predominantly expressed in dental tissues, which include odontoblasts and ameloblasts, during tooth development (Yoshida et al. 2023). Further investigations involving Slc26a2 knockout mice revealed the occurrence of smaller upper incisors and hypoplasia of upper molars (Table 2). These observations concur with recent findings that implicate mutations in SLC26A2 in a wide variety of clinical manifestations in the craniomaxillofacial region. Such manifestations encompass an enlarged upper facial height, micrognathia, a high palate, a cleft palate (seen in 25%–60% of instances), congenital anodontia (30%), and dwarf teeth (Härkönen et al. 2021). It is noteworthy that mutations in the other eight members of the SLC26 family have also been associated with human diseases. However, these mutations, unlike those in SLC26a2, do not induce any abnormalities in the skeletal system or craniofacial region. Therefore, it may be inferred that SLC26A2-mediated sulfate metabolism plays a pivotal role in tooth development.

Future prospective

Based on the knowledge in vitro and animal models, the impact of GAGs in human tooth development should be clarified. Genetic diseases are some of the most valuable sources of information towards understanding the importance of GAGs in human tooth development. With the advancements and increasing availability of gene analysis techniques, genetic mutations have been identified in many individuals with congenital abnormalities. However, the dental phenotypes of patients with these abnormalities may not have been sufficiently investigated thus far. Ongoing advancements in

medical science are expected to enhance patients' quality of life, which, in turn, will increase their access to dental care. Consequently, there is potential for a more comprehensive understanding of dental abnormalities (phenotypes) in these patients in the future. This could further clarify the significance of GAG mutations in human tooth development. Such information is believed to be critically important for the advancement of regenerative dental medicine.

Furthermore, it was considered that GAGs are promising biomaterials for tissue engineering and regenerative medicine owing to their multiple bioactivities. In the case of spinal cord injury, CS is known to strongly inhibit the axonal growth from the neural regeneration (Takeuchi et al. 2013). It was revealed that CS glycosyltransferase, N-acetylgalactosaminyltransferase-1, knockout mice showed quick recovery from the spinal cord injury comparing with the wild type mice. Thus, biomaterials in combination with chondroitinase or siRNA for CS glycosyltransferase could be useful in central nerve regeneration. Our group reported that HS regulates the vasculogenesis of mesenchymal stem cells (Li et al. 2023). Specifically, an antagonist of HS and genetic silencing of EXT1 were found to inhibit endothelial differentiation and vasculature formation in dental pulp stem cells. Since efficient vascularization is crucial for regenerated tissue, the utilization of HS could potentially improve the prognosis of regenerative therapies. Furthermore, we recently reported on Matching Transformation System® (MA-T), a novel chlorinated oxidant, which enhances dentinogenesis by decreasing the sulfation of HS proteoglycan and subsequently activating Wnt signaling (Inubushi 2023).

Further investigations are required to determine the function of specific GAGs for tissues and stem cells, and these findings would call for more in-depth research of the relationship between GAGs and regenerative medicine.

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Author contributions

Toshihiro Inubushi (CRediT contribution not specified), Priyanka Nag (Writing—original draft [equal]), Junichi Sasaki (Conceptualization [supporting], Supervision [supporting], Writing—original draft [supporting], Writing—review & editing [supporting]), Yuki Shiraishi (Writing—original draft [supporting], Writing—review & editing [supporting]), and Takashi Yamashiro (Conceptualization [supporting], Supervision [supporting], Validation [supporting], Writing—review & editing [supporting])

Supplementary material

Supplementary material is available at *Glycobiology Journal* online.

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Data availability

The data presented in this study are available from the corresponding author upon reasonable request.

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