

Figure 2. Potential approach to regeneration therapy for periodontal disease. By using cell sheet-engineering, stem cells obtained from the periodontal ligament (PDL) or dental follicle (DF) of wisdom tooth germ are harvested as temperature sensitive sheets for transplantation into periodontal tissue damaged by periodontal disease (upper panel). Stem cell sheets are also applied to dental implants accompanied by a bioengineered PDL that can recover the loss of periodontal tissue including the PDL, cementum and alveolar bone (lower panel).

the efficacy of FGF-2 in stimulating the regeneration of periodontal tissue. These findings collectively suggest that cytokine therapy has great clinical potential for achieving the partial regeneration of periodontal tissue.

4. Novel approaches to periodontal tissue regeneration using ECM administration therapy

ECM components organized in the PDL not only reflect the functional requirements of this matrix such as mechanical stress and storage of signaling molecules, but also regulate the tissue framework during development and regeneration [21]. Diseases affecting ECM function such as MFS have been shown to increase the susceptibility to severe periodontal disease due to a dysfunction of the PDL through a microfibril insufficiency, suggesting that fibrillin-1 microfibril formation plays a central role in PDL formation [68-74]. In addition, a new therapeutic concept has proposed that a fibrillin-1 microfibril insufficiency can be corrected by the administration of ECM components [23].

4.1 Periodontal disease and MFS

MFS is a severe, systemic disorder of connective tissue formation and can lead to aortic aneurysms, ocular lens dislocation, emphysema, bone overgrowth and severe periodontal

disease [68,75,76]. MFS has an estimated prevalence of 1 in 5000 – 10,000 individuals [77]. Fibrillin-1 comprises one of the major insoluble ECM components in connective tissue microfibrils which provides limited elasticity to tissues and stores cytokines such as TGF- β [78,79] (Figure 3A). Various mouse models of MFS have now been established via gene targeting or missense mutations in which germline mutations in *fibrillin-1* lead to progressive connective tissue destruction due to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation [72,74,75]. Hence, it is largely accepted that MFS is caused by insufficient fibrillin-1 microfibril formation in various connective tissues [76]. The study of PDL provides a useful experimental model not only for investigating the molecular pathogenesis of MFS, but also for evaluating novel therapeutic strategies for the improvement of microfibril disorders. This is because the principal elastic fiber system of the PDL, the oxytalan fiber, is composed of fibrillin-1 microfibrils and does not contain significant amounts of elastin [80-82]. Indeed, an abnormal PDL in association with progressive destruction of microfibrils is an obvious phenotype in the MFS mouse model [23]. Hence, PDLs will likely be more susceptible to breakdown in MFS compared with other elastic tissues composed of both elastin and fibrillin-1 (Figure 3B).

A structural insufficiency of fibrillin-1 microfibrils arises in MFS and leads to activation of TGF- β and its regulatory targets

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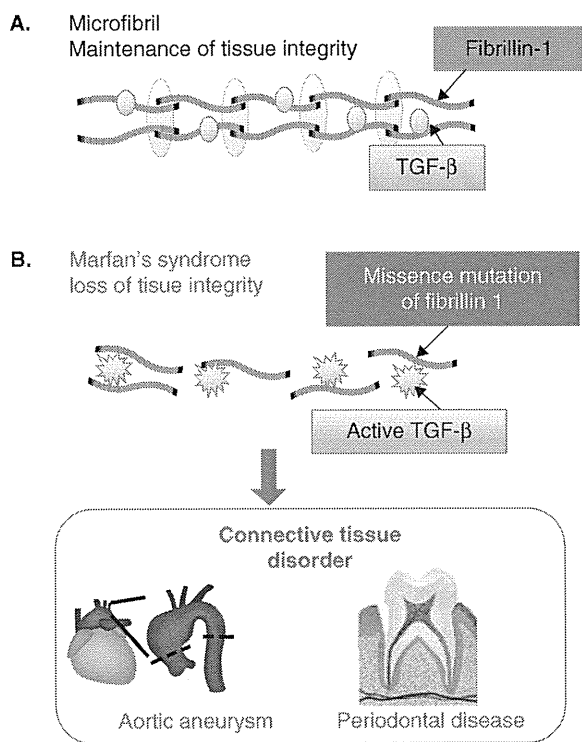


Figure 3. Schematic representation of the pathogenic mechanisms of MFS. A. Fibrillin-1 comprises insoluble extracellular matrix components in connective tissue microfibrils and provides limited elasticity to tissues through fibrillin-1 microfibril formation. B. Missense mutations in the *fibrillin-1* gene lead to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation and the pathogenic activation of TGF- β . These abnormalities cause progressive connective tissue destruction including aortic root aneurysms that are life-threatening and severe periodontal disease.

(Figure 3B) [73]. Recently, deregulation of TGF- β activation has been shown to contribute to pathogenesis and systemic antagonism of TGF- β signaling has been observed to have a beneficial effect on MFS symptoms including alveolar septation and muscle hypoplasia [72]. These observations have indicated that the microfibril network plays an important role in not only PDL function but also in the recovery of periodontal tissue integrity and prevention of the pathogenic activation of TGF- β caused by the fibrillin insufficiency that arises in MFS. However, molecular mechanisms governing fibrillin-1 assembly during organogenesis have been hampered because of unanswered issue of the actual factor that drives microfibril assembly.

4.2 Administration of ADAMTSL6 β serves as a microfibril therapy for repair of the PDL in an MFS mouse model

A disintegrin-like metalloprotease domain with thrombospondin type I motifs (ADAMTS)-like, ADAMTSL, is a subgroup of the ADAMTS superfamily and its members share particular

protein domains with the ADAMTS protease, including thrombospondin type I repeats, a cysteine-rich domain and an ADAMTS spacer, but lack the catalytic and disintegrin-like domains. Among the novel ADAMTSL family molecules, ADAMTSL6 β is essential for the development and regeneration of the PDL [23]. ADAMTSL6 β was recently found to associate with fibrillin-1 microfibrils through its direct interaction with the N-terminal region of fibrillin-1, and thereby promote fibrillin-1 matrix assembly both *in vitro* and *in vivo* [24]. Another study has indicated that fibronectin is an essential component during the assembly of fibrillin-1 through its interaction with the C-terminal region of fibrillin-1, thus suggesting the potential for improved microfibril assembly through the regulation of fibrillin-1-associated proteins including ADAMTSL6 β [83,84]. In an animal model of MFS microfibril disorder [85], ADAMTSL6 β expression can rescue fibrillin-1 microfibril formation through the promotion of fibrillin-1 microfibril assembly (Figure 4A). More importantly, the local administration of ADAMTSL6 β was found to be highly effective in accelerating the wound healing of periodontal tissues through the restoration of microfibrils (Figure 4B). Further evidence for the impact of ADAMTSL6 β on microfibril assembly is its suppression of TGF- β signaling, a pathway which is known to contribute to elastolysis in MFS.

These findings have demonstrated that microfibril assembly induced by ADAMTSL6 β is essential not only for fibrillin-1 microfibril restoration but also for the inhibition of the pathological activation of TGF- β . Thus, ECM administration therapy such as microfibril assembly could form the basis of a novel therapeutic approach to PDL regeneration and the treatment of periodontal disease in MFS patients.

5. Conclusions

Regenerative therapies for periodontal disease that use the cells of the patient to repair the periodontal defect have been proposed in a number of studies [86-88]. PDL-derived stem cells such as PDLSCs can differentiate into all of the periodontal lineages that contribute to cell turnover in the steady-state and would thus be useful cell sources for regenerative therapies to treat periodontal disease following tissue injury [89-91]. Treatments that partially regenerate damaged PDLs through the local application of cytokines have now been established, and such regenerative therapies have provided a very useful and feasible clinical study model for the future design of stem cell and cytokine therapies [15,61,92]. Although partial regeneration of the periodontal tissue has been established, methods to achieve the functional regeneration of large defects caused by severe periodontal disease are still lacking. To address this, it is essential to better understand the molecular mechanisms underlying PDL development and to thereby identify the appropriate functional molecules that induce the differentiation of stem cells into periodontal lineage cells for the successful reconstruction of periodontal tissue [31,32]. Investigations of the molecular mechanisms of fibrillin-1 microfibril assembly via ADAMTSL6 β during

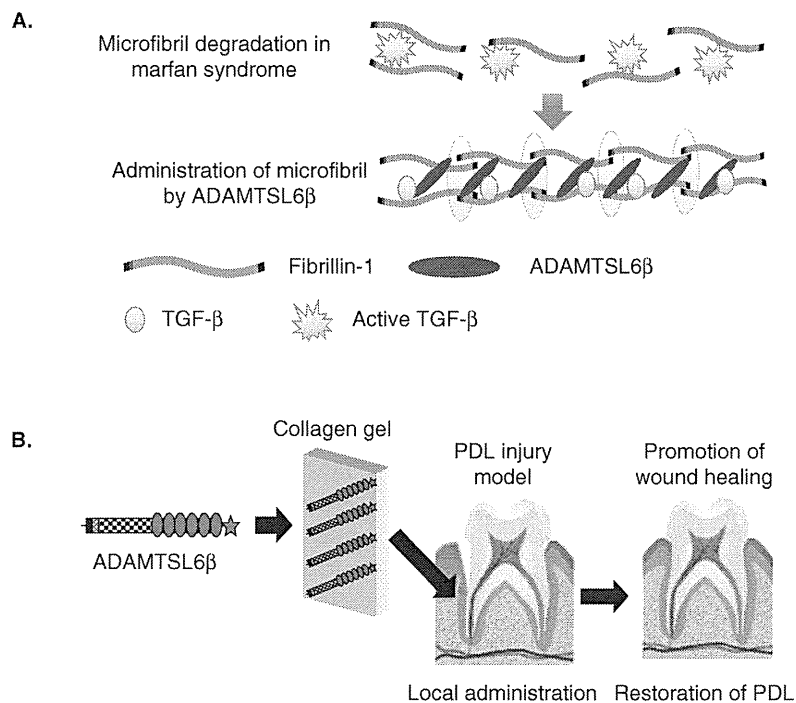


Figure 4. Microfibril administration by administration of ADAMTSL6β. A. Administration of microfibrils by ADAMTSL6β An ECM administration therapy that induces restoration of properly formed microfibrils via ADAMTSL6β is essential not only for improvement of the microfibril disorder, which is a predominant symptom of MFS, but also for the suppression of excessive TGF-β signaling induced by microfibril disassembly. B. ADAMTSL6β promotes wound healing of the PDL. A collagen gel containing recombinant ADAMTSL6β is prepared and locally administrated to the injured PDL of Marfan's syndrome mice established via the gene targeting of *fibrillin-1*. In this model, recombinant ADAMTSL6β restores fibrillin-1 microfibril assembly and enhances wound healing.

PDL formation will make substantial contributions to this endeavor [23]. In addition, since microfibrils play an important role in maintaining connective tissue integrity, including the aorta, lung and skin, we are hopeful the ECM administration therapy will in the future encourage the development of PDL regeneration for the treatment of periodontal disease as well as connective tissue disorders such as MFS [75,77].

6. Expert opinion

As described above, the partial regeneration of connective tissue damaged by pathological microflora has been achieved by regeneration therapy using stem cell transplantation and the local application of cytokines. Identification of the stem cells in the PDL or DF has enabled the development of protocols to regenerate the PDL and these have proved to be useful model systems for the development of connective tissue regeneration therapies [15,17]. One of the major research obstacles in PDL regeneration studies is the identification of all of the key functional molecules that drive PDL development. The establishment of ECM administration therapy such as fibrillin-1 microfibril

assembly is ultimately critical for the development of new therapeutic approaches for periodontal disease and MFS [76]. MFS fibrillinopathies have been explained by the structural insufficiency of fibrillin-1 microfibrils leading to the activation of TGF-β and its regulatory targets [93]. A variety of MFS therapies have been developed to date, including surgical therapy for aortic root aneurysms that are life-threatening [76], traditional medical therapies such as β-adrenergic receptor blockade for slow aortic growth and to decrease the risk of aortic dissection, and novel approaches based on new insights such as the pathogenesis of insufficient fibrillin-1 microfibril formation and the deregulation of TGF-β activation [77]. In the case of periodontal disease in MFS, surgical therapy or regeneration therapy is performed using stem cells or cytokines to recover damaged periodontal tissue (Figure 5, left panel).

In contrast to these approaches, the administration of ADAMTSL6β to fibrillin-1 microfibrils may represent a new ECM administration therapy which is viable for the treatment of the periodontal disease of MFS [23]. The evidence indicates that ADAMTSL6β is capable of enhancing microfibrils even in the case of a fibrillin-1 haploinsufficiency. Hence, ECM

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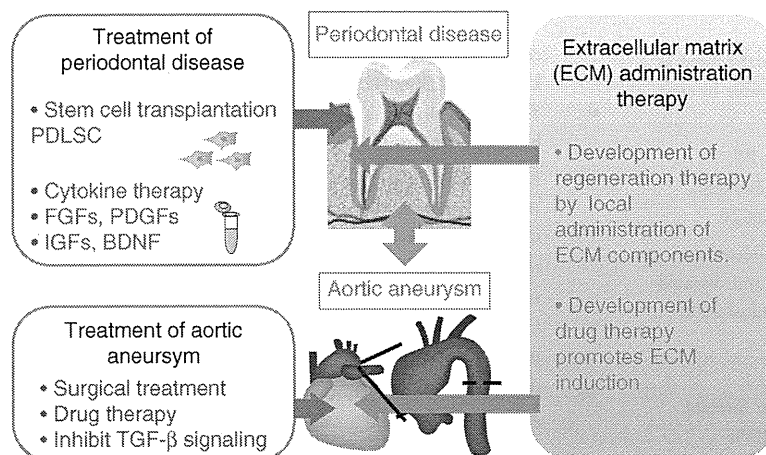


Figure 5. Extracellular matrix (ECM) administration therapy as a novel therapeutic strategy for MFS syndrome. Left panel: A variety of MFS therapies have been developed, including surgical therapy for aortic root aneurysms, traditional medical therapies and mechanisms to deregulate TGF- β activation and thereby decrease the risk of aortic dissection. In the case of periodontal disease, regeneration therapies including stem cell transplantation and cytokine therapy are being performed for the treatment of periodontal disease. Right panel: ECM administration therapy such as ADAMTSL6 β administration which induces microfibril assembly should be considered in the development of future mechanism-based therapeutics for the improvement of periodontal disease in MFS. It will also be beneficial to develop drug therapies that promote ADAMTSL6 β expression for the treatment of aortic aneurysms.

administration therapy through the promotion of microfibril assembly by ADAMTSL6 β may have potentially novel therapeutic benefits for the treatment of periodontal disease and disorders associated with MFS (Figure 5, right panel).

In conclusion, we here introduce the concept that a fibrillin-1-associated protein such as ADAMTSL6 β , which induces microfibril assembly, should be considered as an ECM administration agent for the treatment of periodontal disease and improvement of connective tissue disorders such as MFS. The exogenous application of recombinant ADAMTSL6 β improves fibrillin-1 microfibril assembly, indicating that the reinforcement of fibrillin-1 microfibrils by ADAMTSL6 β may represent a new treatment for periodontal disease which is accessible from oral cavity in MFS patients. Since elastolysis occurs continuously in aortic aneurysms arising in MFS cases, the chronic administration of ADAMTSL6 β may be required for

the stabilization of microfibrils to prevent progressive tissue destruction. It will also be necessary to develop methodologies for the systemic administration of ADAMTSL6 β to induce fibrillin-1 microfibril assembly in connective tissue for the treatment of life-threatening conditions such as an aortic aneurysm (Figure 5, right panel). Hence, an ECM administration therapy involving ADAMTSL6 β has the capacity to facilitate drug discovery for treating periodontal diseases, and MFS-associated disorders.

Declaration of interest

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ADAMTSL6 β Protein Rescues Fibrillin-1 Microfibril Disorder in a Marfan Syndrome Mouse Model through the Promotion of Fibrillin-1 Assembly^{*S}

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Background: The pathology of Marfan syndrome is caused by insufficient fibrillin-1 microfibril formation in connective tissues.

Results: Successful improvement of Marfan syndrome manifestations are induced by the direct administration of recombinant ADAMTSL6 β .

Conclusion: This study demonstrated critical importance of microfibril regeneration in preventing Marfan syndrome.

Significance: Our current data support a new concept that the regeneration of microfibrils using ADAMTSL6 β is essential for improving Marfan syndrome.

Marfan syndrome (MFS) is a systemic disorder of the connective tissues caused by insufficient fibrillin-1 microfibril formation and can cause cardiac complications, emphysema, ocular lens dislocation, and severe periodontal disease. ADAMTSL6 β (A disintegrin-like metalloprotease domain with thrombospondin type I motifs-like 6 β) is a microfibril-associated extracellular matrix protein expressed in various connective tissues that has been implicated in fibrillin-1 microfibril assembly. We here report that ADAMTSL6 β plays an essential role in the development and regeneration of connective tissues. ADAMTSL6 β expression rescues microfibril disorder after periodontal ligament injury in an MFS mouse model through the promotion of fibrillin-1 microfibril assembly. In addition, improved fibrillin-1 assembly in MFS mice following the administration of ADAMTSL6 β attenuates the overactivation of TGF- β signals associated with the increased release of active TGF- β from disrupted fibrillin-1 microfibrils within periodontal ligaments. Our current data thus demonstrate the essential contribution of ADAMTSL6 β to fibrillin-1 microfibril formation. These findings also suggest a new therapeutic strategy for the treatment of

MFS through ADAMTSL6 β -mediated fibrillin-1 microfibril assembly.

Marfan syndrome (MFS)² is a severe, systemic disorder of connective tissue formation and can lead to aortic aneurysms, ocular lens dislocation, emphysema, bone overgrowth, and severe periodontal disease (1–3). MFS has an estimated prevalence of 1 in 5,000–10,000 individuals (3, 4). Fibrillin-1 comprises one of the major insoluble extracellular matrix components in connective tissue microfibrils and provides limited elasticity to tissues through fibrillin-1 microfibril formation (5, 6). Various mouse models of MFS have been established via gene targeting or missense mutations, with germ line mutations in fibrillin-1 leading to progressive connective tissue destruction due to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation (7–10). Hence, it is largely accepted that MFS is caused by insufficient fibrillin-1 microfibril formation in various connective tissues (11, 12).

A variety of MFS therapies have been developed, including surgical therapy for aortic root aneurysms that are life-threatening (12), traditional medical therapies, such as β -adrenergic receptor blockade, for slow aortic growth and to decrease the risk of aortic dissection, and novel approaches based on new insights, such as the pathogenesis of insufficient fibrillin-1

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² The abbreviations used are: MFS, Marfan syndrome; PDL, periodontal ligament; En, embryonic day *n*; Pn, postnatal day *n*; MHPDL, MFS periodontal ligament; HPDL, human periodontal ligament; DF, dental follicle.

microfibril formation and deregulation of TGF- β activation (2). It has been demonstrated also that deregulation of TGF- β activation contributes to MFS pathogenesis and that matrix sequestration of TGF- β is critical for the regulated activation and signaling of the extracellular fibrillin-1 microfibrils of connective tissues (8). These observations predict that the clinical features of MFS-like manifestations are caused by alterations in TGF- β signaling networks (13). Loeys-Dietz syndrome, which is caused by heterozygous mutations in the genes encoding TGF- β receptors 1 and 2, is another autosomal dominant disorder with MFS-like manifestations, such as aortic root aneurysms, aneurysms and dissections throughout the arterial tree, and generalized arterial tortuosity (4). Importantly, systemic antagonism of TGF- β signaling through the administration of a TGF- β -neutralizing antibody or losartan, an angiotensin II type 1 receptor blocker, has been shown to have a beneficial effect on alveolar septation and muscle hypoplasia (8, 10). These observations provide a proof of principle for the use of TGF- β antagonism as a general therapeutic strategy for MFS and other disorders of the TGF- β signaling network. However, another potential therapeutic strategy that remains to be investigated is the reconstruction of the microfibril in connective tissues through the expression or administration of a microfibril-associated molecule that regulates or stabilizes fibrillin-1 microfibril formation. To investigate this concept, it will be necessary to identify molecular mechanisms of microfibril formation and an appropriate fibrillin-1 microfibril-associated molecule.

ADAMTSL (A disintegrin-like metalloprotease domain with thrombospondin type I motifs-like) is a subgroup of the ADAMTS superfamily that shares particular protein domains with the ADAMTS protease, including thrombospondin type I repeats, a cysteine-rich domain, and an ADAMTS spacer, but lacks the catalytic and disintegrin-like domains (14). A recent study has demonstrated that ADAMTSL2 mutations cause geleophysic dysplasia, an autosomal recessive disorder similar to MFS, through the dysregulation of TGF- β signaling (15). A homozygous mutation in ADAMTSL4 also causes autosomal recessive isolated ectopia lentis, another disease similar to MFS that is characterized by the subluxation of the lens as a result of disruption of the zonular fibers (16). The novel ADAMTSL family molecules ADAMTSL6 α and -6 β were recently identified by *in silico* screening for novel ECM proteins produced from a mouse full-length cDNA data base (FANTOM). These proteins are localized in connective tissues, including the skin, aorta, and perichondrocytes. Among the ADAMTSL6 family, ADAMTSL6 β has been shown to associate with fibrillin-1 microfibrils through its direct interaction with the N-terminal region of fibrillin-1 and thereby promotes fibrillin-1 matrix assembly *in vitro* and *in vivo* (17). These findings suggest a potential clinical application of ADAMTSL6 β as a novel MFS therapy by promoting fibrillin-1 microfibril assembly and regulating TGF- β activation.

In our current study, we report that ADAMSL6 β is essential for the development and regeneration of the connective tissue periodontal ligament (PDL), a tooth-supporting tissue located between the root and alveolar bone that is morphologically similar to the ligament tissue that is capable of withstanding mechanical force. Using mgR/mgR mice as an animal model of

MFS microfibril disorder, we demonstrate that ADAMSL6 β expression can rescue fibrillin-1 microfibril formation through the promotion of fibrillin-1 microfibril assembly. PDL provides a useful experimental model not only for investigating the molecular pathogenesis of MFS but also for evaluating novel therapeutic strategies for the improvement of microfibril disorders. This is because the principal elastic fiber system of PDL is composed of fibrillin-1 microfibrils and does not contain significant amounts of elastin (18–20). This composition also suggests that PDL will have an increased susceptibility to breakdown in MFS compared with other elastic tissues composed of both elastin and fibrillin-1. Furthermore, the restoration of fibrillin-1 assembly following administration of recombinant ADAMTSL6 β regulates the overactivation of TGF- β signaling, which is associated with an increased release of active TGF- β from disrupted fibrillin-1 microfibrils. The results of our present study demonstrate for the first time that ADAMTSL6 β is essential for fibrillin-1 microfibril formation and suggest a novel therapeutic approach to the treatment of MFS through the promotion of ADAMTSL6 β -mediated fibrillin-1 microfibril assembly.

EXPERIMENTAL PROCEDURES

Animals—C57BL/6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). mgR/mgR mice were generously provided by Dr. Francesco Ramirez (Mount Sinai Medical Center, New York). All mouse care and handling conformed to the National Institutes of Health guidelines for animal research. All experimental protocols were approved by the Tokyo University of Science Animal Care and Use Committee.

Histochemical Analysis—Frontal sections of C57BL mouse heads at embryonic day 13 (E13), E15, E17, and postnatal day 1 (P1) were prepared as described above. Fresh frozen sections of P7 and P35 mice were prepared using the Kawamoto tape method, according to the manufacturer's instructions (Leica Microsystems, Tokyo, Japan) (21), and 10- μ m sagittal sections were generated. Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA. The primary antibody used was an anti-Adamtsl6 polyclonal antibody (R1–1) (17), anti-fibrillin-1 polyclonal antibody (pAB9543), anti-FIBRILLIN-1 monoclonal antibody (clone 69, Chemicon, Temecula, CA), and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich). The secondary antibodies used were Alexa 488 or Alexa 555 anti-rabbit or anti-mouse IgG (Invitrogen), followed by nuclear staining with DAPI. An anti-Adamtsl6 polyclonal antibody was labeled with Alexa 488 by using the Zenon antibody labeling kit according to the manufacturer's instructions (Invitrogen) for double immunostaining with an anti-fibrillin-1 polyclonal antibody. For visualization of oxytalan fibers, sections were oxidized for 15 min in 10% Oxone (Merck) and subsequently stained with aldehyde fuchsin as described previously (20). Fluorescence images were sequentially collected using a confocal microscope featuring 403-, 488-, and 543-nm laser lines (LSM510; Carl Zeiss MicroImaging, Jena, Germany). The *in situ* hybridization methodology and probe design are described in the supporting information.

ADAMTSL6 β cDNA—As described previously (17), ADAMTSL6 β or *Adamtsl6 β* was cloned into p3XFLAG-

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CMV-14 to generate p3XFLAG-CMV-ADAMTSL6 β . The coding sequence of the cDNA was confirmed to be identical to the published sequence. The ADAMTSL6 β coding sequence containing the Kozak consensus sequence and tagged with the FLAG epitope at its C terminus end was then subcloned into the pcDNA4 expression vector (Invitrogen) or into the pDONR221 vector via a BP reaction (Invitrogen) to generate adenovirus or lentivirus, respectively.

Generation of Adenovirus—Recombinant adenovirus was constructed by homologous recombination between the expression cosmid cassette (pAxCawt) and the parental virus genome in 293 cells (Riken, Tsukuba, Japan) as described previously (22) using an adenovirus construction kit (Takara, Ohtsu, Japan).

Generation of Lentivirus—Recombinant lentivirus carrying ADAMTSL6 β was constructed via the recombination of pDONR221-containing ADAMTSL6 β segments into CSII-CMV-RfA using a LR reaction to generate CSII-CMV-ADAMTSL6 β . CSII-CMV-RfA was kindly provided by Dr. Hiroyuki Miyoshi (Riken, Tsukuba, Japan). Lentiviruses were produced essentially as described previously (23). Next, a 500- μ l aliquot of producer cell culture fluid was added to human periodontal ligament (HPDL) (passage 7) or MFS periodontal ligament (MHPDL) (passage 7) cells in the presence of Polybrene (7.5 μ g/ml). Stably transduced cells were maintained in the medium described above.

For knockdown experiments, miRNA expression vectors were constructed according to the manufacturer's protocol (Invitrogen). Two sets of Adamtsl6 β miRNAs to target sense (5'-TGCTGAATAACAGGTAGCTGACAAACGTTTTGGC-CACTGACTGACGTTTGTTCATACCTGTTATT-3') and antisense (5'-CCTGAATAACAGGTATGACAAACGTCAGTCAGTGGCCAAAACGTTTGTTCAGCTACCTGTTATT-3') transcripts were used to generate lentiviruses for the knockdown of Adamtsl6 β . A control miRNA was purchased from Invitrogen.

Infection of Developing Tooth Germ with Adenovirus—To investigate the effects of Adamtsl6 β on PDL formation in mgR/mgR mice, developing tooth germs were dissected from E14.5 mgR/mgR mouse embryos as described above and then infected with adenovirus that had been concentrated using the Adeno-X Maxi purification kit (Clontech) at 4 °C for 48 h in accordance with the manufacturer's recommendations. The adenovirus-infected tooth germs were then further incubated at 37 °C for 6 days in an *in vitro* organ culture as described previously (24).

Expression and Purification of Recombinant Adamtsl6 β —The expression and purification of recombinant Adamtsl6 β was performed using 293F cells (Invitrogen) and nickel-agarose (Qiagen, Hilden, Germany) as described previously (17). Briefly, pSecTag2A containing an Adamtsl6 β segment fused with Myc and His tags at its C terminus was transfected into 293F cells, which were cultured for 3 days. Conditioned medium was applied to a nickel-agarose column for the purification of recombinant Adamtsl6 β . The purified protein was dialyzed against PBS and stored at -80 °C.

Tissue Culture and *In Vitro* Microfibril Assembly Assay—The establishment of immortalized human periodontal cells and

MHPDL cells has been described previously (25). Cells were incubated with α -minimum essential medium (Sigma) containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 50 μ g/ml ascorbic acid, and 100 units/ml streptomycin and penicillin in a humidified atmosphere of 5% CO₂ at 37 °C. HPDL or MHPDL cells were plated onto 12-mm-thick coverglass coated with poly-L-Lys (Iwaki, Tokyo, Japan) placed in 24-well plates at 6×10^4 cells/well and incubated for 14 days. For the addition of purified recombinant mouse Adamtsl6 β , C-terminal histidine-tagged mouse Adamtsl6 β was prepared as described previously (17). Adamtsl6 β protein (10, 5, 2.5, 1.25, or 0.625 μ g) and the cells were incubated for 3 days. The cells were fixed with 4% paraformaldehyde and immunostained as described above.

RNA Preparation and Real-time RT-PCR—Total RNA was isolated from cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) as described previously (26). cDNAs were synthesized from 1- μ g aliquots of total RNA in a 20- μ l reaction containing 10 \times reaction buffer, 1 mM dNTP mixture, 1 unit/ μ l RNase inhibitor, 0.25 unit/ μ l reverse transcriptase (M-MLV reverse transcriptase; Invitrogen), and 0.125 μ M random 9-mers (Takara, Tokyo, Japan). The mRNA expression levels were determined using Power SYBR[®] Green PCR Master Mix (Applied Biosystems), and products were analyzed with an AB 7300 real-time PCR system (Applied Biosystems). Specific primers for human fibrillin-1 (forward, 5'-AATGAGCTGAATGGCTGTTACAA-3'; reverse, 5'-ACCATATGCTATATATTCTTCGATAACAAT-3'), mouse fibrillin-1 (forward, 5'-AAGGGGTTAATGTCATGATGTCAC-3'; reverse, 5'-CCACACAAGAACATAAAACCAAGG-3'), and mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward, 5'-ACTGAGCAAGAGAGGCCCTATCC-3'; reverse, 5'-CCTAGGCCCTCTCTGTTATTATGG-3') were used for real-time PCR. The primers for human *GAPDH* have been described previously (27).

Pull-down Assay—Pull-down assays to demonstrate direct interactions between Adamtsl6 β and TGF- β 1 proteins were performed as described previously by Nakajima *et al.* (28). Briefly, purified recombinant mouse Adamtsl6 β (5 μ g) was incubated with 0.1 μ g of recombinant TGF- β 1 proteins (Wako, Osaka, Japan) for 1 h at 4 °C in 0.3 ml of binding buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100). We next added 12.5 μ l of nickel-magnet (Promega, Madison, WI) to the reactions and incubated them for 30 min at 25 °C. The precipitates were washed three times with binding buffer, eluted by 250 mM imidazole, and subjected to SDS-PAGE. The proteins were blotted and visualized with the corresponding antibody.

Cell Culture—The method used to culture the mouse dental follicle cells has been described previously (29). To examine the effects of Adamtsl6 β upon the TGF- β 1-induced expression of periostin, mouse dental follicle cells were cultured in a 12-well plate at a density of 5×10^4 cells/well in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS until they reached confluence. At this point, the medium was replaced with DMEM containing 0.2% FBS. After 12 h, the cells were treated with recombinant mouse Adamtsl6 β . After 12 h, the

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cells were treated with TGF- β 1 (10 ng/ml) for 3 days and subjected to real-time PCR analysis.

Tooth Replantation Model—The tooth replantation experiments were performed as described previously (30). Briefly, the upper first molar from 4-week-old C57BL/6(SLC) mice was extracted under deep anesthesia. Extracted teeth were then replanted into the original cavity to allow the natural repair of the PDL. The replanted teeth were collected at 3, 7, and 14 days after transplantation and subjected to immunohistochemical analysis using the Kawamoto tape method or *in situ* hybridization as described in the supplemental Methods.

Generation of Transgenic Bioengineered Tooth Germ—Molar tooth germs were dissected from the mandibles of E14.5 mice. The isolation of mesenchyme and epithelium and the dissociation of mesenchymal cells have been described previously (24). Dissociated cells were cultured on tissue culture plates in DMEM containing 10% fetal calf serum and then infected with AdamtSL6 β adenovirus for 8 h. After incubation for 24 h, mesenchymal cells overexpressing AdamtSL6 β were collected via trypsin digestion and precipitated by centrifugation in a siliconized tube, and the supernatant was completely removed. The cell density of the precipitated, adenovirus-infected mesenchymal cells after removal of supernatant reached a concentration of 5×10^8 cells/ml, as described previously (24). Transgenic bioengineered tooth germ was reconstituted with dissociated adenovirus-infected mesenchymal cells and epithelial tissue using our previously described three-dimensional cell manipulation system, the organ germ method (24). The transgenic bioengineered tooth germs were incubated for 10 min at 37 °C, placed on cell culture inserts (0.4- μ m pore diameter; BD Biosciences), and then further incubated at 37 °C for 6 days in an *in vitro* organ culture as described previously (24). Mesenchymal cells infected with lentiviruses carrying AdamtSL6 β miRNAi were used to generate AdamtSL6 β miRNAi-transgenic bioengineered tooth germ by incubation for 10 min at 37 °C, placement on cell culture inserts (0.4- μ m pore diameter; BD Biosciences), and then a further incubation at 37 °C for 12 days in an *in vitro* organ culture as described previously (24).

Local Administration of ADAMTSL6 β Using a PDL Injury Model—To create gels for injection, 2 μ l of recombinant AdamtSL6 β (10 μ g/ μ l) (see supplemental Methods) and 1.5 μ l of PKH67 green fluorescent cell linker (Sigma-Aldrich) were suspended in a 9- μ l gel drop of collagen liquid Cellmatrix type I-A (Nitta Gelatin) composed of acid-soluble collagen isolated from pig tendon. The lower first molars of 4-week-old C57BL/6(SLC) mice were extracted under deep anesthesia. Following blood coagulation, at 3 days after tooth extraction, the alveolar bony wall of the proximal site of the lower second molar tooth was surgically removed to expose the PDL. The PDL was then disrupted by dislocation of the second molar tooth with lingual to buccal side movement. The collagen drop containing recombinant AdamtSL6 β was then inserted into the damaged PDL. Mouse mandibles were collected 17 days after insertion, and immunohistochemical analysis was performed using the Kawamoto tape method as described in the supplemental Methods.

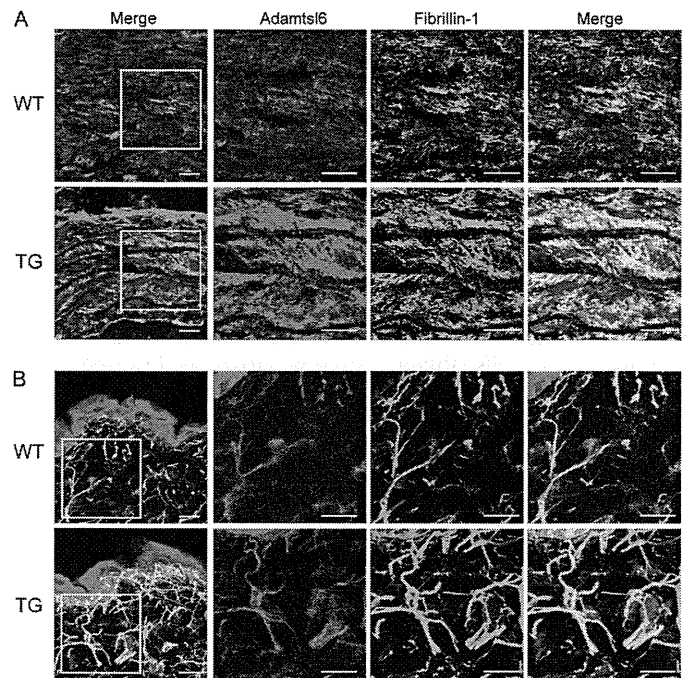


FIGURE 1. Immunohistochemical analysis of *Tsl6 β -TG* mice. Immunofluorescence detection of ADAMTSL-6 proteins and fibrillin-1. Frontal cryosections were prepared from the skin (A) or aortas (B) of wild type (top) or *Tsl6 β -TG* (bottom) littermates and subjected to double immunostaining with antibodies against AdamtSL6 (red) and fibrillin-1 (green). The boxed areas in the leftmost panels are shown at higher magnification in the rightmost panels. Immunohistochemical analysis indicated that the expression of AdamtSL6- and fibrillin-1-positive microfibrils was markedly increased in the aorta and skin of *Tsl6 β TG* mice compared with WT mice. The merged images illustrate that these fibrils are colocalized in the skin and aorta.

RESULTS

ADAMTSL6 β Regulates Microfibril Assembly in Various Connective Tissues—To investigate whether AdamtSL6 β plays a critical role in microfibril assembly in connective tissues, we generated AdamtSL6 β -transgenic mice (*Tsl6 β -TG* mice) in which the transgene is expressed in the whole body. Because AdamtSL6 has been shown to be expressed in the aorta and skin, we investigated microfibril assembly of these tissues in the *Tsl6 β -TG* mice. Immunohistochemical analysis revealed that AdamtSL6-positive microfibril assembly was barely detectable in WT mice but strongly induced in the aorta of *Tsl6 β -TG* mice (Fig. 1A). Confocal microscopy analysis further revealed that AdamtSL6- and fibrillin-1-positive microfibrils are clearly increased in the aorta and that microfibril assembly is also induced in the skin of *Tsl6 β -TG* mice. This confirmed that AdamtSL6 induces fibrillin-1 microfibril assembly in connective tissue, such as the aorta and skin (Fig. 1B).

ADAMTSL6 β Is Involved in Microfibril Formation during PDL Development and Wound Healing—To investigate whether AdamtSL6 β contributes to connective tissue formation, we first examined its expression patterns during embryonic tooth germ development and in the PDL formation stage after birth as a model of connective tissue formation. Development of the PDL proceeds as follows: 1) the dental follicle (DF), the origin of the PDL, is formed at the CAP stage of tooth germ formation; 2) the DF differentiates during the tooth root-forming stage; and 3) the DF differentiates into the PDL to be

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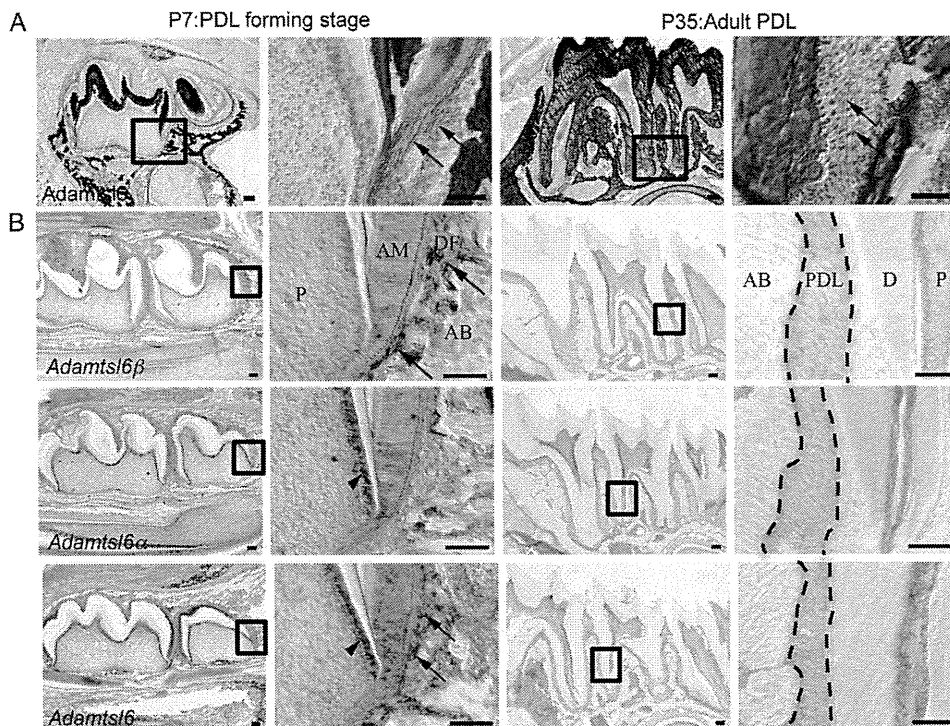


FIGURE 2. Expression patterning of Adamtsl6 α and - β during the PDL-forming stage. *A*, sagittal sections of lower molar at P7 and P35 were immunostained with anti-Adamtsl6 antibody. The image in the box on the left is shown at higher magnification on the right. Note that Adamtsl6 protein was deposited as microfibril aggregates in DF at the PDL-forming stage (arrows) and formed a mature microfibrillar assembly in adult PDL (arrows). Bar, 100 μ m. *B*, *in situ* hybridization analysis using a specific probe for Adamtsl6 α and - β and control probe are shown. Adamtsl6 β mRNA at the P1-late bell stage of dental follicle formation in the tooth germ is indicated by arrows. Expression of Adamtsl6 α was detected in the odontoblast (arrowheads) by specific probes. In contrast, control probes that detected the conserved region of Adamtsl6 recognized both odontoblast (arrowhead) and DF (arrows). Bar, 100 μ m. AB, alveolar bone; AM, ameloblast; D, dentin; P, pulp; DF, dental follicle; PDL, periodontal ligament.

inserted into collagen fibers known as Sharpey's fibers in the cementum matrix and alveolar bone, which resembles tendinous tissue (26, 31). *In situ* hybridization analysis revealed that Adamtsl6 β is barely expressed in the DF, the origin of PDL formation in the surrounding tooth germ (supplemental Fig. S1B), but was very strongly expressed in the PDL-forming stage of the DF at P7 (Fig. 2B). However, Adamtsl6 β expression was significantly down-regulated in the adult PDL at P35 (Fig. 2B). In contrast to Adamtsl6 β , Adamtsl6 α was found to be expressed in odontoblasts but not to be expressed in either the DF during the PDL-forming stage or in the adult PDL (supplemental Fig. S1B and Fig. 2B). Immunohistochemical analysis further revealed that Adamtsl6 is only weakly expressed in the early stage (bell stage) DF (supplemental Fig. S1A) but became detectable in assembled microfibril-like structures during the PDL-forming stage of the DF and in organized microfibrils in the adult PDL (Fig. 2A). Using confocal microscopy analysis, Adamtsl6 was observed to colocalize with fibrillin-1 to form immature microfibrillar-like structures at the PDL-forming stage of the DF, which were then observed as fully assembled mature microfibril structures in the adult PDL (Fig. 3A).

Using an Adamtsl6 antibody, positively stained fibers were observed in the adult PDL that were almost identical to those marked by aldehyde fuchsin staining and are indicative of microfibrils (supplemental Fig. S2). This suggested that Adamtsl6 was a component of microfibrils. Because developmental processes involve similar mechanisms to wound healing, we next determined whether Adamtsl6 β is involved in PDL

microfibril assembly during wound healing using a tooth replantation model (supplemental Fig. S3A) (30). Histochemical analysis revealed an injured PDL with an irregular architecture at 3 days after replantation, although gradual healing then occurred at between 7 and 14 days after replantation (Fig. 3B and supplemental Fig. S3B). During these processes, Adamtsl6 β and fibrillin-1 mRNA expression were found to be clearly induced in the PDL at 3–7 days after replantation but to decrease again by 14 days after replantation (Fig. 3B).

Similar to these gene expression patterns, Adamtsl6- and fibrillin-1-positive microfibrillar-like structures resembling those seen in the DF during the PDL-forming stage were markedly increased in the damaged PDL at 3–7 days after replantation. These structures had evolved into mature microfibrils by 14 days after replantation (Fig. 3C and supplemental Fig. S3B). In contrast to these gene expression patterns, the expression of periostin, a PDL differentiation marker, was detected at 7 days after replantation (supplemental Fig. S3C). These data indicate that fibrillin-1 microfibril formation is induced in the early stages of both PDL development and wound healing and that Adamtsl6 β is involved in these processes.

ADAMTSL6 β Regulates PDL Formation through Fibrillin-1 Microfibril Assembly—We have recently developed a new three-dimensional single cell processing technique, the organ germ method, which can be used to generate bioengineered tooth germ reconstituted from E14.5 molar tooth germ-derived epithelial and mesenchymal cells (24). Utilizing this system, we developed a transgenic bioengineered tooth germ by overex-

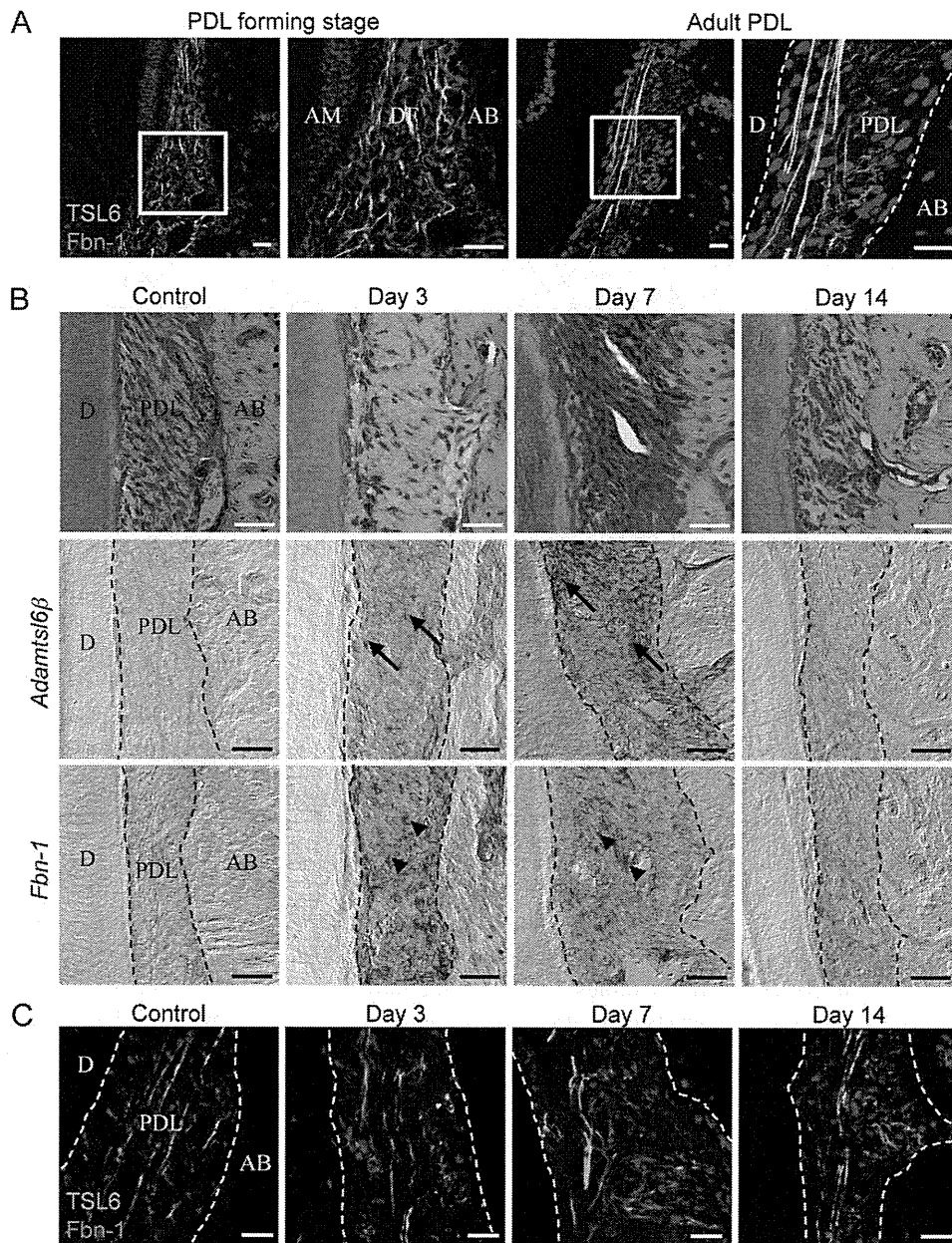


FIGURE 3. Adamtsl6 β is involved in fibrillin-1 microfibril formation during PDL development and wound healing. *A*, localization of fibrillin-1- and Adamtsl6-positive microfibrils were analyzed during the PDL formation stage (*left*) and in adult PDL (*right*) using antibodies against Adamtsl6 (*TSL6*; *green*) and fibrillin-1 (*Fbn-1*; *red*). Formation of microfibrils positive for anti-Adamtsl6 β and anti-fibrillin-1 was detectable during the process of PDL formation. *B*, frontal section of control side PDL and injured PDL 3, 7, and 14 days after replantation of the tooth were analyzed by hematoxylin and eosin staining (*top*) and *in situ* hybridization analysis of Adamtsl6 β (*middle*) or fibrillin-1 (*Fbn-1*; *bottom*) expression in PDL. Cells positive for Adamtsl6 β and fibrillin-1 mRNA expression are indicated by *arrows* or *arrowheads*, respectively. *C*, immunohistochemical analysis using anti-Adamtsl6 (*TSL6*; *green*) and anti-fibrillin-1 (*Fbn-1*; *red*) antibodies indicated that expression of Adamtsl6- and fibrillin-1-positive microfibrils was markedly increased 3 and 7 days after injury. *A merged image* illustrates that these fibrils were colocalized during the wound healing processes.

pressing exogenous genes in mesenchymal cells derived from tooth germ using adenovirus (supplemental Fig. S4, *A* and *B*). Because transgenic bioengineered tooth germ was found to accurately reproduce PDL development (supplemental Fig. S4, *C* and *D*), we generated Adamtsl6 β -transgenic bioengineered tooth germ to examine the contributions of Adamtsl6 β to PDL formation. Following immunohistochemical staining, Adamtsl6 β -transgenic bioengineered tooth germs showed clear colocalization between fibrillin-1 microfibrils and Adamtsl6 β (Fig. 4A) after 6 days of culture. Conversely, fibril-

lin-1 microfibrils were barely detectable in control *LacZ*-transgenic bioengineered tooth germ (Fig. 4A).

To confirm the role of Adamtsl6 β in regulating microfibril formation in the DF from bioengineered tooth germ, we generated Adamtsl6 β miRNAi-transgenic bioengineered tooth germ to suppress Adamtsl6 β expression. Immunohistochemical analysis subsequently revealed that the Adamtsl6 β miRNAi-transgenic germ exhibited poor Adamtsl6- and fibrillin-1-positive microfibril formation after 12 days of culture. However, no changes were observed in control miRNAi-transgenic bioengi-

ADAMTSL6 β Rescues Disorder in Marfan Syndrome

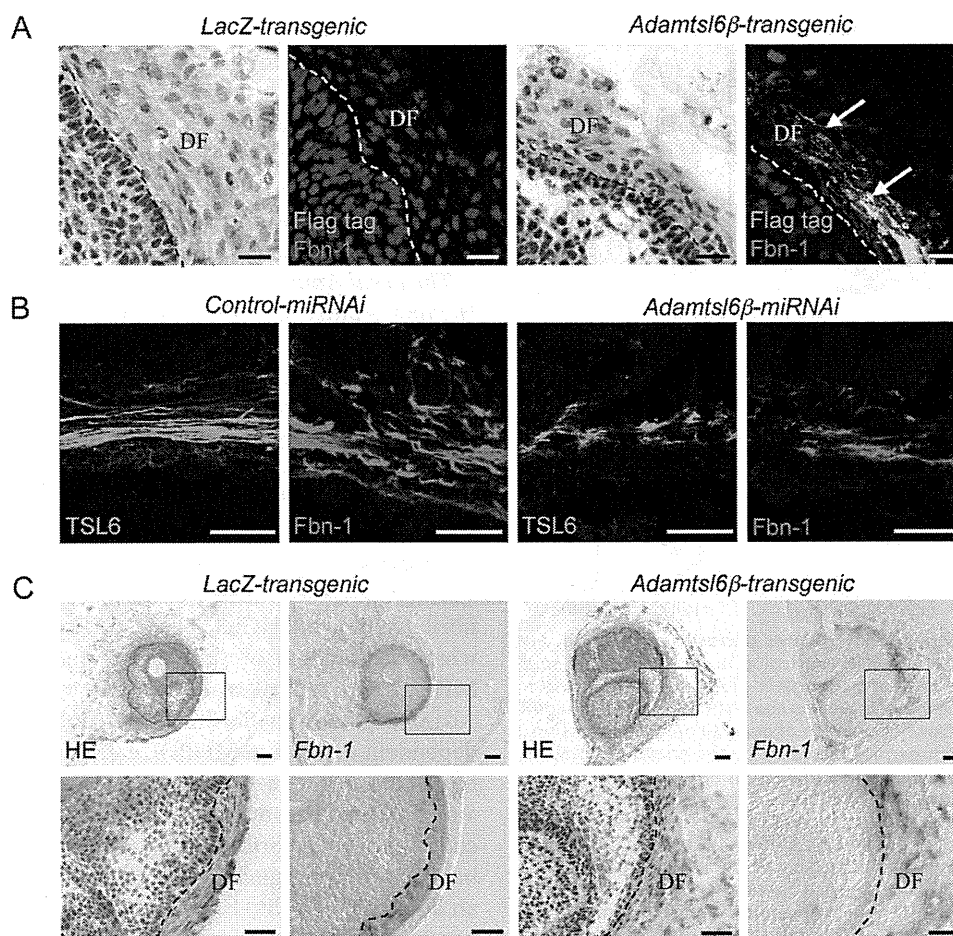


FIGURE 4. AdamtSL6 β contributes to PDL formation through regulation of fibrillin-1 microfibril assembly. A, immunohistochemical analysis of *LacZ*-transgenic tooth germ (*LacZ*-transgenic) or *AdamtSL6 β* -transgenic bioengineered tooth germ (*AdamtSL6 β* -transgenic) using double immunostaining with anti-FLAG (Flag tag; green) and anti-fibrillin-1 (Fbn-1; red). Microfibrils positive for AdamtSL6 β and fibrillin-1 expression are indicated by arrows. B, immunohistochemical analysis of control miRNAi-transgenic bioengineered tooth germ (*Control miRNAi*) or *AdamtSL6 β* miRNAi-transgenic tooth germ (*AdamtSL6 β -miRNAi*) using double immunostaining with anti-AdamtSL6 (TSL6; green) and anti-fibrillin-1 (Fbn-1; red). C, hematoxylin and eosin staining (HE) and *in situ* hybridization analysis of fibrillin-1 mRNA expression (Fbn-1) in *LacZ*-transgenic or *AdamtSL6 β* transgenic bioengineered tooth germ (*AdamtSL6 β* -transgenic). The image in the box at the top is shown at higher magnification at the bottom (C).

neered tooth germ, further indicating that AdamtSL6 β regulates microfibril assembly during PDL formation (Fig. 4B). We next evaluated whether the promotion of fibrillin-1 microfibril assembly was the result of increased mRNA expression. *In situ* hybridization analysis revealed that fibrillin-1 mRNA expression was similar in *LacZ*- and *AdamtSL6 β* -transgenic bioengineered tooth germ (Fig. 4C). These data indicate that AdamtSL6 β is capable of recruiting fibrillin-1 to assembling microfibrils without increasing the fibrillin-1 transcript levels.

ADAMTSL6 β Negatively Regulates TGF- β -induced Periostin Gene Expression during PDL Formation—To investigate whether AdamtSL6 β regulates PDL formation, we analyzed the expression of genes that function in PDL formation, including type I collagen, type XII collagen, periostin, and f-spondin (27). Among these genes, periostin, the protein product of which is known to be induced by TGF- β (32, 33), was markedly down-regulated in *AdamtSL6 β* -transgenic bioengineered tooth germ (Fig. 5B and supplemental Fig. S5). *In situ* hybridization analysis further revealed the strong expression of periostin in the DF from *LacZ*-transgenic tooth germ when compared with the DF from *AdamtSL6 β* -transgenic tooth germ (Fig. 5A). Real-time

PCR analysis confirmed the suppression of periostin expression in *AdamtSL6 β* -transgenic tooth germ (Fig. 5B).

To evaluate whether AdamtSL6 β negatively regulates periostin gene expression in our MFS model system, we analyzed *AdamtSL6 β* adenovirus-infected tooth germ obtained in MFS mice that were homozygous for a targeted hypomorphic allele (mgR/mgR) of fibrillin-1 (7). *In situ* hybridization analysis showed that periostin expression was remarkably reduced in developing tooth germ from mgR/mgR mice after infection with *AdamtSL6 β* -adenovirus compared with *LacZ*-adenovirus-infected tooth germ (Fig. 5C). We next investigated the effects of ADAMTSL6 β on human PDL cells obtained from an MFS patient with severe periodontitis (MHPDL) (25). As expected, ADAMTSL6 β overexpression in these MHPDL cells clearly reduced periostin expression when compared with mock-infected cells. Interestingly, the level of periostin expression in MHPDL cells with ADAMTSL6 β overexpression was comparable with that of normal HPDL cells (Fig. 5D), raising the possibility that ADAMTSL6 β negatively regulates TGF- β and thereby reduces periostin expression. We evaluated this possibility by testing the ability of His-tagged recombinant AdamtSL6 β to bind to TGF- β 1. Interactions between

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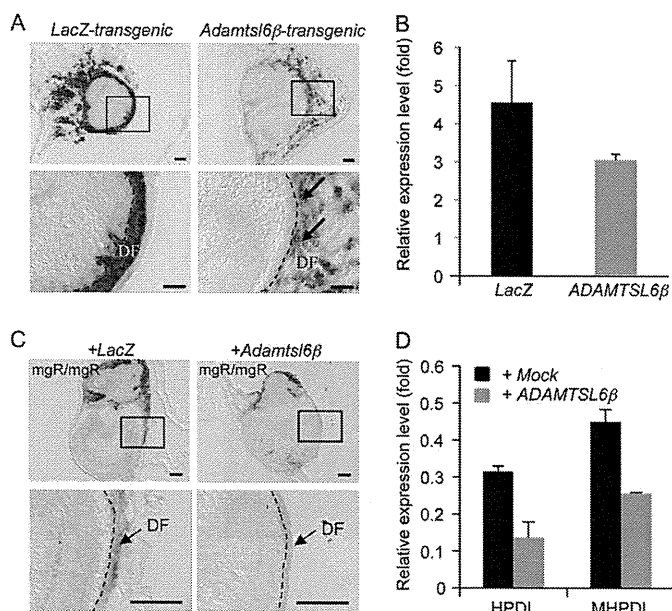


FIGURE 5. ADAMTSL6 β negatively regulates periostin expression. *A*, *in situ* hybridization for periostin mRNA expression in *LacZ* transgenic tooth germ (*LacZ*-transgenic) or *AdamtSL6 β* transgenic bioengineered tooth germ (*AdamtSL6 β* -transgenic). The image in the top box is shown at higher magnification in the bottom box. Down-regulation of periostin mRNA expression in *AdamtSL6 β* transgenic tooth germ is indicated by the arrows. *B*, total RNA extracted from *LacZ* (*LacZ*-) or *AdamtSL6 β* (*AdamtSL6 β*)-transgenic bioengineered tooth germ. cDNA was synthesized and subjected to quantitative real-time PCR for the expression of periostin and *GAPDH* transcripts. Levels of *GAPDH* transcript were used to normalize cDNA levels. Levels of *GAPDH* were set at 1, and relative expression levels are shown. Data are presented as triplicates, and the means \pm S.D. are shown. *C*, *in situ* hybridization analysis of *AdamtSL6 β* -adenovirus-infected mgR/mgR mouse tooth germ showed reduced periostin expression when compared with *LacZ*-adenovirus-infected mgR/mgR mouse tooth germ. Bar, 100 μ m. *D*, real-time PCR analysis of periostin mRNA in HPDL and MHPDL cells transduced with mock or *AdamtSL6 β* . Periostin mRNA expression was down-regulated in HPDL and MHPDL cells overexpressing *AdamtSL6 β* as compared with expression in mock-overexpressed HPDL and MHPDL cells.

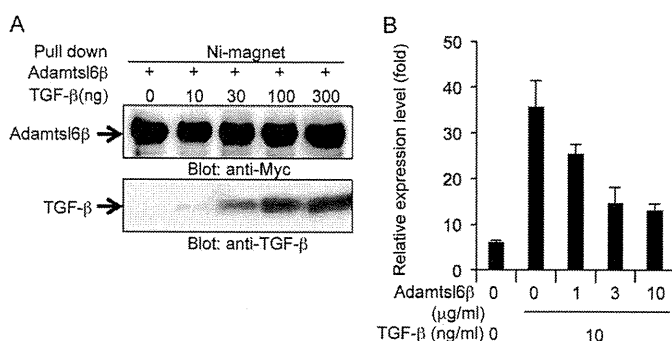


FIGURE 6. AdamtSL6 β binds TGF- β to negatively regulate periostin gene expression. *A*, His tag recombinant *AdamtSL6 β* was incubated with TGF- β 1 at the indicated concentrations followed by treatment with nickel-magnetic beads. Co-precipitates were detected using the corresponding antibodies, and *AdamtSL6 β* was found to bind to TGF- β 1 directly. *B*, mouse dental follicle cells were cultured with TGF- β 1 for 3 days in the presence of recombinant mouse *AdamtSL6 β* . Periostin mRNA levels were quantified by real-time PCR analysis. *AdamtSL6 β* inhibited expression of periostin in a dose-dependent manner. Error bars, S.D.

AdamtSL6 β and TGF β 1 were barely detectable at the 10 ng/ml concentrations, but strong associations between these proteins could be detected in a dose-dependent manner at 30, 100, or 300 ng/ml by pull-down analysis using nickel-magnetic beads (Fig. 6A). To then test the effects of recom-

binant *AdamtSL6 β* on TGF- β activity, we measured the periostin expression levels in mouse dental follicle cells treated with *AdamtSL6 β* in the presence or absence of TGF- β 1. Recombinant *AdamtSL6 β* inhibited TGF- β -induced periostin expression in a dose-dependent manner (Fig. 6B). These data suggest that *AdamtSL6 β* directly binds to TGF- β to reduce periostin expression during the PDL-forming stage in both normal and MFS model settings.

The Local Administration of ADAMTSL6 β Improves Wound Healing Ability in an MFS Model—We next investigated whether ADAMTSL6 β alleviates fibrillin-1 microfibril disorder in MHPDL cells, which exhibit reduction in fibrillin-1 microfibril assembly (Fig. 7). The overexpression of ADAMTSL6 β strongly induced fibrillin-1 microfibril assembly in MHPDL cells compared with the mock-infected controls (Fig. 7, top and middle). Merged images revealed that ADAMTSL6 β colocalizes with fibrillin-1 in MHPDL cells that overexpress ADAMTSL6 β (Fig. 7, bottom). We have previously shown that recombinant *AdamtSL6 β* induces fibrillin-1 microfibril assembly in MG63 cells (17). Thus, we next investigated whether recombinant *AdamtSL6 β* improves the symptoms of MHPDL microfibril disorder. We found that recombinant *AdamtSL6 β* induces fibrillin-1 microfibril assembly in a dose-dependent manner in MHPDL cells during a 3-day incubation in culture (Fig. 8, top and middle). Staining with an anti-*AdamtSL6* polyclonal antibody indicated that exogenous *AdamtSL6* colocalizes with fibrillin-1 (Fig. 8, bottom). Endogenous fibrillin-1 was only marginally detectable in MHPDL cells. However, an abundant fibrillin-1 network formation was evident in the presence of high concentrations (10 μ g/ml) of recombinant *AdamtSL6 β* (Fig. 8, top). These results indicate that *AdamtSL6 β* improved fibrillin-1 MHPDL microfibril assembly.

To investigate whether *AdamtSL6 β* was capable of improving microfibril assembly *in vivo*, we investigated the PDL from mgR/mgR mice and by histochemical analysis observed a disorganized structure with a disrupted cell alignment, both of which are characteristic MFS morphologies (Fig. 9A). Immunohistochemical analysis clearly revealed fragmented *AdamtSL6 β* - and fibrillin-1-positive microfibrils when compared with wild type mice (Fig. 9A, arrows). We next infected *AdamtSL6 β* adenovirus into the DF of developing tooth germ isolated from mgR/mgR mouse embryos at E14.5 to evaluate the improvements in fibrillin-1 microfibril disorder during PDL formation. By histochemical analysis, we found that the overexpression of *AdamtSL6 β* resulted in an improved DF morphology with compact and aligned cells (Fig. 9B). DF tooth germ infected with *LacZ* showed low cell numbers and an irregular architecture. Immunohistochemical analysis subsequently revealed that *AdamtSL6 β* overexpression strongly induces fibrillin-1 microfibril assembly in the tooth germ from mgR/mgR mice, whereas no assembly was observed in *LacZ*-infected tooth germ (Fig. 9B, arrows). These data indicate that *AdamtSL6 β* can indeed restore the impaired microfibrils in mgR/mgR mice.

We next investigated whether *AdamtSL6 β* might be developed as a novel therapeutic for MFS microfibril disorder. Collagen gel containing recombinant *AdamtSL6 β* was locally administrated into an experimentally damaged PDL in mgR/

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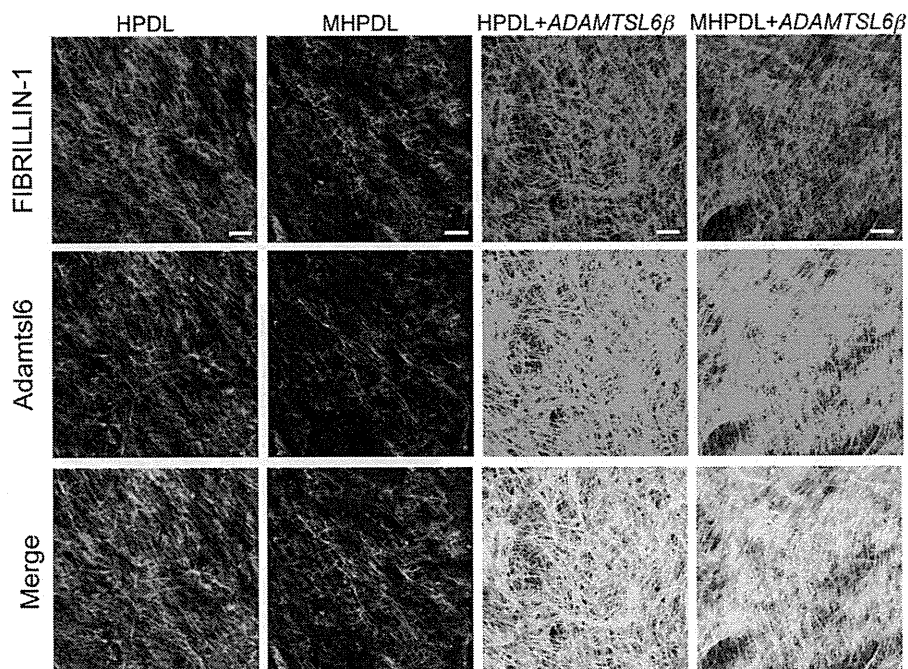


FIGURE 7. Overexpression of ADAMTSL6 β improves microfibril disorder in PDL from an MFS patient. Immunohistochemical analysis of HPDL or MHPDL cells transduced with mock or ADAMTSL6 β using anti-fibrillin-1 (*top*) and anti-Adamtsl6 (*middle*) antibodies. The data show that ADAMTSL6 β induces fibrillin-1 microfibril assembly in MHPDL cells. The *bottom* images were produced by superimposition of the *upper* and *middle* images, together with DAPI nuclear staining.

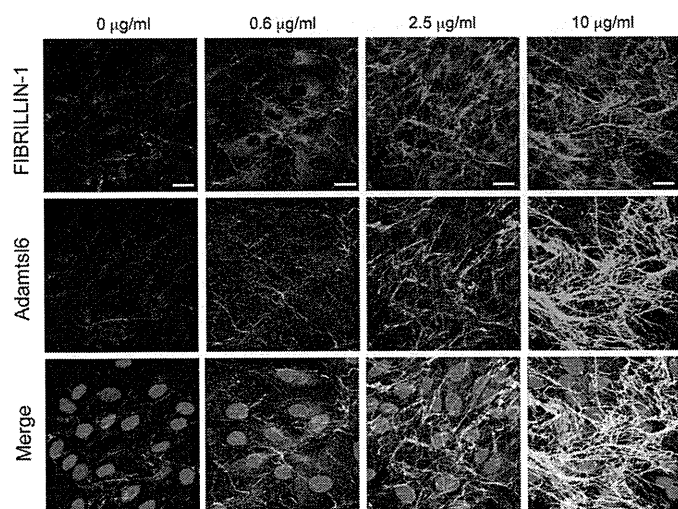


FIGURE 8. Recombinant Adamtsl6 β improves microfibril disorder in PDL from MFS patients. Immunohistochemical analysis with anti-fibrillin-1 (*top*) and anti-Adamtsl6 (*middle*) antibodies reveals a marked improvement in fibrillin-1 microfibril assembly (*arrows*) in MHPDL cells incubated with purified recombinant Adamtsl6 β at 0.6, 2.5, and 10 μ g/ml for 3 days. The *bottom* images were produced by superimposition of the *upper* and *middle* images, together with DAPI nuclear staining.

mgR mice (supplemental Fig. S6, *A* and *B*). Fluorescence microscopic analysis revealed that the collagen gel implanted in damaged PDL was still present at 17 days after injection (supplemental Fig. S6C). Histochemical analysis showed that a damaged PDL could still be observed at 7 days after injection of the collagen gel containing recombinant Adamtsl6 β (supplemental Fig. S6D). However, healing and improved cell alignment were apparent in the PDL of wild type mice at 17 days after injection (Fig. 9C, *asterisk*). Immunohistochemical analysis further revealed that the reorganization of fibril-

lin-1- and Adamtsl6-positive microfibril assembly could be observed after 17 days of incubation (Fig. 9C (*arrowheads*) and supplemental Fig. S6E). In contrast, the administration of control collagen gel failed to induce PDL healing, and an irregular cell morphology and poor fibrillin-1 microfibril formation could still be observed (Fig. 9C and supplemental Fig. S6E).

The enhanced activation of TGF- β has been suggested to directly contribute to tissue destruction in MFS (12). Ligand-activated TGF- β receptors induce the phosphorylation of Smad2 and Smad3 (pSmad2/3), which form a heteromeric complex with Smad4 that translocates to the nucleus and mediates the expression of target genes (34). The nuclear accumulation of pSmad2/3 has been detected in affected tissues in an MFS mouse model, including the aorta and skeletal muscle (10, 35). Consistent with these results, we observed the nuclear accumulation of pSmad2/3 in PDL from mgR/mgR mice 17 days after injection of control collagen gel in our current experiments (Fig. 9D). However, the local administration of Adamtsl6 β markedly suppressed the nuclear localization of pSmad2/3. Further evidence for the Adamtsl6 β suppression of TGF- β signaling is derived from the previous analysis of matrix metalloprotease (MMP)-9, which is known to be induced by TGF- β and is expressed in abnormal smooth muscle cells in the early vascular lesions that contribute to elastolysis (36, 37). In contrast to control collagen gel administration, the expression of MMP-9 was markedly suppressed by the administration of collagen gel containing recombinant Adamtsl6 β (Fig. 9D). These results illustrate that reorganization of microfibrils by recombinant Adamtsl6 β prevents the pathological activation of TGF- β by structurally damaged fibrillin-1 in MFS.

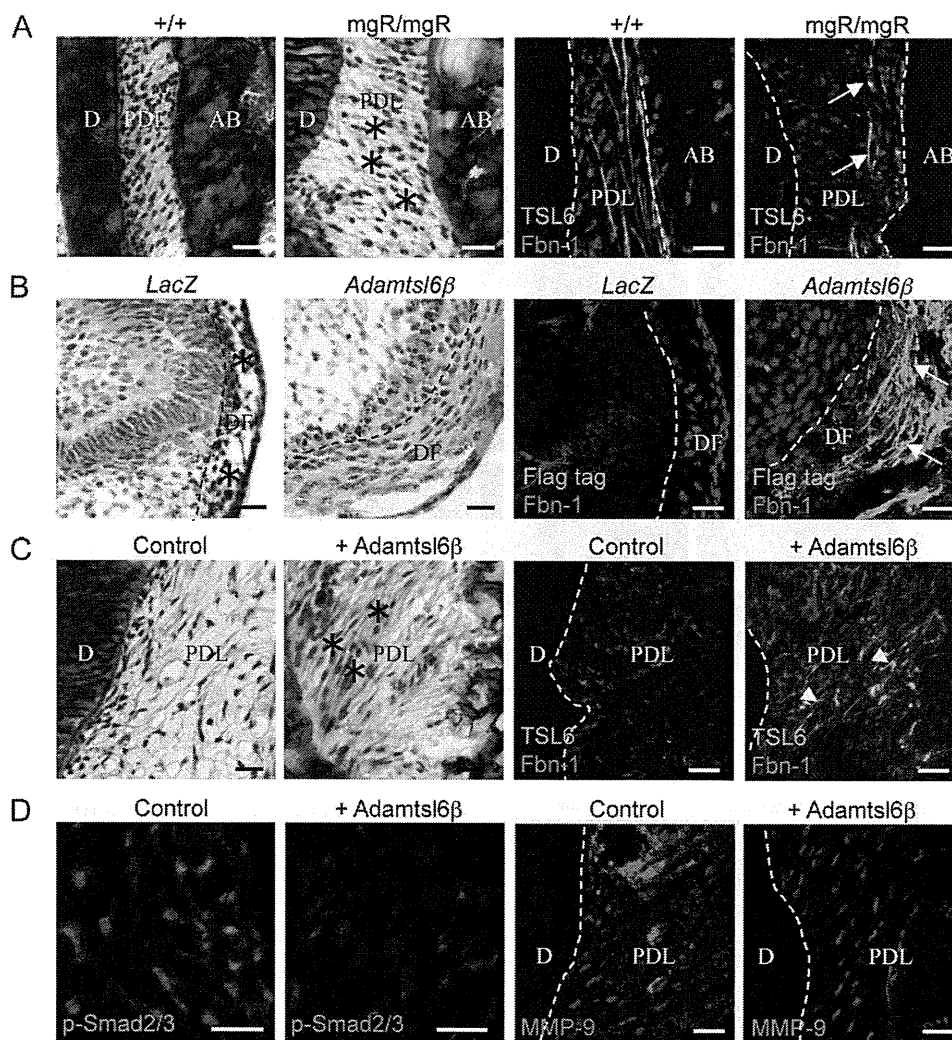


FIGURE 9. Local administration of AdamtSL6 β improves microfibril disorder and attenuates TGF- β signaling in PDL from an MFS model. *A*, hematoxylin and eosin staining of a PDL revealing a markedly abnormal architecture in mgR/mgR mice when compared with wild type. Notable is the loosening of the PDL with an irregular cell alignment and an expanded cell-cell distance (*asterisks*). Immunohistochemical analysis using AdamtSL6 (*TSL6*; *green*) and fibrillin-1 (*Fbn-1*; *red*) antibodies revealed a clear disruption of fibrillin-1- and AdamtSL6-positive microfibrils in the PDL from mgR/mgR mice (*arrows*). *B*, hematoxylin and eosin staining of *LacZ*-infected mgR/mgR mouse tooth germ (*LacZ*) revealing an abnormal architecture with an irregular cell alignment (*asterisks*) when compared with *AdamtSL6 β* -infected mgR/mgR mouse tooth germ (*AdamtSL6 β*). Immunohistochemical analysis using FLAG (*Flag-tag*; *green*) and fibrillin-1 (*Fbn-1*; *red*) antibodies showed an improvement in fibrillin-1 microfibril assembly in *AdamtSL6 β* -infected mgR/mgR mouse tooth germ (*arrows*). *C*, histological analysis of the injured PDL in mgR/mgR mice after the local administration of control gel or gel containing recombinant AdamtSL6 β for 17 days. Hematoxylin and eosin staining revealed PDL healing after the injection of gel containing recombinant AdamtSL6 β (*asterisks*) compared with the control. Immunohistochemical analysis further showed an improvement in fibrillin-1 microfibril assembly (*arrowheads*) induced by the injection of recombinant AdamtSL6 β . *D*, immunohistochemical analysis of pSmad2/3 and MMP-9 expression in the injured PDL of mgR/mgR mice after the local administration of control gel or gel containing recombinant AdamtSL6 β for 17 days. The suppression of nuclear accumulation of pSmad2/3 and MMP-9 expression is evident after injection of recombinant AdamtSL6 β compared with the control gel.

DISCUSSION

Our current experiments successfully demonstrate that ADAMTSL6 β has an essential role in PDL development and regeneration through the promotion of fibrillin-1 assembly and the negative regulation of TGF- β signaling. We also demonstrate in our present analyses that the local administration of ADAMTSL6 β can rescue the disease manifestations of MFS in a mouse model, raising the possibility that this extracellular matrix protein could be used as a novel therapeutic agent for the treatment of MFS. Hence, our data show for the first time that the restoration of properly formed microfibrils by ADAMTSL6 β is essential not only for improvement of the predominant symptoms of MFS but also for the suppression of excessive TGF- β signaling induced by microfibril disassembly.

To clarify the role of ADAMTSL6 β in PDL formation, experiments were performed to determine whether ADAMTSL6 β -mediated fibrillin-1 microfibril assembly is critical for PDL development and regeneration. The formation of fibrillin-1 microfibril networks has been shown to be essential for the development and growth of individual organ systems (38). Vascular smooth muscle cells are gradually organized via the formation of elastic fibers and interconnecting fibrillin-1 microfibrils during aortic media generation, resulting in the organization of elastic lamellae as the main determinant of arterial function (39). In addition to providing mechanical stability, previous studies have demonstrated that the organization of fibrillin-1 microfibril assemblies contributes to the regulation of the activities of signaling molecules, such as TGF- β and

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BMP-7 (40, 41). During digit formation, fibrillin-1 may be a positive regulator that dictates the functional sites for cytokine concentration. In other tissues, fibrillin-1 acts as a negative regulator of signaling through cytokine sequestration (2, 7). Thus, the importance of microfibril network formation has been demonstrated in several disparate settings. However, the importance of the molecular mechanisms governing fibrillin-1 assembly during organogenesis has been hampered by the unanswered issue of the actual factor that drives microfibril assembly. Our present study demonstrates that ADAMTSL6 β , an inducing factor for microfibril assembly (18), regulates development and regeneration of PDL. Furthermore, ADAMTSL6 β -mediated fibrillin-1 microfibril assembly may accelerate the sequestration of large latent complexes of TGF- β or active TGF- β , thereby negatively regulating the expression of TGF- β regulatory targets, such as periostin (35, 42). Hence, our data provide significant insight into the molecular mechanisms by which ADAMTSL6 β controls fibrillin-1 microfibril assembly and TGF- β signaling during organogenesis.

The establishment of fibrillin-1 microfibril assembly mechanisms is ultimately critical for the development of new MFS therapeutic approaches (12). The pathogenetic relevance of MFS is highlighted by the fact that microfibril assembly is frequently disrupted in patients with various fibrillinopathies (43). MFS fibrillinopathies have been explained by the structural insufficiency of fibrillin-1 microfibrils, leading to activation of TGF- β and its regulatory targets (7, 44). Although many recent publications have addressed the organization of fibrillins in microfibrils (45–48), relatively little information has been available regarding the mechanisms and components involved in microfibril formation. A previous study reported that the transgenic expression of wild-type fibrillin-1 alleles in a missense mutation (C1039G) heterozygous mouse model of MFS effectively rescues the aortic phenotype (11). From these data, essential improvements in fibrillin-1 microfibril formation represent a productive therapeutic strategy for the reduction of MFS disease severity (3).

In contrast to our present findings, another recent study has indicated that fibronectin is an essential component in the assembly of fibrillin-1 through its interaction with the C-terminal region of fibrillin-1, thus suggesting the possibility of improved microfibril assembly through regulation of fibrillin-1-associated proteins (49, 50). Our present data demonstrate, however, that the exogenous application of recombinant ADAMTSL6 β improves fibrillin-1 microfibril assembly in an MFS mouse model. Hence, ADAMTSL6 β reinforcement of fibrillin-1 microfibrils may represent a new, viable treatment for MFS. Although the mechanisms by which ADAMTSL6 β accelerated fibrillin-1 microfibril assembly remain to be determined, in another study using the MFS mouse model and MHPDL cells, which are PDL cells obtained from an MFS patient, ADAMTSL6 β seems to recruit available normal fibrillin-1 molecules and induce microfibril assembly with a resulting improvement in microfibril mechanical stability. These findings indicate that ADAMTSL6 β is capable of enhancing microfibrils even in animals with a fibrillin-1 haploinsufficiency. Thus, ADAMTSL6 β is potentially a novel therapeutic target for the treatment of MFS.

Recent evidence has suggested that restoration of microfibril assembly plays an important role in the prevention of pathological activation of TGF- β signaling in MFS (12). TGF- β is secreted from cells as a large latent complex consisting of TGF- β , latency-associated peptide, and LTBP-1 to be sequestered by fibrillin-1 (51). The promotion of fibrillin-1 microfibril assembly is therefore critical for the prevention of tissue destruction mediated by abnormal TGF- β signaling in MFS. In the present study, we have demonstrated that the reinforcement of fibrillin-1 microfibril assembly and the inhibition of TGF β 1 function by ADAMTSL6 β facilitate wound healing in the PDL of mgR/mgR mice.

In conclusion, we provide evidence for the contributions of ADAMTSL6 β -mediated fibrillin-1 microfibril assembly to PDL development, regeneration, and alleviation of MFS manifestations. We thereby introduce the concept that a fibrillin-1-associated protein, such as ADAMTSL6 β , which induces microfibril assembly, should be considered in the development of future mechanism-based therapeutics for the improvement of connective tissue disorders, such as MFS. Our data suggest that the reinforcement of fibrillin-1 assembly by ADAMTSL6 β accelerates the sequestration of newly synthesized large latent complexes into fibrillin-1. Further studies will help to clarify the nature of the interactions between ADAMTSL6 β , fibrillin-1, TGF- β , and LTBP-1 and reveal how ADAMTSL6 β expression suppresses TGF- β signaling. It will also be necessary to develop methodologies for the systemic administration of ADAMTSL6 β to induce fibrillin-1 microfibril assembly in connective tissue for the treatment of life-threatening conditions, such as aortic aneurysm. Because elastolysis occurs continuously in aortic aneurysms in MFS, chronic administration of ADAMTSL6 β may be required for the stabilization of microfibrils to prevent progressive tissue destruction. This approach will facilitate drug discovery for treating MFS and related connective tissue disorders.

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Functional Tooth Regeneration Using a Bioengineered Tooth Unit as a Mature Organ Replacement Regenerative Therapy

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Abstract

Donor organ transplantation is currently an essential therapeutic approach to the replacement of a dysfunctional organ as a result of disease, injury or aging *in vivo*. Recent progress in the area of regenerative therapy has the potential to lead to bioengineered mature organ replacement in the future. In this proof of concept study, we here report a further development in this regard in which a bioengineered tooth unit comprising mature tooth, periodontal ligament and alveolar bone, was successfully transplanted into a properly-sized bony hole in the alveolar bone through bone integration by recipient bone remodeling in a murine transplantation model system. The bioengineered tooth unit restored enough the alveolar bone in a vertical direction into an extensive bone defect of murine lower jaw. Engrafted bioengineered tooth displayed physiological tooth functions such as mastication, periodontal ligament function for bone remodeling and responsiveness to noxious stimulations. This study thus represents a substantial advance and demonstrates the real potential for bioengineered mature organ replacement as a next generation regenerative therapy.

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Introduction

Donor organ transplantation is currently essential to replace a dysfunctional organ and to restore organ function *in vivo* [1,2]. This approach is problematic for clinicians however as donor organs are constantly in short supply [2,3]. An attractive new concept in current regenerative therapy that may possibly replace conventional transplantation in the future is stem cell transplantation therapy [4,5] or a two-dimensional uniform cell sheet technique [6,7] to repair the local sites of the damaged tissues and organs [8]. The ultimate goal of regenerative therapy in the future is to develop organ replacement regenerative therapies that will restore lost or damaged tissues following disease, injury, or aging with a fully functioning bioengineered organ [9,10,11]. To construct a bioengineered organ, one of two major concepts is to construct fully functional artificial organs using three-dimensional tissue-engineering technology, involving biodegradable materials and various cell types, that can immediately function after transplantation *in vivo* [12,13,14]. However, further technological developments are required to create such artificial organs which can immediately function [15].

For the regeneration of ectodermal organs such as a tooth, hair follicle or salivary gland [16,17], a further concept has been proposed in which a bioengineered organ is developed from bioengineered organ germ by reproducing the developmental processes that take place during organogenesis [11,18]. Tooth regenerative therapy is thought to be a very useful study model for organ replacement therapies [11,19,20]. The loss of a tooth causes fundamental problems in terms of oral functions, which are achieved in harmony with the teeth, masticatory muscles and the temporomandibular joint under the control of the central nervous system [21]. It has been anticipated that a bioengineered tooth could restore oral and physiological tooth functions [19]. We have previously developed a three-dimensional cell manipulation method, designated the organ germ method, for the reconstitution of bioengineered organ germ, such as a tooth or whisker follicle [22]. This bioengineered tooth erupted with the correct structure, occluded at the lost tooth region in an adult mouse. It also showed sufficient masticatory performance, periodontal functions for bone remodeling and the proper responsiveness to noxious stimulations [20]. This previous study thus provided a proof of concept that