Expression, localization and synthesis of small leucine-rich proteoglycans in developing mouse molar tooth germ

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The gene expression and protein synthesis of small leucine-rich proteoglycans (SLRPs), including decorin, biglycan, fibromodulin, and lumican, was analyzed in the context of the hypothesis that they are closely related to tooth formation. In situ hybridization, immunohistochemistry, and organ culture with metabolic labeling of [35S] were carried out in mouse first molar tooth germs of different developmental stages using ICR mice at embryonic day (E) 13.5 to postnatal day (P) 7.0. At the bud and cap stage, decorin mRNA was expressed only in the surrounding mesenchyme, but not within the tooth germ. Biglycan mRNA was then expressed in the condensing mesenchyme and the dental papilla of the tooth germ. At the apposition stage (late bell stage), both decorin and biglycan mRNA were expressed in odontoblasts, resulting in a switch of the pattern of expression within the different stages of odontoblast differentiation. Decorin mRNA was expressed earlier in newly differentiating odontoblasts than biglycan. With odontoblast maturation and dentin formation, decorin mRNA expression was diminished and localized to the newly differentiating odontoblasts at the cervical region. Simultaneously, biglycan mRNA took over and extended its expression throughout the new and mature odontoblasts. Both mRNAs were expressed in the dental pulp underlying the respective odontoblasts. At P7.0, both mRNAs were weakly expressed but maintained their spatial expression patterns. Immunostaining showed that biglycan was localized in the dental papilla and pulp. In addition, all four SLRPs showed clear immunostaining in predentin, although the expressions of fibromodulin and lumican mRNAs were not identified in the tooth germs examined. The organ culture data obtained supported the histological findings that biglycan is more predominant than decorin at the apposition stage. These results were used to identify biglycan as the principal molecule among the SLRPs investigated. Our findings indicate that decorin and biglycan show spatial and temporal differential expressions and play their own tissue-specific roles in tooth development.

Key words: Decorin; biglycan; tooth germ; in situ hybridization; organ culture.
Introduction

Small leucine-rich proteoglycans (SLRPs) have been identified as important components in the extracellular matrix (ECM) and are involved in several biological and pathological processes in various tissues. They are proteoglycans (PGs) with small core proteins of tandem leucine-rich repeats that carry one or more glycosaminoglycan (GAG) chains, such as chondroitin sulfate (CS), dermatan sulfate (DS), or keratan sulfate (KS). Eighteen SLRP genes have been identified to date and are classified into five categories based on their characteristics at both the genomic and protein levels.\(^1\) It has been reported that these SLRPs play significant roles in collagen fibrillogenesis and in preventing premature mineralization,\(^7\) whereas biglycan is more significant in knockout studies, the absence of osteoblast differentiation and matrix mineralization.\(^8\) In gene-knockout studies, the absence of decorin gives rise to skin fragility and dysregulation of lateral fibil growth.\(^9\) Likewise, the absence of biglycan results in osteoporosis-like phenotypes.\(^10\)

Class II SLRPs include fibromodulin and lumican, which are close relatives of decorin and biglycan in terms of their function. They have KS chains and are found in tissues such as tendons, cartilage, sclera, and the cornea.\(^6\) Goldberg et al.\(^11,12\) identified two different molecular weights for fibromodulin present in bone and dentin, the former of which is larger. Lumican is also involved in collagen fibrillogenesis and determines the collagen fiber diameter and spacing.\(^11\) It is also recognized as a marker for the identification of differentiating and mature osteoblasts from undifferentiated osteoblasts.\(^14\)

Tooth development is a complex process regulated by epithelial-mesenchymal interactions and continues through different developmental stages known as bud, cap, bell, and apposition stages.\(^13\) Throughout the tooth developmental process, the involvement of versican, a large CS- PG;\(^16\) perlecan, a large heparan sulfate (HS)-PG;\(^17\) and syndecans, small cell surface HS-PGs,\(^18\) have been thoroughly investigated. Several studies have also confirmed the involvement of SLRPs in tooth development in humans\(^11,12,20\) and other species.\(^21-23\)

SLRPs have been proposed to play several roles in tooth development, including cellular differentiation, collagen fibrillogenesis, dental mineralization, and tooth eruption.\(^24\) To elucidate the exact roles of SLRPs in tooth formation, gene-knockout studies have been carried out. The absence of decorin has been found to affect dentin mineralization, whereas biglycan has been shown to have a multifunctional involvement, the knockout of which affects both amelogenesis and dentinogenesis, together with collagen fibrillogenesis in predentin.\(^25\) Fibromodulin deficiency has been found to affect enamel formation and increases the collagen diameter in predentin, as well as causing dentin hypomineralization in newborn mice, in contrast to the self-repair observed in adult mice.\(^11,12\) Lumican deficiency results in periodontal ligament phenotypes.\(^26\) Many immunohistochemical studies in tooth germs have also been performed in rodents\(^25,26\) and humans.\(^13,20\) Several in situ hybridization studies in mice have also been carried out.\(^28-30\) These studies, however, have not examined the temporal changes in SLRP expression during the process of identical tooth germ formation. Thus, our hypothesis that SLRPs are closely involved in tooth formation led us to perform an in situ hybridization study to elucidate their specific functions by investigating the formation of identical tooth germs (of the lower first molar) from the early stage to the postnatal stage. Simultaneously, the synthesis of SLRP proteins by tooth germs was investigated by immunohistochemistry and an organ culture system using metabolic labeling of \(^{35}\)S.

Materials and Methods

Animals

A total of 16 pregnant Institute of Cancer Research (ICR) mice at embryonic day (E) 13.5 to E18.5 (08:00 h on the day of the vaginal plug was designated as stage E0), and a total of 12 postnatal day (P) 1.0, 3.0, and 7.0 mice were used in this study. All animals were housed in facilities approved by Tokyo Medical and Dental University. Our animal use protocol and experimental system were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (2016-009A, A2017-151A, A2018-005A, and A2019-009A).

Tissue preparation

Pregnant and postnatal mice were sacrificed by cervical dislocation under CO\(_2\), asphyxiation. The heads and lower limbs of the fetal and postnatal mice were separated and were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C for 1 day. The E18.5, P1.0, P3.0, and P7.0 specimens were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) at 4°C for 10 days. All specimens were then embedded in paraffin. Frontal or sagittal sections of 5 µm in thickness were obtained for toluidine blue (TB) general staining, immunohistochemistry, and in situ hybridization.

In situ hybridization

All primer sequences used are listed in Table 1. The cDNA

### Table 1. Primer sets for RNA probes.

| Gene       | Primer sequence (5’-3’) | Product size | Accession number |
|------------|-------------------------|--------------|------------------|
| Decorin    | Forward: GCTCACCGCATGAAACCTTAG Reverse: GCGCAATTCGCGCAAGC | 754 bp | NM_001190451.2 |
| Biglycan   | Forward: ACTCATTGATCCTGTTGCCC Reuse: CGAGTTTCCAGGTACCCC | 721 bp | NM_001190451.2 |
| Fibromodulin | Forward: GGTTGAGGGGAATGGGTGAG Reverse: AGTCCACCTACTACGACCCC | 878 bp | NM_001190451.2 |
| Lumican    | Forward: GCTCACCGCATGAAACCTTAG Reuse: GCGCAATTCGCGCAAGC | 774 bp | NM_001190451.2 |
products were subcloned into a pCR II vector (Stratagene, La Jolla, CA, USA), and antisense and sense RNA probes were synthesized. Each probe was then labeled with [³⁵S]-UTP using the Riboprobe in vitro Transcription System (Promega, Madison, WI, USA). In situ hybridization using [³⁵S]-UTP labeled probes was performed as previously described. Following hybridization and RNase treatment, the sections were dipped in emulsion (NTB; Kodak, Rochester, NY, USA) and exposed for 2 weeks at 4°C for autoradiography. Sense probes were used as negative control samples. We examined three different samples for each embryonic day to ensure that consistent results were obtained.

**Immunohistochemistry**

Rabbit polyclonal antibodies against decorin, biglycan, and fibromodulin were obtained from Biozzi Antibodies (Boston, MA, USA). A rabbit polyclonal antibody against human lumican was obtained from Funakoshi (Tokyo, Japan). Immunohistochemical staining was performed as previously described. Briefly, the sections were deparaffinized and digested with testicular hyaluronidase (25 mg/mL in phosphate buffered saline; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C. Then, the sections were immersed in methanol containing 1% hydrogen peroxide, followed by 1% bovine serum albumin to block endogenous peroxidase activity and non-specific binding, respectively. The primary antibodies diluted for decorin (1:25), biglycan (1:50), fibromodulin (1:100), and lumican (1:100) were applied and incubated overnight at 4°C. This was followed by biotin-labeled rabbit IgG and peroxidase-labeled streptavidin application. 3-amino-9-ethylcarbazole was used for protein visualization after counterstaining with hematoxylin. The samples were observed under a light microscope. The negative controls were incubated with normal anti-rabbit IgG instead of the primary antibodies. We examined three different samples for each embryonic day to ensure that consistent results were obtained.

**Organ culture and metabolic labeling with [³⁵S]**

An organ culture system was created according to a previous study. Based on the results of in situ hybridization/immunohistochemistry, we selected the lower first molar tooth germs of E16.0, E18.0, and P3.0 as representatives of the late cap stage, predentin formation stage, and enamel and dentin formation stage, respectively. A total of 36 explants of tooth germs were cultured on cell culture inserts (Millicell, Merck, Tokyo, Japan) containing BGB medium (Gibco: Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) for 2 h in a humidified atmosphere of 5% CO₂ in air at 37°C. Three tooth germs were placed on a cell culture insert. These explants were labeled with [³⁵S]Na₂SO₄ (3.7 MBq/mL, American Radiolabeled Chemicals, St Louis, MO, USA) for another 12 h under the same culture conditions.

**Extraction of proteoglycan and gel filtration**

After labeling, the explants were extracted with 4 M guanidine HCl, 50 mM sodium acetate, pH 6.0, containing 2% (w/v) Triton X-100 and 1% protease inhibitors (Sigma-Aldrich) at 4°C for 24 h. Each extract was subjected to a Sephadex G-50 column (Amersham Pharmacia Biotech, Tokyo, Japan) and eluted with 4 M guanidine HCl, 50 mM sodium acetate (pH 6.0) containing 0.5% (w/v) CHAPS to remove any unincorporated radioactive precursors. The collected void volume fractions were counted with a liquid scintillation counter and estimated as the total [³⁵S]-labeled macromolecules per dish. To assess changes of synthesized [³⁵S]-labeled macromolecules in amounts associated with tooth germ growth, data analyses were performed using one-way analysis of variance (ANOVA) for several group comparisons. Significance was determined using Tukey’s comparison test. A P-value of <0.05 was considered statistically significant.

The [³⁵S]-labeled macromolecules from the Sephadex G-50 columns were then injected into prepacked Superose 6 columns (Amersham Pharmacia Biotech) and eluted with 4 M guanidine HCl, 0.05 M sodium acetate (pH 6.0) containing 0.5% (w/v) Triton X-100 as previously described. The fractions (0.4 mL each) were collected and counted.

**Enzymatic treatment of PGs**

[³⁵S]-labeled macromolecules from the Sephadex G-50 columns were dialyzed against 0.1 M Tris-acetate buffer (pH 7.3) and digested with chondroitinase ABC (Seikagaku, Tokyo, Japan) (10 mU/µL) with protease inhibitors (10 mM EDTA, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, 0.36 mM pepstatin A). One part of the chondroitinase ABC-digested samples was further digested with heparitinase (Seikagaku, Tokyo, Japan) (10 mU/µL) and/or keratanase (Seikagaku, Tokyo, Japan) (10 mU/µL) at 37°C for an additional 2 h. Finally, the enzyme-treated samples were subjected to the Superose 6 column.

**Polyacrylamide gel electrophoresis (SDS-PAGE)**

Heparitinase-treated samples were applied to precast 5-20% gradient gels (Atto, Tokyo, Japan) and run at a constant current of 30 mA. Pre-stained molecular weight markers were used as standards (Life Technologies: Thermo Fisher Scientific). After electrophoresis, the gels were dried for 2 h at 60°C, then placed on an imaging plate (Fuji IP IN cassette) and analyzed by FCR PRIMA T2 (Fujifilm Medical, Tokyo, Japan).

**Results**

**In situ hybridization in mouse molar tooth germ**

All terms related to tooth germ morphogenesis are according to Pieco and Avery. At E13.5, the lower first molar tooth germ consisting of an epithelial tooth bud and condensed mesenchyme (future dental papilla) was at the bud stage, and mandibular bone started to surround it (Figure 1 a,f). Decorin, biglycan, and fibromodulin mRNAs were expressed in various tissues in the mandible (Figure 1 b-d). Focusing on tooth germ, decorin mRNA was expressed in the surrounding mesenchyme, but not within the tooth germ (Figure 1g). Biglycan mRNA was well expressed in the condensing mesenchyme of the tooth germ, but was not expressed within the epithelial tooth bud (Figure 1h). Fibromodulin mRNA did not show significant expression within the tooth germ (Figure 1i).

Lumican mRNA did not show significant expression in any regions at this stage (Figure 1e). This was similar for lumican mRNA at all stages examined in the present study (data not shown)). Since each mRNA showed similar expression patterns at E15.0 and E16.0, we describe results at E16.0 as representative. At E16.0, the masseter muscle was attached to the developing mandibular bone, which surrounded the tooth germ in the late cap stage (Figure 2 a,e). Decorin mRNA was expressed in the mesenchyme around the tooth germ, although not within it (Figure 2 b,f). Biglycan mRNA showed expression patterns similar to those of decorin; however, it was additionally expressed in the dental papilla (Figure 2 c,g). Fibromodulin mRNA did not show significant expression within the tooth germ (Figure 2h). Note that each mRNA was expressed in the epimysium of the masseter muscle (Figure 2 b-d). At E18.5, the tooth germ was in the apposition stage (late bell stage), and predentin formation with differentiating odontoblasts was evident at the cusp tip (Figure 3a). Decorin mRNA was well expressed in...
differentiating odontoblasts, as well as in the dental papilla beneath the odontoblastic layer at the cusp region (Figure 3b). However, biglycan mRNA was expressed at a lesser intensity than decorin and was only localized to odontoblasts at the cusp tips (Figure 3c). Fibromodulin mRNA was expressed in the epimysium of the masseter muscle, although not within the tooth germ (Figure 3d). Note that the epimysium also showed decorin and biglycan mRNA (Figure 3b,c). At P1.0, dentin formation was clearly evident (Figure 3e).

Decorin mRNA expression was reduced in mature odontoblasts facing dentin; however, it remained well expressed in the newly differentiating odontoblasts towards the cervical part of the tooth germ (Figure 3f). In contrast, biglycan expression was enhanced in the mature odontoblasts, but remained weakly expressed in the newly differentiating odontoblasts (Figure 3g). Decorin and biglycan mRNA were also detected in the dental papillae beneath the odontoblastic layers; however, the expression of the former was slightly stronger than the latter (Figure 3f,g). The epimysium was found to still express every mRNA examined at this stage (Figure 3f-h). At P3.0, dentin, as well as enamel formation, were evident (Figure 4a). Strong decorin mRNA expression was localized in the newly differentiating odontoblasts at the cervical region (Figure 4b). Meanwhile, biglycan mRNA was expressed strongly throughout the odontoblastic layer (Figure 4c). The dental papillae showed similar expression patterns for decorin and biglycan to those at P1.0 (Figure 4b,c). Fibromodulin mRNA was expressed in the epimysium, but it was not expressed within the tooth germ (Figure 4d). Notably, not every mRNA was expressed at all stages in the ameloblasts (Figure 4b-d).

At P7.0, crown formation was found to have advanced, with Hertwig's epithelial root sheath having started to form (Figure 4e). Decorin mRNA expression was completely reduced, although it remained at the cervical tip, that is, where the new odontoblasts were being differentiated (Figure 4f). Biglycan mRNA expression was also reduced, although it remained weakly expressed throughout the odontoblastic layer (Figure 4g). The dental papilla at the coronal region weakly expressed decorin and biglycan mRNAs (Figure 4f,g).

In situ hybridization in mouse incisor tooth germ and in lower limb tendons and epimysium

The incisor tooth germ at P7.0 cut at the sagittal plane showed every stage of differentiating odontoblast (Figure 5a). Decorin mRNA expression was reduced in mature odontoblasts facing dentin; however, it remained well expressed in the newly differentiating odontoblasts towards the cervical part of the tooth germ (Figure 5b). In contrast, biglycan expression was enhanced in the mature odontoblasts, but remained weakly expressed in the newly differentiating odontoblasts (Figure 5c). Decorin and biglycan mRNA were also detected in the dental papillae beneath the odontoblastic layers; however, the expression of the former was slightly stronger than the latter (Figure 5b,g). The epimysium was found to still express every mRNA examined at this stage (Figure 5b-f). At P12.0, dentin, as well as enamel formation, were evident (Figure 6a). Strong decorin mRNA expression was localized in the newly differentiating odontoblasts at the cervical region (Figure 6b). Meanwhile, biglycan mRNA was expressed strongly throughout the odontoblastic layer (Figure 6c). The dental papillae showed similar expression patterns for decorin and biglycan to those at P1.0 (Figure 6b,c). Fibromodulin mRNA was not expressed in the epimysium of the masseter muscle (arrows in b-d). Scale bars: 50 μm.

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mRNA expression was detected in newly differentiating odontoblasts, although it was gradually reduced towards the coronal region (Figure 5b). *Biglycan* mRNA expression started to be detected in odontoblasts at a similar position to that showing *decorin* mRNA expression; however, the odontoblasts maintained *biglycan* mRNA expression more towards the tip region compared to *decorin* (Figure 5c). The tendon of the quadriceps femoris muscle was continuous to the epimysium of the tibialis anterior muscle at P1.0.
The tendon, the epimysium, and the perimysium all expressed decorin, biglycan, and fibromodulin mRNA, although the expression of decorin mRNA was weak in the epimysium while strong in the perimysium (Figure 5d). The tendon, the epimysium, and the perimysium all expressed decorin, biglycan, and fibromodulin mRNA, although the expression of decorin mRNA was weak in the epimysium while strong in the perimysium (Figure 5d).

### Immunohistochemistry in mouse molar tooth germ

At E16.0, although biglycan immunoreactivity was detected in the dental papilla, other immunoreactivities were not detected within the tooth germ (Figure 6a-d). Despite this, decorin immunoreactivity was detected in the lamina propria of the oral mucosa around the tooth germ (Figure 6a). These findings correlated with mRNA expression patterns; however, in contrast to the mRNA findings, lumican immunoreactivity was detected in the osteoid of the mandibular bone (Figure 6d). At E18.5, immunoreactivity for four SLRPs was detected in the predentin and osteoid (Figure 6c-h). These results were in accordance with mRNA expression for decorin and biglycan in odontoblasts, indicating that these molecules were mainly synthesized by odontoblasts, but not in accordance with the mRNA expression for fibromodulin and lumican.

Biglycan immunoreactivity was also detected in the central region of the dental pulp; however, it was weakly expressed in the peripheral region beneath the odontoblastic layer where its mRNA expression was clearly detected (Figure 6f). From P1.0 (data not shown) to P3.0, immunoreactivities showed similar expression patterns to those at E18.5. Notably, immunoreactivity was detected neither in the dentin nor in the enamel (Figure 6i-l).

### Analysis of SLRPs synthesized by tooth germ explants

In the explant cultures, the amount of [35S]-labeled macromolecules released into the explants at E18.0 was significantly larger than that at E16.0, and that at P3.0 was further significantly larger than that at E18.0, indicating that the amount of synthesized macromolecules increased in association with tooth germ growth (Figure 7a). A [35S]-labeled tissue sample from each day was analyzed by Superose 6 gel chromatography (Figure 7b). Intact samples from E16.0 and E18.0 showed similar elution profiles. In both samples, a major peak eluted at V0 (peak 1) and a broad peak eluted at Kd = 0.23 (peak 2) were observed. The ratios of [35S] radioactivity in peaks 1 and 2 to total radioactivity were ~69% and ~29% at E16.0 and ~51% and ~39% at E18.0, respectively. Peaks 1 and 2 at E16.0 were somewhat susceptible to chondroitinase ABC, while only ~5% of [35S] radioactivity remained in the regions of peaks 1 and 2 and was not susceptible to digestion by heparitinase or keratanase, indicating small amounts of synthesized HS-PGs and KS-PGs. At E18.0, ~27% of [35S] radioactivity remained in peak 1 and 2 regions after chondroitinase ABC digestion. About 10% of [35S] radioactivity in the remaining peaks was susceptible to heparitinase, however, it was not...
susceptible to subsequent digestion by keratanase, indicating that a significant amount of HS-PGs was synthesized, while KS-PGs was synthesized at low levels. In addition, a third peak was disclosed at $K_d = 0.68$ after chondroitinase ABC digestion and was not susceptible to either heparitinase or keratanase. The remaining peaks at the 1 and 2 regions and peak 3 may represent sulfated proteins or sulfated glycoproteins. However, the necessary analyses for their elucidation did not fall within the scope of this study. At P3.0, peak 1 became small, while other peaks were recognized at $K_d = 0.48$ (peak 4) and $K_d = 0.70$ (peak 5). Since these peaks were not susceptible to enzymatic treatments, they may represent sulfated proteins or glycoproteins. Peaks 1 and 2 were susceptible to chondroitinase ABC. The remaining peak (~11%) was further susceptible to heparitinase, but was not susceptible to keratanase, indicating that significant amounts of HS-PGs were also synthesized, while KS-PGs were synthesized at low levels.

**SDS-PAGE analyses**

Since the specimens investigated above were found to potentially contain significant amounts of HS-PGs, they were subjected to SDS-PAGE after heparitinase digestion. One band outside the gel (band 1) and two broad bands (~250 kDa (band 2) and ~100 kDa (band 3)) were observed in all three stages (Figure 7c). Compared to E16.0 and E18.0, band 2 of P3.0 was significantly thicker than band 3. According to previous studies, bands 1, 2, and 3 correspond to versican-type large PG, biglycan-type small PG, and decorin-type small PG, respectively. Hence, the tooth germ at P3.0 synthesized higher amounts of biglycan-type PG than decorin-type PG.

**Discussion**

**Expression of decorin and biglycan mRNAs in odontoblasts with the formation of predentin/dentin**

In the present study, decorin and biglycan mRNAs were more highly expressed than fibromodulin and lumican mRNAs in developing mouse molar tooth germ. In addition, the explant cultures indicated that KS-PGs were synthesized at low levels by tooth germs from E16.0 to P3.0, suggesting that the involvement of KS-PGs (e.g., fibromodulin and lumican) in tooth formation was less than that of CS-PGs (e.g., versican, decorin, and biglycan). Although a previous study reported that fibromodulin mRNA is expressed in the outer enamel epithelium and lumican mRNA in the dental papillae of E14.5 mice incisors, our results suggest that tooth germs express fibromodulin and lumican mRNAs in small amounts,
as they were observed at a low intensity during our procedure. Thus, the relationship of decorin and biglycan to tooth formation was the main focus of the present study.

As biglycan mRNA was expressed at every developmental stage of the mouse molar tooth germ, we considered it to be the most abundant molecule among the four SLRPs examined. Notably, decorin and biglycan mRNAs were found to be differentially expressed in odontoblasts. At the predentin formation stage (E18.5), decorin mRNA was well expressed in the differentiating odontoblasts at the cusp tip, while the expression of biglycan mRNA was low. With the formation of dentin (P1.0 to 3.0), decorin mRNA expression was decreased in that area, being more localized to the newly differentiating odontoblasts towards the cervical part of the tooth germ. Simultaneously, biglycan mRNA expression became more intense, being expressed throughout the new and mature odontoblasts. This change in the pattern of expression strongly supports the idea that they play different roles in tooth formation by regulating odontoblast differentiation, as we hypothesized.

The expression of decorin and biglycan mRNAs in odontoblasts has been previously reported. Hikake et al. reported changes in the expression of decorin and biglycan mRNAs in the odontoblasts of mouse molar tooth germs from E18.5 to P4.5. Although their findings partially correlate with the results presented here, they mainly focused on the co-expression of decorin/biglycan mRNA with OASIS mRNAs, without referring to the distinct expression patterns between decorin and biglycan. Matsuura et al. found that decorin and biglycan show distinct expression patterns, proposing that these molecules have different roles in odontoblast differentiation. Although we agree with their hypothesis, the expression patterns described in their study were slightly different from our own findings. Matsuura et al. demonstrated that biglycan mRNA is expressed in presecretory odontoblasts and is weak in secretory odontoblasts, whereas decorin mRNA is highly expressed in secretory odontoblasts. They used an upper first molar at E19.0 (newborn) as the dentin-secretory stage, with their results being mostly consistent with ours at E18.5. Furthermore, they used a lower incisor at E19.0 as the later stage (dentin/ameloblast secretory stage), obtaining the above results. We investigated changes in the temporal expression patterns in identical tooth germs (lower first molars), resulting in the expression patterns described above. The incisor results correlated with the fact that biglycan mRNA is expressed in mature odontoblasts at later stages.

The differentiation of dental papillae cells at the interface with the enamel organs results in the appearance of odontoblasts, which subsequently secrete predentin matrix. To assess the function of decorin and biglycan in tooth formation, Goldberg et al. analyzed both decorin-deficient and biglycan-deficient mice. They performed immunohistochemistry for decorin and biglycan in molar tooth germs of wild-type mice at P1.0; they described strong immunoreactivities for both molecules, and our in situ hybridization results confirmed that they are mainly secreted by odontoblasts.

Based on analyses of gene-knockout mice, Goldberg et al. suggested the involvement of decorin and biglycan in enamel formation; the former accelerates it, but the latter inhibits it. Our results indicate that the abnormality of enamel formation in their gene-knockout mice seems to have been due to an impairment of dentin/predentin formation, since we recognized no significant mRNA expression for either molecule in ameloblasts. In contrast to our results, Matsuura et al. reported biglycan mRNA expression in ameloblasts. While Matsuura et al. used digoxigenin-labeled probes for cryo-sections, we used RI-labeled probes for paraffin sections. As such, this discrepancy may be the result of the use of different probes and/or labeling methods. Since significant immunoreactivity was not detected in the enamel matrix or ameloblasts, we believe that ameloblasts synthesize and secrete only small amounts of SLRPs, including biglycan.

Goldberg et al. also demonstrated that biglycan, but not decorin, controls collagen fibrogenesis in predentin, and that decorin, to a lesser extent biglycan, promote dentin mineralization. The functions of decorin and biglycan in these phenomena are somewhat controversial according to previous studies. Some studies suggest that decorin regulates collagen fibrillogenesis by binding to many types of collagen, including types I, II, and III. In the absence of decorin, irregular collagen contours and diameters arise in various types of tissues, including the skin and tendons, as well as in the periodontal ligaments. An in vivo study indicated that decorin is a negative regulator of mineralization, but an in vitro study indicated that decorin had no effect on mineral accumulation. Decorin-deficient mice do not show osteoporosis-like phenotypes. Biglycan however has been suggested to be an accelerator of matrix mineralization, and biglycan-deficient mice show osteoporosis-like phenotypes and also have hypomineralized dentin.

Immunohistochemistry analysis showed both decorin and biglycan immunoreactivity in the predentin, although not in mineralized dentin. Similar results have been reported by previous studies in mouse molars, mouse incisors, and rat molars. These results also support the hypothesis that both molecules are involved in dentin formation in terms of predentin formation and/or its mineralization.

These complicated results may indicate discrepancies between in vitro and in vivo experiments or between dentin and bone. In any case, our histological study cannot confirm whether the proposed hypotheses are correct or not, but at least it indicates that predentin/dentin formation may progress via two steps: a decorin-prevailing initial step and a biglycan-prevailing subsequent step. It is well known that the two mechanisms achieve mineralization; the first involves matrix vesicles, and the second involves heterogeneous nucleation along with collagen fibrils, and this is used in dentin mineralization. The present results can classify the second step into further two steps. Further studies, however, are required to clarify this hypothesis while taking the relationship between decorin/biglycan and small integrin-binding ligand N-linked glycoproteins (SIBLINGs) into consideration, since SIBLINGs are known to be involved in dentin mineralization.

Furthermore, Goldberg et al. found that dentin hypomineralization observed in newborn mice with decorin and biglycan gene-knockout had undergone self-repair in adult mice, suggesting that decorin and biglycan may be involved in the initial process of mineralization, but that it is subsequently taken over by other molecules. In the present study, mRNA expression for both molecules was reduced at P7.0 despite dentin formation being a continuous process, which further supports this hypothesis.

Expression of decorin and biglycan mRNA/proteins in dental papilla/dental pulp

The present study showed that both decorin and biglycan mRNA were expressed in the dental papillae beneath the odontoblastic layers at the cusp regions, in line with their strong expression in odontoblasts. However, immunohistochemistry analyses indicated that only biglycan immunoreactivity was
consistently present in the dental papillae/pulp. Goldberg et al.25 did not report biglycan immunoreactivity in the dental papillae of wild-type mice at P1.0. This may be due to pretreatment of sections with hyaluronidase in the present study, since GAG chains of proteoglycans often block immunoreactivity of core proteins.44 At E18.5, biglycan was strongly expressed in the central region, but was weak in the peripheral region beneath the odontoblastic layer, indicating that the dental pulp cells beneath the odontoblastic layer may secrete biglycan protein into the central region.

The distribution patterns of biglycan immunoreactivity in the dental pulp were similar to those of versican46 or perlecan.17 Filling spaces for the development of organs appears to be an important function of large PGs, such as versican.44 Thus, biglycan may be related to the development of dental papilla/pulp together with other PGs. The region immediately below the odontoblast layer is known as the cell-rich zone.16 Cells in this zone are capable of differentiating into odontoblasts when the initial cells are damaged, and may be involved in the transport and reabsorption of fluid and ions from the pulp to odontoblastic layer.46,47 Thus, decorin and/or biglycan may also exert such functions. Future experimental studies that induce damage to the dental pulp and accelerate tissue repair may clarify this hypothesis.

**Protein synthesis analyzed by organ culture with metabolic labeling of [35S]**

In the present study, compared to CS-PGs, KS-PGs were found to be synthesized at low levels by tooth germs from E16.0 to P3.0. These results support the in situ hybridization results, wherein the expression of fibromodulin and lumican mRNA were not detected at any stage of the tooth germs examined. Significant amounts of HS-PGs, however, were synthesized by tooth germs at E18.0 and P3.0. Ida-Yonemochi el al.17 investigated the expression and synthesis of perlecan, a representative large HS-PG, and demonstrated its involvement in tooth formation. Furthermore, the expression of the syndecan family, which are principal cell-surface HS-PGs, has also been reported in the developing tooth germ.48,49 In situ hybridization results correlated with those reported by previous studies in which decorin, biglycan, and fibromodulin expression were not highly expressed in other epimysia of muscles in the craniofacial region (data not shown), indicating that the expression of these three SLRPs has muscle-specific variations.

To conclude, the present study suggests that SLRPs show different expression patterns in each stage of tooth development, suggesting that SLRPs may have their own tissue-specific roles. In particular, biglycan was found to be the most abundant molecule among the four SLRPs examined.

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