A Novel Role of Periostin in Postnatal Tooth Formation and Mineralization*

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Periostin plays multiple functions during development. Our previous work showed a critical role of this disulfide-linked cell adhesion protein in maintenance of periodontium integrity in response to occlusal load. In this study, we attempted to address whether this mechanical response molecule played a direct role in postnatal tooth development. Our key findings are 1) periostin is expressed in preodontoblasts, and odonto-blasts; and the periostin-null incisor displayed a massive increase in dentin formation after mastication; 2) periostin is also expressed in the ameloblast cells, and an enamel defect is identified in both the adult-null incisor and molar; 3) deletion of periostin leads to changes in expression profiles of many non-collagenous protein such as DSPP, DMP1, BSP, and OPN in incisor dentin; 4) the removal of a biting force leads to reduction of mineralization, which is partially prevented in periostin-null mice; and 6) both in vitro and in vivo data revealed a direct regulation of periostin by TGF-β1 in dentin formation. In conclusion, periostin plays a novel direct role in controlling postnatal tooth formation, which is required for the integrity of both enamel and dentin.

It is well known that mechanical loading stimulates new bone formation, whereas unloading or disuse of bone (e.g. longtime bed rest, spaceflight, or cast immobilization) accelerates bone resorption. This adaptation is critical for bone modeling and remodeling (1). The anabolic degree to which the bone responds to physical activity has being associated with the intensity and loading magnitude of the exercise. Under normal conditions, strenuous exercise such as weightlifting yields thicker and denser bone compared with jogging and swimming; the latter two types of exercise are less forceful and produce less mechanical stimulus (2). A well-designed study by Tatsumi et al. (3) demonstrated that osteocytes are the key sensor controlling both bone formation and bone resorption in the unloading animal model. The accumulated evidence supports a recommendation by the National Osteoporosis Foundation: regular weight-bearing and muscle strengthening exercise is an effective countermeasure to fight against osteoporosis, a silent bone loss in the elderly population worldwide.

The development and maintenance of the dental and periodontal structures are also directly influenced by mechanical stimuli (4). This stimulation can be the result of normal occlusal function or orthodontic treatment. It is well documented that during orthodontic treatment, tension on the side from which a tooth moves away results in the formation of new bone (i.e. osteogenic), while compression on the opposite site leads to bone resorption. This relationship seems contrary to the situation in the long bone where the loaded site is osteogenic and the unloaded site is resorptive (5). One of the interpretations regarding this difference between the alveolar bone and long bone is that the periodontal ligament (PDL), a soft tissue located between the teeth and alveolar bone, plays a unique role in this transformation, because the PDL contains osteogenic cells that are able to derive or generate bone cells and produce cementum (6). Melsen proposed that resorption at compression sites could be considered a consequence of lowering the normal strain from the periodontal ligament, whereas osteogenesis at tension sites could reflect loading of the periodontal ligament (7).

Enamel and dentin are highly mineralized components of a matured tooth. A great amount of research has been focused on determining the processes that initiate tooth development. Numerous genes have been found to be associated with human diseases affecting the integrity of teeth and the craniofacial region. In contrast, the mechanical effects on tooth structure and mineralization are largely unknown. Yet, early studies using the tooth trimmed incisor model (in which the lower incisor is repeatedly trimmed to prevent it from touching the upper incisor) showed rapid tooth growth as well as a reduction in dentin thickness, enamel maturation and mineralization (8–11).

In search of possible molecules that might be critical in the response of the periodontium to mechanical loading response, we studied the roles of periostin both in vitro and in vivo (12–13). Our previous studies showed that periostin, an adhesive molecule highly expressed in the PDL, is essential for...
the integrity and function of the periodontal ligament during occlusal loading; and that loss of this protein lead to defects predominantly in the PDL, followed by enamel and alveolar bone defects (12, 13). However, it is unknown that periostin has a direct role during postnatal tooth development.

The aims of this study were 1) to examine the expression pattern of periostin in the dentoalveolar complex; 2) to characterize the periostin-null tooth phenotype; 3) to determine the response differences of enamel and dentin to tooth trimming in wild type (WT) and periostin-null mice; and 4) to explore the regulation of periostin by mechanical loading/unloading or TGF-β1. The results in this study led us to conclude that masticatory force is essential for the maintenance of the tooth structures (enamel and dentin) and for mineralization in which periostin plays a vital direct role.

EXPERIMENTAL PROCEDURES

Mice Maintenance, Genotyping, and Occlusal Hypofunction
Model Preparation—All animal studies were conducted in accord with the guidelines of the Institutional Animal Care and Use Committee of Baylor College of Dentistry. The periostin−null mice were generated as described previously (13). Transgenic mice overexpressing TGF-β1 under the control of the dentin sialophosphoprotein gene were reported by Haruyama et al. (15). The TGF-β1-null mice were generated by Kulkarni et al. (16). Both periostin-null and wild type mice used for tooth trimming were maintained in a C57BL/6 background. Genotyping was determined by polymerase chain reaction (PCR) analysis of genomic DNA with primers p01: 5′-AGTGTGCAAGTTGTGCTTG-3′ and p02: 5′-ACGAAAATACAGTTGCTATCC-3′ to detect the wild-type allele (∼300 base pairs [bp]); and primers p01: 5′-AGTGTGCAAGTTGTGCTTG-3′ and primers p03: 59-CAGCGCATCAGCCTTCTATCG-39 to detect the targeted periostin allele (∼700 bp). To eliminate occlusal force on the lower incisors, the mandibular incisors were trimmed twice a week, starting at 4 weeks of age for 4 weeks, using surgical clippers (trimming 2–3 mm from the occlusal surface).

Extraction and Separation of Non-collagenous Proteins (NCPs) from Dentin—NCPs were extracted from 2-month-old incisor dentin obtained from 4 groups (WT control, WT trimming, KO control, and KO trimming; 4 animals a group) of mice by 4 M guanidinium chloride/0.5 M EDTA, as previously described (17). The extracted NCPs were separated by Q-Sepharose (Amersham Biosciences, Uppsala, Sweden) chromatography as previously described (17). The Q-Sepharose column separated dentin matrix NCPs into 120 0.5-ml fractions.

Detection of SIBLING Protein Members (18–20)—Each of the chromatographic fractions that potentially contained any of the four SIBLING family members was assayed by Stains-All staining and subsequently by Western immunoblotting. The chromatographic fractions were analyzed by sodium docyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Stains-All staining and Western immunoblotting. Stains-All staining was employed for detecting SIBLING family members in individual fraction from 120 fractions. For Western immunoblotting, we used four types of well-validated antibodies against the SIBLING family members. For detection of DMP1, anti-DMP1-C-857, an affinity-purified polyclonal antibody, which recognizes the C-terminal region of DMP1 were used at a dilution of 1:1000 (21). For detection of DSPP/DSP, an anti-DSP monoclonal antibody (anti-DSP-2G7.3) (22) was used at a dilution of 1:2000. For detection of BSP, an anti-BSP monoclonal antibody (anti-BSP-10D9.2) (23) was employed at a dilution of 1:2000. For detection of OPN, an anti-OPN monoclonal antibody (Santa Cruz Biotechnology) was used at a dilution of 1:2000. Blots were washed three times in phosphate-buffered saline (PBS) containing 0.3% Tween-20, followed by incubation in the alkaline phosphate-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma-Aldrich) at a dilution of 1:5000. Last, the blots were incubated with the chemiluminescent substrate CDP-star (Ambion, Austin, TX) for 5 min and exposed to x-ray films.

Histologic Preparation—The maxillary and mandibular specimens were isolated and immediately immersed in fixative (4% paraformaldehyde) overnight at 4 °C for 48 h. The tissues for in situ hybridization and H&E were decalcified with 15% EDTA solution (pH 7.4) at 4 °C for 3 weeks, then dehydrated and embedded in paraffin. The tissue blocks were cut into 4-μm thick mesio-distal serial sections and mounted on glass slides. The tissue pulp area was measured by Bioquant software.

Backscattered Scanning Electron Microscopy (SE) Imaging—The mandibles were dissected, fixed in 70% ethanol at room temperature for 24 h. The tissue specimens were dehydrated in ascending concentrations of ethanol (from 70 to 100%), embedded in methyl-methacrylate (MMA, Buehler, Lake Bluff, IL) without decalcification and sectioned through the center of the first molar using a water-cooled diamond-imregnated circular saw (Isomet, Buehler). The cut surface was polished using 1, 0.3, and 0.05 μm alumina alpha micropolish II solution (Buehler) in a soft cloth rotating wheel separately. Each sample was placed in an ultrasonic bath between steps and immediately following the polishing steps. The dehydrated specimens were then sputter coated with carbon and scanned with a backscatter electron detector using a FEI/Philips XL30 Field emission environmental scanning electron microscope. The parameters were kept constant while the BSE micrographs were taken.

Double Fluorochrome Labeling of the Teeth—To analyze the changes in the dentin apposition rate, double fluorescence labeling was performed as described previously with one minor modification (26). Briefly, a calcein label (5 mg/kg intraperitoneal; Sigma-Aldrich) was administered to 7-week-old mice, followed by injection of an Alizarin Red label (20 mg/kg intraperitoneal; Sigma-Aldrich) 7 days later. The mice were sacrificed 48 h after the second injection, and the mandibles were removed and fixed in 70% ethanol for 48 h. The specimens were dehydrated through a graded series of ethanol (70–100%) and embedded in MMA without decalcification. 50-μm sections were cut using a Leitz 1600 saw microtome. The unstained sections were viewed under epifluorescent illumination using a Nikon E800 microscope interfaced with the Bioquant software. The mean distance between the two fluorescent labels was determined and divided by the number of
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days between labels to calculate the apposition rate (μm/day). These analyses were performed on the lower incisors.

In Situ Hybridization—The digoxigenin (DIG)-labeled mouse cRNA probes of periostin (0.6 kb) (27) were prepared with the use of an RNA labeling kit (Roche, Indianapolis, IN). In situ hybridization on paraffin sections was carried out as described previously (28). The hybridization temperature was set at 55 °C and washing temperature at 70 °C so that the endogenous alkaline phosphatase (AP) would be inactivated. DIG-labeled nucleic acids were detected in an enzyme-linked immunoassay with a specific anti-DIG-AP antibody conjugate and an improved substrate that produces a red signal (Vector, Burlingame, CA) according to the manufacturer’s instructions.

Radiography—To measure changes in the incisors, radiographs were taken using a Faxitron model MX-20 Specimen Radiography System with a digital camera attached (Faxitron X-ray Corp., Lincolnshire, IL).

Cell Culture—Preodontoblast cell line was kindly provided by Dr. A. Poliard, France (29). The cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal bovine serum, penicillin G (100 IU/ml), and streptomycin (100 mg/ml), and incubated at 37 °C and 5% CO₂ in air. For the mineralization assay, 5 mM β-glycerophosphate and 50 mg/ml ascorbic acid were added to the medium after reaching confluence.

Application of Stress to the Preodontoblast—The preodontoblast cells were cultured on the flexible membrane base that was subjected to cyclic strain produced by a Flexercell FX-4000T (Flexercell International Inc., Hillsborough, NC), as described by Banes et al. (30). The cells were cultured (1 × 10⁵ cells/well) on flexible-bottomed culture plates for 24 h until they became subconfluent. They were then stretched (elongated) at six cycles/min and 8% elongation of the cell bodies for 24 h. Fresh medium was added every 24 h until the application of the mechanical stress. Non-stretched cells were used as controls.

Western Assay—For testing the effects of the recombinant periostin on AKT signaling, an odontoblast cell line (29) was grown to 70% confluency. The cells were serum-deprived for 16 h prior to recombinant periostin treatment. The cells were treated in triplicates with 100 ng/ml periostin, and harvested at different time points (5, 15, 30, 60 min) for AKT phosphorylation measurement. The concentration of cell lysates was determined by using a BCA kit (Pierce). 10 μg of total protein was subjected to 10% SDS-PAGE gel, followed by blotting onto polyvinylidene difluoride membranes and probing with the following antibodies: anti-phosphorylated-AKT or anti-non-phosphorylated-AKT according to the manufacturer’s protocol (R&D). A Western blot was performed using chemiluminescence (Perkin Elmer) of horseradish peroxidase (HRP) linked to a second antibody (Cells Signaling Inc.). The membranes were then exposed to X-Omat Nulldak film to visualize the bands.

Real-time Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)—To investigate the expression of periostin, TGF-β1 mRNA in preodontoblast cell lines and odontoblast cells isolated from TGF-β1 transgenic mice, total RNA was extracted using Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer’s recommended protocol. The cDNA was synthesized with the reverse transcription kit II (Invitrogen). SYBR green real-time RT-PCR was then performed. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using the Primer Express (version 2.0.0, Applied Biosystems). The primer sequences are listed in supplemental Table S1. Real-time PCR was performed using 1 μl of cDNA and 12.5 μl SYBR Green Master mix in a 25-μl reaction volume with ABI PRISM 7700 (Applied Biosystems Inc.). GAPDH was used as a housekeeping gene.

Assay of Alkaline Phosphatase Activity—After reaching confluence, cells in 24-well plates were rinsed three times with PBS, and 600 μl of distilled water were added to each well and sonicated. ALP activity was assayed by a method modified from that of Bowers et al. (31). In brief, the assay mixtures contained 0.1 x 2-amino-2-methyl-1-propanol, 1 mM MgCl₂, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates. After 4 min of incubation at 37 °C, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol. Each value was normalized to the protein concentration.

Mineralization Assay—Mineralization of the odontoblast cells was determined in 24-well plates using Alizarin red staining. The cells were fixed with ice-cold 70% ethanol/30% 1 mM Hepes and stained with Alizarin red to detect calcification. For quantification, cells stained with Alizarin red were destained with ice-cold 10 mM HCl/70% ethanol, and then the extracted stain was transferred to a 96-well plate. The absorbance at 550 nm was measured using a microplate reader.

RESULTS

Periostin Was Predominantly Expressed in the Subodontoblast Layer with a Low Level in the Ameloblast and Odontoblast Cell Layer—Molecules normally expressed in the PDL complex, such as cbfa-1, insulin-like growth factor-I, its receptor (IGF-IR), and insulin receptor substrate 1 are potential modulators constituting the PDL responses to mechanical loading. Periostin, an adhesive molecule that possesses the ability to organize the extracellular matrix in response to mechanical loading, is a potential modulator highly expressed in PDL. To address whether periostin is critical for postnatal tooth formation, we first studied the expression pattern of periostin in incisor using an in situ hybridization method. At the age of 4 weeks, periostin mRNA was expressed in a subodontoblast layer with a relatively low level of expression in both the ameloblast and odontoblast cells (Fig. 1a). At the age of 10 weeks, periostin was detected only in the subodontoblast cells at a similar level as that in the PDL (Fig. 1b). Note that periostin is undetectable in the molars at this stage, suggesting that periostin is a good marker to reflect the activity of preodontoblasts in the incisors which are active during postnatal development.

Periostin-null Mice Displayed an Enormous Increase in the Incisor Dentin Mass and the Worn-out Cusps of Molars—Previously we reported that periostin-null mice developed enamel abnormalities in the incisors only after mastication,
which was thought to be secondary to the PDL defects (13). In the present study, we re-examined the radiographs of the upper incisors from postnatal day 7 to 5 months (Fig. 2a). One of the key surprising findings was that the periostin-null dentin became thicker and thicker, and the null pulp space gradually became narrower and narrower with age (Fig. 2a–c, white arrows). The quantitative data showed approximately an 80% reduction of periostin-null pulp space compared with that of the age-matched control (Fig. 2d). Furthermore, both the photograph of the tooth (Fig. 2e, upper panel), and the radiographic images (Fig. 2e, lower panel) showed worn-out cusps of the 3-month-old lower null molars, suggesting a functional failure of the enamel structure upon occlusion. Note that there is no apparent defect in the periostin-null tooth before the weaning stage when mice start to masticate (data not shown).

To probe the effects of the absence of mechanical force on enamel and dentin, we prevented the left lower incisor from touching the upper incisor for 4 weeks using a tooth trimming method. We observed a number of changes related to the lack of biting force exerted. First, tooth trimming led to sharp reductions in enamel mineral content as reflected by the following observations: (a) The backscattered SE technique revealed a loss of white color in the enamel; this change in color (from white to dark) reflects a decrease in the mineralization level (Fig. 3a, right panels); (b) SE images of an acid-etching method to remove enamel rods (25) showed that there were empty spaces in the control where rods were previously located, indicating the formation of new enamel rods (Fig. 3b, left panel) but there was no sign of rod formation in the outer layer of the unloaded enamel (Fig. 3b, right panel). In addition, the space in the honeycomb-like structure was much smaller in the unloaded incisor.

Removal of Biting Force-induced Reduction of Mineralization Was Partially Prevented in Periostin-null Mice—The mouse incisor is a good model for studies of mechanical effects on tooth formation and mineralization, as the dentin and enamel forms continuously throughout the lifetime of the rodents. An alternative strategy of testing the potential role of periostin in postnatal tooth development is to determine whether there is a difference in mechanical responses between the periostin-null and the age-matched control mice in the absence of biting forces using a tooth trim method. It is known that there is a decrease in mineralization when incisor is repeatedly trimmed, which is thought to be lack of enough time for the enamel layer to mature (8, 10). A representative backscattered SE image showed that normal enamel rods, which have the highest mineral content in the body, were sharply reduced in the mineral content and the size of the WT enamel in the absence of occlusion (Fig. 3a, right panel) compared with the loading side in the same animal (Fig. 3a, left panel). In contrast, removal of occlusal forces by tooth trimming led to mild changes in both the mineral content and
the prism size in the periostin-null incisor (Fig. 3b, right panel). Similarly, the double-labeling data showed a reduction of the dentin formation rate in the trimmed WT incisor (Fig. 3, upper right panel) but an increase of the dentin formation rate in the trimmed KO incisor (Fig. 3c, lower right panel). The quantitative data showed that the reduction of the dentin formation rate in the trimmed WT incisor was significant (Fig. 3d). As a whole, this data showed that periostin-null mice were partially resistant to the dentin and enamel intrinsic structural changes induced by tooth trimming.

Deletion of Periostin Changed the Expression Profiles of SIBLING Proteins in Dentin—For a better understanding of the mechanism by which periostin controls dentin formation in occlusal and non-occlusal condition, we compared expression profiles of non-collagenous proteins by Stains All Staining, and the SIBLING protein (DSP, DMP1, OPN, and BSP) expression profiles by Western blotting. As shown in Fig 4a, non-collagen protein levels were lower overall in the periostin-null incisor (either the non-trimmed or the trimmed group). On the other hand, the expression profiles of SIBLING protein levels are totally different in the WT and the periostin-null incisor (Fig. 4b). DSP, the major SIBLING protein in dentin, was increased in the periostin-null mice compared with the WT incisor. DMP1 bands are largely undetectable by Stains-All assay but detected by Western-blot (see below). Western immunoblotting results. The control DSP (0.3 μg) was isolated from rat incisor dentin. The fraction 33 (rich of DSP) was used for DSP antibody staining using anti-DSP-2G7.3 antibody. The control DMP1 (1 μg) was extracted from rat long bone. The fraction 45 (rich of DMP1) was used for Western immunoblotting using the anti-DMP1 C-857 antibody. The control BSP (1 μg) was isolated from rat long bone. The fraction 54 (rich of BSP) was used for Western immunoblotting using the anti-BSP-10D9.2 antibody. The control OPN (1 μg) was isolated from rat long bone. The fraction 39 (rich of OPN) was used for Western immunoblotting using the anti-OPN antibody. Note there is no commercial antibody against DPP available. The above assays were repeated two times.
pared with the WT incisor. But the DMP1 level was decreased in the null mice. The protein levels of other 2 SIBLING members (BSP and OPN) were largely unchanged in the perisotin-null mice. Furthermore, the SIBLING protein expression profiles are different between the WT and the periostin-null groups in the absence of occlusion. DSP was increased in the WT-trimmed group, whereas its expression level was largely unchanged in the null group. DMP1 was reduced in both groups. Both BSP and OPN were either slightly increased (OPN) or largely unchanged (BSP) in the WT group. However, both protein levels were dramatically reduced in the periostin-null group. Taken together, deletion of periostin leads to dramatic changes of SIBLING protein expression profiles in both the control and the trimmed group, suggesting periostin regulates these proteins.

Periostin Negatively Regulates Odontoblast Cell Proliferation and Mineralization via the Akt Signaling Pathway—Next, we addressed the potential roles of periostin in odontoblast cell proliferation and mineralization in vitro. Recombinant periostin (100 or 500 ng/ml) was added into a preodontoblast cell line (29), and results displayed an inhibition of cell proliferation in a dose-dependent manner (Fig. 5a). To address whether periostin affects mineralization, a periostin expression vector (32) was transiently transfected into an odontoblast cell line, followed by culturing for an additional 10 days. Alizarin red stain showed that periostin inhibited mineralization (Fig. 5b). To further address the molecular mechanisms, recombinant periostin (100 or 500 ng/ml) was added into the same cell line, followed by a Western blot assay to measure the changes in Akt signaling with an antibody against the specific phosphorylation of Akt1/PKBα on Ser-473. As shown in Fig. 5c (right panel), a potent inhibition of periostin on Akt1 phosphorylation was observed at the time points of 5, 15, and 30 min after the addition of recombinant periostin protein.

Periostin Is Regulated by TGF-β1 in Dentinogenesis Both in Vitro and in Vivo—Recently, we showed that periostin responded to mechanical loading in a PDL cell line and that the periostin expression was regulated by TGF-β1, a potent growth factor for mechanical response (12) and enamel maturation (33). In this experiment, we showed that 1 day of stretching using Flex 4000 (leading to an 8% elongation of the cell bodies) reduced ~70% of periostin expression, whereas the same stretching force resulted in an ~2-fold increase in alkaline phosphatase in the preodontoblast cell line (Fig. 6a). We also showed that there was a dose-dependent increase of periostin mRNA by recombinant TGF-β1 in the same preodontoblast cell line in vitro (Fig. 6b). To investigate whether the regulation of periostin by TGF-β1 has a physiological sig-
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The current studies on periostin expressions in the ameloblast and subodontoblast layers, characterizations of periostin-null enamel and dentin phenotype, tooth trimming (unloading) of incisors, and mechanism investigations of the periostin roles and regulation reveal a key role of periostin in postnatal dentinogenesis and amelogenesis, which is responsible, in part, by mechanical loading.

Both odontoblasts in dentin and osteocytes in bone contain dendritic processes. To test if their dendrites share a common feature, we previously compared their cellular morphology as visualized using SE. Analysis of our data showed that both cells share an alike dendritic canicular system and express extensive processes forming a complex network within the mineralized matrix (1). We have also proposed a concept of “odontocyte” partly based on the structure similarity and the gene expression pattern (2). However, we had no direct evidence to prove this concept till the periostin-null work described in this work. The expression of periostin in the subodontoblast and odontoblast cells of the incisor was not expected, since the periostin-null mice display no apparent changes in molar crown dentin or in early incisor dentin (up to 4 weeks). Immediately after the null animal starts chewing food, there is a progressive increase in dentin accumulation in the periostin-null incisors (Fig. 2, a–d). This increase is correlated with an increase of DSP (a key dentin non-collagen protein, Fig. 4b). Furthermore, the dentin formation rate was significantly increased in the absence of mastication (the trimmed incisor) in the periostin-null mice, whereas the dentin formation rate was significantly reduced in the WT-trimmed incisor (Fig. 3c). These lines of evidence support an inhibitory role of periostin in postnatal dentin formation, which is closely linked to mechanical loading in vivo. We also demonstrated the inhibitory role of periostin in odontoblast cells in vitro, including 1) the recombinant periostin inhibited cell proliferation and mineralization in an odontoblast cell line (Fig. 5, a and b); and 2) addition of recombinant periostin sharply reduced the phosphorylation of Akt signaling (Fig. 5c).

In parallel, we documented that periostin is able to respond to the mechanical stimulus in vitro. Our data clearly showed that the mechanical challenging force greatly inhibited the periostin expression in the odontoblast cell line, whereas the same force increased ALP expression (Fig. 6a), supporting the specificity of this reaction. Furthermore, we showed that TGF-β1, a potent growth factor for mechanical response, regulated periostin expression in a dose-dependent manner (Fig. 6b). Importantly, we demonstrated that the periostin level was 3-fold higher in a transgenic mouse line where TGF-β1 both the ameloblast and odontoblast cells (14, 35–37). Conversely, the periostin level was inhibited by more than 80% in the TGF-β1-null mice (Fig. 6, c and d). These data support the role of periostin in the mechanical response in the tooth (see Fig. 7 for detail), which is in agreement with its role in the control of cell integrity and the mechanical response in periodontal ligament cells (12, 13).

We previously reported enamel defects in the periostin-null incisors and attributed this defect to a secondary effect of the loss of integrity in the PDL cells (12, 13). Identification of the expression of periostin in the ameloblasts (Fig. 1) and documentation of the worn-out cusps in periostin-null molars (Fig. 2c) support the direct role of periostin in maintaining enamel integrity. However, this direct role is relatively mild in comparison with its role in dentin where there is a striking increase in dentin mass when periostin is deleted (Fig. 2), as both light and electronic microscopic images did not reveal an apparent difference in morphology between the control and the periostin-null enamel (data not shown). Currently, we

![Figure 6](image-url)

**Figure 6.** Periostin, a mechanical responsible molecule, was regulated by TGF-β1 both in vitro and in vivo. a, one day stretching using Flex 4000 significantly reduced periostin expression resulting in an ~2-fold increase in alkaline phosphatase in the odontoblast cell line (*, *p < 0.05; **, *p < 0.01; n = 4). b, periostin mRNA expression was induced by recombinant TGF-β1 (2.5, 10, and 20 ng/ml) in a dose-dependent manner in the predentoblast cell line in vitro. c, whole tooth mRNAs isolated from 1-month-old transgenic (Tg) mice overexpressing TGF-β1 in the odontoblasts were used for quantitative analyses of periostin expression by the real-time RT-PCR method. The increase was ~3-fold compared with the age-matched control mice. d, in contrast, the periostin mRNA level in the 2-week-old TGF-β1-null incisors were isolated for quantitative comparison with the WT controls, which showed a ~75% reduction with a P value less than 0.01 (n = 4).
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Periostin, expressed in both preodontoblast (high) and odontoblast, is negatively responsible for mechanical stimulation either through TGFβ or other molecules. As a negative control molecule, periostin inhibits cell proliferation and differentiation partly via Akt signaling. We do not know exactly how periostin works in amelogenesis and only speculate that periostin plays a negative role.

In summary, we identified periostin expressions in ameloblast, subodontoblast, and odontoblast cells. An enormous increase of dentin mass in periostin-null incisor plus defects of enamel in these null molars support its direct role in modulating postnatal tooth formation. Our data also document a decrease of dentin mass in periostin-null incisors plus defects of enamel in these null molars support its direct role in modulating postnatal tooth formation. A future goal will be to clarify the mechanism by which periostin functions in controlling the postnatal tooth response to mechanical force.

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