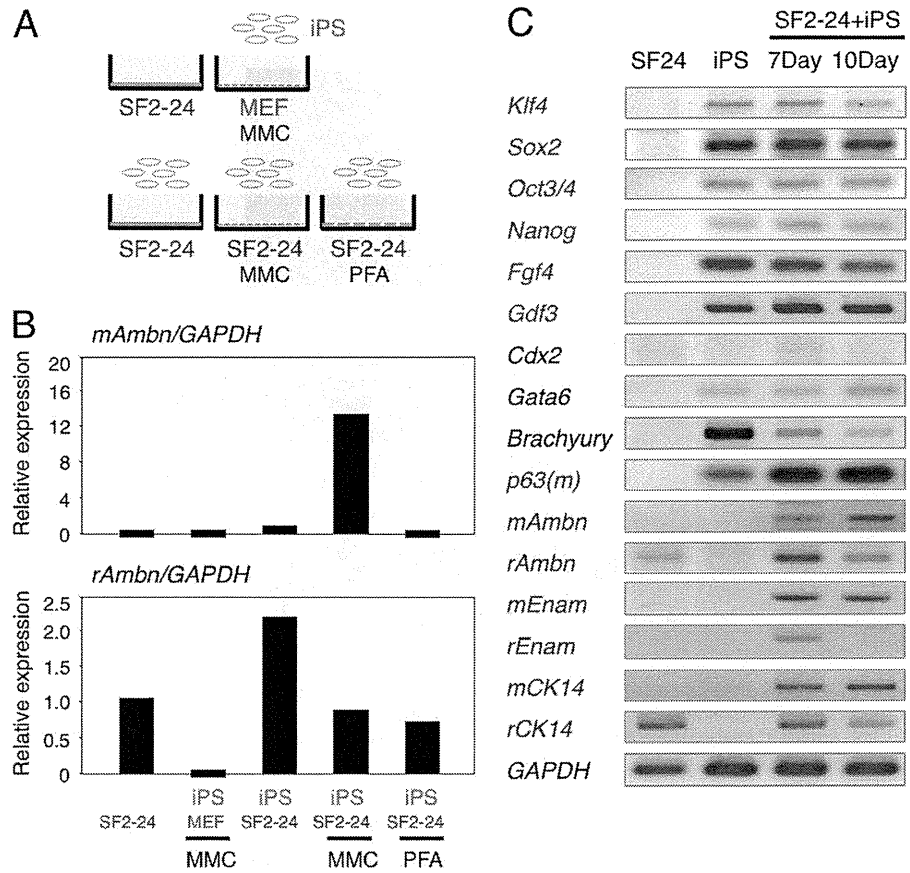

FIGURE 7. **Effects of culture conditions on ameloblast induction of iPS cells.** *A*, iPS cells were co-cultured with SF2-24 cells, MMC-treated (*MMC*) MEFs, MMC-treated SF2-24 cells or PFA-treated SF2-24 cells. *B*, Ambn expression in mouse iPS (*upper panel*) and rat-derived SF2-24 (*bottom panel*) cells in different co-culture conditions for 10 days. *C*, time course analysis of gene expressions of stem cell (*blue*), endo/mesoderm (*black*), and ameloblast (*red*) markers in iPS cells co-cultured with SF2-24 cells for 7 (*7Day*) and 10 days (*10Day*).

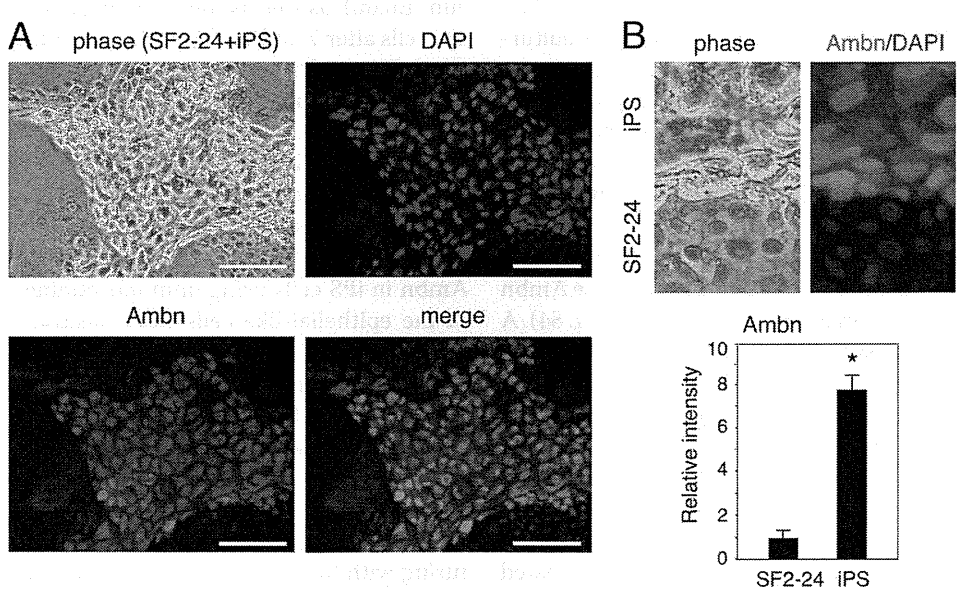

FIGURE 8. **Expression of Ambn, an ameloblast specific protein, in iPS cells co-cultured with SF2-24 cells.** *A*, phase micrographs of iPS cell colonies cultured with mitomycin C-treated SF2-24 cells. Hoechst staining (*blue*), Ambn staining (*red*), and merged images. *B*, high magnifications of phase and merged images in *A*. *Bottom panel*, relative expression levels of Ambn protein in SF2-24 and iPS cells cultured in ameloblast induction system. *, $p < 0.05$; Bar, 100 mm.

proteins (AB1, -2, or -3) were added to cultures of iPS cells. Conditioned media from SF2-24 cells and full-length AMBN-expressing cells, but not from other transfectants or recombinant Ambn proteins, induced Ambn expression in iPS cells (Fig. 9*D*), indicating that Ambn may be necessary for differentiation of iPS cells into dental epithelium. Previously, we showed that neurotrophic factor NT-4 is important for the differentiation of ameloblasts (29). To examine the effect of NT-4

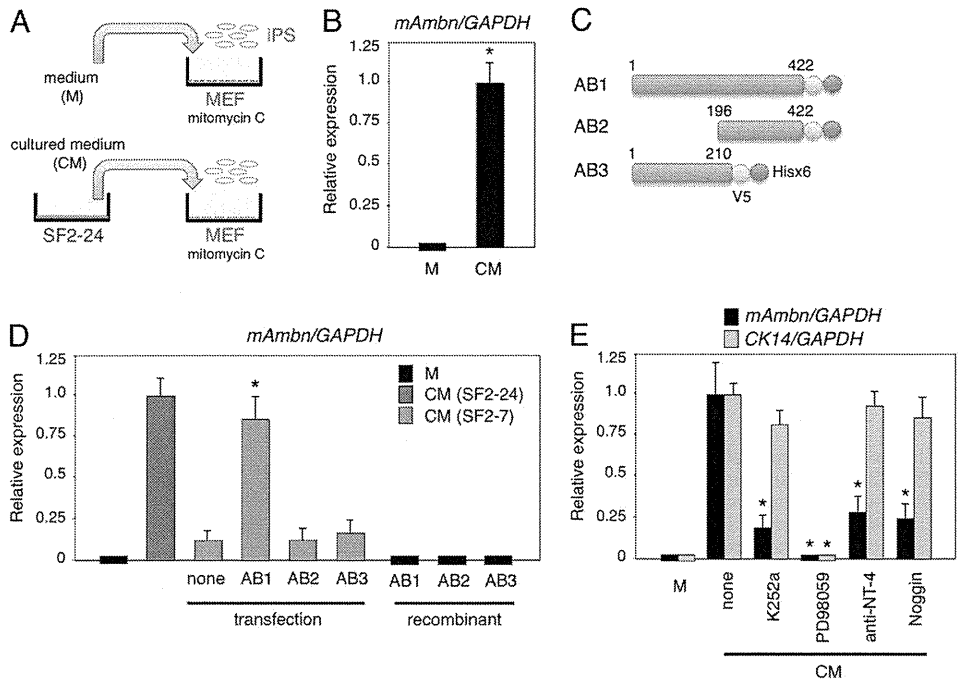

FIGURE 9. **Promotion of ameloblast induction of iPS cells using conditioned SF2-24 cells.** *A*, iPS cells were cultured on mitomycin C-treated MEFs in iPS cell culture medium supplemented with (*CM*) or without (*M*) conditioned medium from SF2-24 cells. *B*, expression of mouse Ambn gene in iPS cells cultured in iPS cell culture medium supplemented with (*CM*) or without (*M*) conditioned medium from SF2-24 cells. *C*, creation of Ambn deletions. All recombinant Ambn proteins have V5 and His tags at the C terminus. *D*, expression of mouse Ambn gene in iPS cells cultured in iPS cell culture medium supplemented with (*CM*) or without (*M*) condition medium from SF2-24 cells, recombinant Ambn-expressing SF2-7 cells or recombinant Ambn proteins. *, $p < 0.05$ (compared with non-transfected SF2-7 cells). *E*, expression of mouse Ambn and CK14 genes in iPS cells cultured in SF2-24 conditioned medium supplemented with K252a, PD98059, anti-NT-4, or Noggin. *, $p < 0.05$ (compared with CM only).

on dental epithelial cell differentiation by iPS cells, we analyzed the expressions of Ambn and CK14 in iPS cells cultured with SF2-24-conditioned medium in the presence of K252a (inhibitor of neurotrophic receptor Trk), PD98059 (MEK inhibitor), anti-NT-4 neutralizing antibody, or Noggin (BMP antagonist). K252a, PD98059, anti-NT-4, and Noggin each inhibited the expression of Ambn in iPS cells. Furthermore, CK14 expression in iPS cells was not inhibited by K252a, anti-NT-4, or Noggin (Fig. 9*E*). These results indicate that NT-4 and BMP signaling are important for differentiation into dental epithelial cells, but not CK14-positive epithelial cells.

DISCUSSION

Tooth development progresses through a number of stages, and the differentiation of dentin matrix-secreting odontoblasts and enamel matrix-producing ameloblasts results in formation of the crown. Ameloblasts and odontoblasts are central cell types involved in tooth development. In developing molars, restricted dental mesenchymal cells interact with the inner dental epithelium through the matrix and differentiate into odontoblasts. In the present study, we established an SP cell line from dental papilla mDP cells using cell sorting with Hoechst staining. SP cells are known to retain multipotency characteristics and can differentiate into various cell types, such as odontoblasts, osteoblasts, adipocytes, and neural cells. Our method for obtaining multipotent SP cells from a single cell line may be useful for development of novel therapeutic strategies that aim at regeneration of oral tissues.

Our co-culture assay of SP cells with dental epithelial cells showed that dental epithelial cells promote SP cell differentiation into DSPP-expressing cells via BMP2 and BMP4, which are secreted from dental epithelial cells (Fig. 5*B*, 5*D*, and 10*A*). Because BMP2 is not highly expressed in dental epithelium, BMP4 may be the dominant signaling regulator during odontoblast differentiation. In the early stages of tooth development, BMP4 is expressed in dental epithelium and induces the transcription factor Msx1 (30). The expression of DSPP is induced via the BMP signaling pathway in cooperation with Runx2, Dlx5, and Msx1 in undifferentiated mesenchymal cells (31). Previously, a bead soak assay of mandibular organ culture showed that BMP4 induced dental mesenchymal cell differentiation (32). Also, a transgenic approach revealed that inhibition of BMP4 by Noggin overexpression, driven by a keratin 14 promoter (K14-Noggin), resulted in the absence of all molars in the mandible. This indicates that BMP4 is essential for tooth bud formation by inducing dental mesenchymal cells (33). As demonstrated, in the present study odontoblastic differentiation of SP cells is completely disturbed by the blocking of BMP signaling. Thus, our finding strongly support the notion that BMP4 signaling is a key factor in induction of dental mesenchymal cells and their differentiation.

Differential synchronization between dental epithelial and mesenchymal cells has been observed during tooth development. Dental epithelial and mesenchymal cells are separated by a basement membrane, which is an essential regulator for epithelial-mesenchymal interaction (34). Both crown and root odontoblasts are induced by interactions with epithelial cells, such as those of the inner dental epithelium, epithelial rest, and epithelial diaphragm (35). Similar to *in vivo* situations, physical

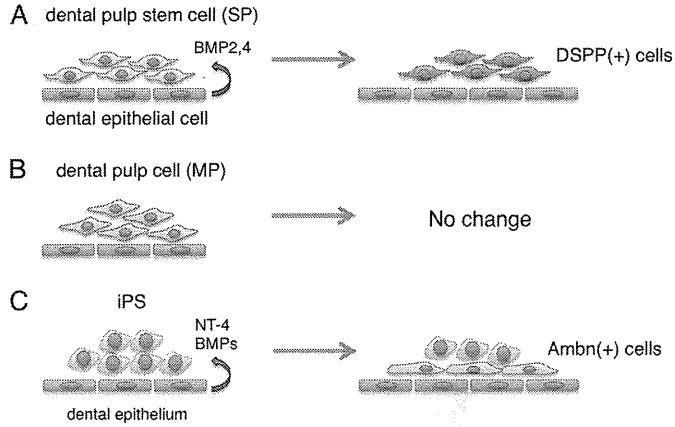

FIGURE 10. **Proposed models of odontogenic induction from dental mesenchymal stem cells and iPS cells by co-culturing with dental epithelial cells.** *A*, dental epithelial cells induce DSPP-expressing odontoblasts from SP cells. *B*, no odontogenic induction was observed in differentiated (*MP*) cells co-cultured with dental epithelial cells. *C*, dental epithelial cells induce Ambn-expressing ameloblasts from iPS cells.

cell attachment of dental epithelial cells was not required for odontogenic induction of SP cells in our experiments, indicating that soluble factors including BMPs are important for odontogenic induction by dental epithelial cells in culture. We also found that MP cells from dental papilla did not differentiate into DSPP-expressing cells, indicating that epithelial-mesenchymal interactions are important for cell fate determination of dental pulp stem cells, but not for differentiated dental pulp cells (Fig. 10, *A* and *B*). It was recently reported that Ambn protein, or a synthetic peptide based on the N-terminal region of the Ambn protein, induced osteoblastic cell differentiation (36). In addition to BMPs, Ambn may also be one of the factors involved in the odontogenic induction process, because the sharing of signaling pathways underlies the mechanism of odontoblastic and osteoblastic induction.

Ameloblasts secrete enamel-specific extracellular matrices including Ambn, which are lost upon tooth eruption. This makes it impossible to repair or replace damaged enamel in an erupted tooth. Therefore, identifying alternative sources of these cells becomes important. Bone marrow-derived cells can give rise to different types of epithelial cells. In mixed cultures with c-Kit+-enriched bone marrow cells, embryonic dental epithelial cells, and dental mesenchyme, bone marrow cells might be reprogrammed to give rise to ameloblast-like cells (37). Our strategy to create ameloblasts from mouse iPS cells may have direct application in tooth regeneration. We succeeded in establishing a co-culture system using cells derived from two different species, mouse iPS cells and rat derived enamel matrix secreting ameloblasts. This is the first demonstration of differentiation of iPS cells into ameloblasts through interactions with dental epithelium (Fig. 10*C*). However, a set of stem cell markers was continuously expressed in iPS cells after 7 days of co-culturing (Fig. 7*C*), indicating that a portion of the iPS cells had differentiated into enamel-secreting ameloblasts and some still retained stem cell potential. Thus, the efficacy of iPS cell differentiation into ameloblasts by enamel-secreting ameloblasts feeder cells must to be improved prior to for clinical application.

A number of factors are thought to give iPS cells the capacity for direct or indirect differentiation into ameloblasts. Possible direct effectors include gap junctions, intercellular binding molecules, adhesion factors, and extracellular matrices secreted by dental epithelium. Growth factors might also be involved, because conditioned medium from SF2-24 cells induced Ambn expression in iPS cells. Ambn is also a candidate factor for dental cell differentiation of iPS cells, as SF2 cells expressing low levels of Ambn did not induce differentiation of iPS cells. Furthermore, overexpression of full-length Ambn in cells expressing low levels of Ambn induced iPS cells into ameloblast-like differentiation (Fig. 9*D*). Ambn has diverse functions in various cellular physiologies, such as cell growth, differentiation, cell polarization, and attachment, though the detailed mechanisms of Ambn signaling require additional investigation. Ambn-null mice display severe enamel hypoplasia due to impaired dental epithelial cell proliferation, polarization, and differentiation into ameloblasts, as well as loss of cell attachment activity with immature enamel matrix (2). These results suggest that Ambn, especially full-length, is necessary for both *in vivo* and *in vitro* ameloblast differentiation.

There were differences in cell lineage determination of the dental pulp stem cells and iPS cells when co-cultured with dental epithelial cells. RT-PCR analysis showed that co-culturing induced SP cells to form odontoblastic cells, whereas iPS cells were induced to form ameloblastic cells. In addition, the expression of Brachyury, a mesodermal marker, in iPS cells was down-regulated by co-culturing with SF2-24 cells (Fig. 7*C*). Conversely, expressions of the epithelial markers p63 and CK14, as well as the dental epithelial marker epiprofin/Sp6 were up-regulated (Fig. 7*C*, supplemental Fig. S5) (28). These results suggest that the cell lineage of the iPS cells in our co-culturing system was effectively guided into an epithelial cell lineage. It has been reported that the default cell lineage of ES cells is the ectodermal cells, except when cultured in the presence of BMP antagonists (38, 39). Because BMPs promote ectodermal differentiation of ES cells, the expression of BMP observed in SF2 cells (Fig. 5*D*) may also contribute to dental epithelial cell differentiation of iPS cells. A previous our reported that NT-4 induced Ambn expression in dental epithelium, while NT-4 knock-out mice showed delayed expression of enamel matrices in the early stage of ameloblast differentiation (29). In the present study, the presence of the anti-NT-4 neutralizing antibody or Noggin in conditioned medium from SF2-24 cells inhibited Ambn expression, but not that of CK14 (Fig. 9*E*). On the other hand, SP cells strongly expressed the endogenous Sox2 protein, one of the reprogramming factors involved in generation of iPS cells (data not shown). Recently, iPS cells were generated from human dental pulp cells with a high level of efficiency in comparison to dermal fibroblasts, possibly due to a high expression level of Sox2 in dental pulp stem cells. However, additional reprogramming factors are required for creation of iPS cells from dental pulp cells. Thus, SP cells themselves did not have the same degree of multipotency as seen with ES and iPS cells. SP cells are considered to be mesenchymal stem cells that originate from dental pulp cells, which are derived from cranial neural crest cells. Neural crest cells can differentiate into several different cell lineages, such as neuron, glia, melanocyte,

osteoblast, chondrocyte, and odontoblast cells (40, 41). We believe that SP cells are not able to gain multipotency beyond the potential of neural crest cells. Thus, SP cells preserve some degree of multipotency that is different in an undifferentiated state as compared with ES and iPS cells. In co-cultures with SF2-24 cells, SP cells did not differentiate into ameloblasts, whereas iPS cells did (Fig. 10). Comparative analysis between SP and iPS cells is essential to clarify the mechanisms involved in directional cell fate determination.

In this study, we sought to clarify the role of dental epithelium and stem cell interactions by culturing rat dental epithelium with mouse iPS cells and SP cells. Rodent incisors grow throughout the lifespan of the animal by maintaining stem cells in the cervical loop, located at the end of incisor. A dental epithelial cell niche also exists in the cervical loop of the incisor. Analysis of gene knock-out mice for epiprofin/Sp6, an essential transcription factor for dental epithelial cell differentiation and enamel formation, has revealed that supernumerary teeth are formed by interactions between dental mesenchyme and undifferentiated dental epithelium (4, 42). In addition, those studies showed continuous signals from dental epithelial cells of mutant mice induced the continued differentiation of dental mesenchymal cells into odontoblasts (4, 42). Together these findings suggest that dental epithelial cells can induce dental mesenchymal cells to differentiate into odontoblasts. Therefore, rat dental epithelial cells may provide an *in vitro* niche environment for surrounding mouse iPS cells and SP cells. Elucidation of the mechanism of cell fate determination by dental epithelial cells may facilitate development of novel therapeutic approaches for regenerative dentistry.

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Expert Opinion

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Extracellular matrix administration as a potential therapeutic strategy for periodontal ligament regeneration

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Introduction: The current strategies employed for the treatment of connective tissue disease include the application of stem cells, the use of functional molecules that can reorganize tissue integrity and cellular activities to recover connective tissue function. Approaches to the regeneration of periodontal tissue, which is the tooth-supporting connective tissue, have made some progress recently and provide a useful experimental model for the evaluation of future strategies to treat connective tissue diseases such as periodontal disease.

Areas covered: The ultimate goal of periodontal tissue regeneration is to reconstruct the ligament structure that will sustain the required mechanical force to connect with mineralized tissues such as cementum and alveolar bone. In this review, we discuss the proposed use of extracellular matrix (ECM) administration therapy as an additional therapeutic strategy to stem cell transplantation and cytokine administration in the current field of periodontal tissue regeneration therapy.

Expert opinion: Although various available tissue engineering technologies can now achieve periodontal tissue regeneration, ECM administration therapy is likely to play an essential future role in the development and regeneration of periodontal tissue and attenuate the signaling events that mediate tissue degradation. Hence, ECM administration could serve as a novel technology in periodontal tissue regeneration and also as a viable approach to alleviating connective tissue disorders such as Marfan's syndrome.

Keywords: connective tissue, microfibril, PDL, regenerative therapy

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1. Introduction

Periodontal tissue is a tooth-supporting tissue comprising periodontal ligament (PDL), cementum and alveolar bone. This tissue thereby plays an important role in the maintenance of the occlusion system. Among the components of periodontal tissue, the PDL consists mainly of an extracellular matrix (ECM) that provides the physical properties to withstand mechanical stress in cooperation with the cementum and alveolar bone. Dysfunction of the PDL occurs as a result of periodontal disease, an inflammatory disorder involving the irreversible destruction of periodontal tissues and requiring the regeneration of PDL as a treatment for recovering occlusion function [1,2]. Periodontal disease is caused by pathogenic microflora including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, and the resulting inflammation extends deep into the periodontal tissue and causes the loss of PDL, cementum and alveolar bone [3]. Chronic periodontal disease is the most common form of this disorder, showing a prevalence rate of > 90% in adults

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Article highlights.

- Periodontal tissue regeneration aims to reconstruct the ligament structures in the tooth-supporting connective tissue.
- Periodontal ligament (PDL) stem cells can be used to reconstitute the PDL structure, including extracellular components.
- PDL stem cells (PDLSCs) express mesenchymal stem cell markers STRO-1 and CD146/MUC18 and show a similar phenotype to dental follicle stem cells (DFSCs).
- PDL cell sheets may induce periodontal regeneration, including reforming the PDL and cementum, and could provide an *in vivo* treatment for periodontal disease.
- The local application of human recombinant cytokines such as fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP)-2 and TGF-β stimulates and promotes the regeneration of periodontal tissues in animal models.
- Fibrillin-1 microfibril network is also important for PDL function and maintaining connective tissue integrity. Targeting the extracellular matrix (ECM) and fibrillin-1 microfibrils may offer a new administration therapy for periodontal disease.

This box summarizes key points contained in the article.

over 60 years of age [4]. Furthermore, this disorder is the major cause of tooth loss in adults of over 40 years and its more severe forms has a worldwide prevalence of up to 20% according to the World Health Organization [5]. Blocking the progression of periodontal disease has been achieved by mechanically removing bacterial biofilm with conventional periodontal and/or surgical treatments. These treatments can reduce the destruction of periodontal tissue and diminish inflammation in the affected region. However, achieving adequate periodontal tissue regeneration remains a problem, particularly in cases where the disease has caused large defects in the periodontal tissue.

The current advances in future regenerative therapies have been influenced by many previous studies of embryonic development, stem cell biology and tissue engineering technologies [6-9]. To restore the partial loss of organ functions and to repair damaged tissues, attractive concepts that have emerged in regenerative therapy is stem cell transplantation into various tissues and organs [10] and cytokine therapy, which has the potential toinduce the activation and differentiation of tissue stem/progenitor cells [11]. Tooth tissue stem cells and the cytokine network that regulates tooth development, and dental tissue cell growth and differentiation, have been well characterized at the molecular level [12,13]. The regeneration of periodontal tissues is being made clinically possible by the transplantation of mesenchymal stem cells which can differentiate into PDL cells, cementoblasts and osteoblasts, or through the local application of cytokines to stimulate the proliferation and differentiation of these stem cells [14-17]. Although these therapies are effective and contribute to periodontal tissue repair, these interventions will likely be improved by an enhanced understanding of the development of periodontal tissues, particularly those involved in the formation of PDL, cementum and alveolar bone.

The ECM is a biologically active molecule composed of a complex mixture of macromolecules that, in addition to serving a structural function, profoundly affect the cellular physiology of an organism [18]. Previous findings have revealed that ECM components including type I collagen, type III collagen, lumican, decorin, periostin, f-spondin, tenascin-N and PLAP1/aspirin are highly expressed during PDL formation [19,20]. Since the ECM is regulated in a tissue-specific manner, these structures could enhance periodontal regeneration by promoting the differentiation of cells required for the synthesis of PDL, bone and cementum [21,22]. Among the ECM formations in the PDL, fibrillin-1, a major component of the microfibrils that regulate tissue integrity and elasticity, has been shown to contribute to the formation and maintenance of this ligament. An abnormal PDL structure in association with the progressive destruction of microfibrils has been observed in a Marfan's syndrome (MFS) mouse model and has characteristics that are similar to those of fibrillin-1 dysfunction [23]. These findings have strongly suggested that microfibril formation through fibrillin-1 assembly plays an important role in PDL formation and function. However, the molecular mechanisms of fibrillin-1 microfibril assembly remain unclear as the microfibril-associated molecule that regulates or stabilizes fibrillin-1 microfibril formation has not yet been identified. Recent findings have revealed that ADAMSL6β is essential for the development and regeneration of the PDL through the direct interaction of fibrillin-1 to promote microfibril assembly [23,24]. These findings have also suggested that the administration of fibrillin-1 microfibrils provides a novel therapeutic strategy for the treatment of periodontal disease.

We here review the present status of the periodontal tissue regeneration technologies that focus on the molecular mechanisms underlying development, regeneration and tissue engineering of periodontal tissue, and also discuss the potential of ECM administration therapy through the promotion of microfibril assembly as a novel therapeutic strategy for the essential functional recovery of periodontal tissue.

2. Development processes in periodontal tissue

The PDL has essential roles in tooth support, homeostasis and repair, and is involved in the regulation of periodontal cellular activities such as cell proliferation, apoptosis, the secretion of extracellular matrices, resorption and repair of the root cementum and remodeling of the alveolar bone [25-27]. To develop future methods to regenerate damaged PDLs, it will be important to understand the molecular basis of PDL development.

2.1 Molecular mechanisms underlying periodontal tissue development

The PDL is derived from the dental follicle (DF), which is located within the outer mesenchymal cells of the tooth germ and can generate a range of periodontal tissues including

the PDL, cementum and alveolar bone [21]. The DF is formed during the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from the cranial neural crest cells [28]. Given the critical role that the progenitor cell population in the DF appears to play in the development of periodontal tissue, the developmental processes in this tissue are of considerable interest in terms of further understanding the biology of these cells (Figure 1). The differentiation of the DF proceeds as follows: i) during the tooth root-forming stage, the Hertwig's epithelial root sheath (HERS) comprising the inner- and outer-dental epithelia that initiate tooth root dentin formation is fragmented into the Malassez epithelium resting on the tooth root surface; ii) the DF migrates to the surface of the tooth root and differentiates into cementoblasts to form the cementum matrix [29,30]; iii) at almost the same time, the DF differentiates into the PDL on the cementoblasts in order to insert collagen fibers, known as Sharpey's fibers, into the cementum matrix. Fiber insertion also takes place along the alveolar bone and iv) both bone- and PDL-derived fibers finally coalesce in the PDL to form the intermediate plexus, which resembles tendinous tissue [31-33].

The DF has long been considered to be a source of multipotent stem cells (DFSCs), since these cells have the ability to migrate onto the tooth root surface to form periodontal tissue including cementum, PDL and alveolar bone during the tooth root-forming stage [32,34-37]. Previous studies have indicated that DF cells can form PDL-like tissues and cementum/bone-like structures after implantation into immunodeficient mice [38,39], supporting the notion that stem cells which can differentiate into PDL, cementoblast, osteoblast lineages are present in the DF [34,35]. To regenerate periodontal tissue, functional molecules which promote the differentiation of DFSCs into PDL need to be elucidated to enable a proper understanding of the mechanisms underlying periodontal tissue formation, including the pathways pertaining to PDL cell, cementum and alveolar bone differentiation.

2.2 Functional molecules involved in DF differentiation

Although the molecular mechanisms of DF development and differentiation remain to be determined, previous gene expression studies of mouse molar root development have suggested that some growth factors, including bone morphogenetic protein (BMP) 4, growth and differentiation factors (GDFs) 5, 6 and 7 [40-43], epidermal growth factors [44], *Shh* [45-47] and insulin-like growth factor (IGF)-1 [48], are involved in the growth or differentiation of the DF. Transcriptional factors such as *Scleraxis*, *Gli*, *Msx1*, *Msx2* and *Runx2* have also been shown to be involved in the differentiation of the DF into cementoblasts and in the mineralization of cementum [39,43,46,49]. Among these factors, GDFs and scleraxis are the most well characterized that are involved in tendon/ligament morphogenesis, suggesting that PDL development shares similar molecular mechanisms to those of tendon/ligament morphogenesis. With regard to cementogenesis/osteogenesis of the DF, treatment of this tissue with BMP-2 and BMP-7 has been found to induce mineralization ability. In addition, previous findings suggest that PDL cells harbor mineralization inhibitory mechanisms that enable them to maintain a ligament structure across the mineralized tissue, including the alveolar bone and cementum, during PDL development [50-52]. These observations strongly suggest that the tendon/ligament-related cytokines, the BMPs, and inhibitors of mineralization are linked to the restoration of the tendinous structure of the PDL. The mechanisms involving these factors may also have a role in preventing ankylosis of the PDL.

3. Regeneration therapies for PDL defects

A partial restoration of periodontal tissue has been achieved previously using a guided tissue regeneration (GTR) technique which provides an adequate space and favorable niche for the repair of periodontal defects using barrier membrane [53]. From the results of these GTR therapies, regeneration of the PDL has been shown to be critical for recovering the connection between the cementum on the root surface and the alveolar bone.

To regenerate periodontal tissue that has been destroyed by periodontal disease requires the recruitment of PDL stem cells (PDLSCs) to properly reconstitute the PDL structure including its extracellular components such as the collagen and elastic fibril systems [32,33]. Recent studies of stem/progenitor cells have provided considerable new insights that have furthered our understanding of PDLSCs, which can differentiate into periodontal tissue cell lineages such as PDL, cementum and alveolar bone [14,54]. PDLSCs will have utility for the future development of stem cell transplantation therapies and tissue engineering applications to restore periodontal organ function as they replace damaged areas with enriched and purified stem cells and thereby achieve PDL repair (Figure 2) [14]. The biological potential of PDLSCs to stimulate the regeneration of periodontal tissue can now be realized by the local application of human recombinant cytokines.

3.1 Stem cell therapies

PDLSCs have been isolated from human PDL tissue by single-colony selection and magnetic activated cell sorting. PDLSCs express the mesenchymal stem cell markers STRO-1 and CD146/MUC18, and can differentiate into cementoblast-like cells, adipocytes and fibroblasts [14]. In addition, PDLSCs show the capacity to generate a cementum/PDL-like structure and contribute to periodontal tissue repair on transplantation into immunocompromised rodents. Clonal PDLSC analysis has further revealed that these cells show a similar phenotype to DFSCs since they also express *RUNX-2*, *Col I*, *ALP*, *OPN*, *OCN*, *RANKL*, *OPG*, *scleraxis*, *periostin*, *Col XII* and *alpha-SMA* mRNAs [54]. Importantly, PDL tissue collected from one tooth can give rise to many stem cells because of their high proliferation capacity *ex vivo*. Recently also, it has been shown that the transplantation of autologous PDLSCs obtained from the

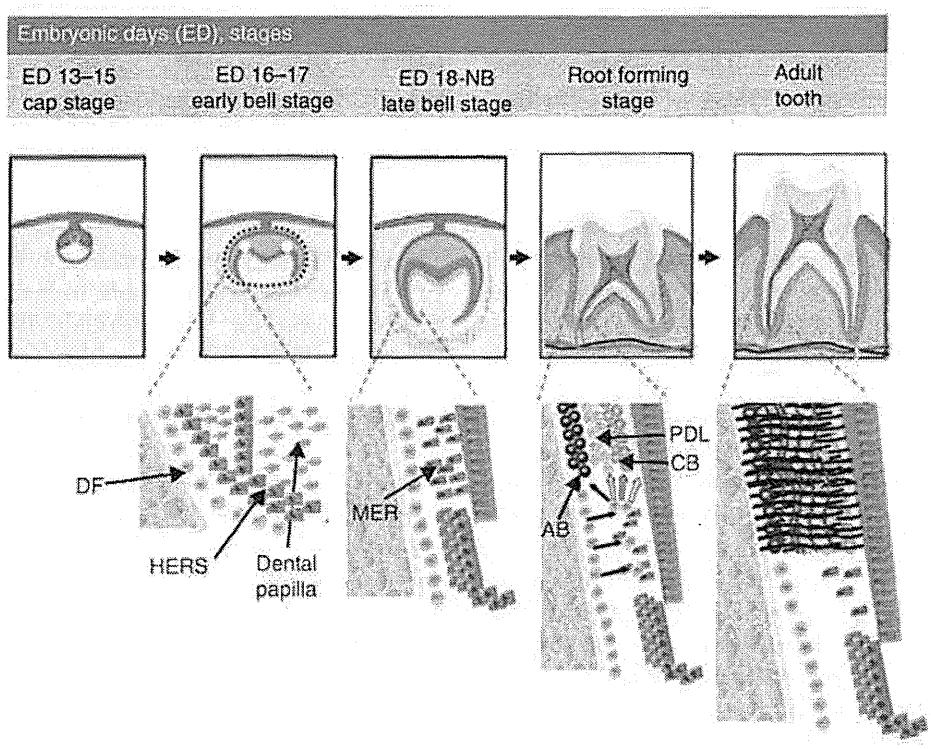

Figure 1. Developmental processes in the PDL (upper panel). PDL development originates from the DF located on the periphery of the tooth germ (arrows). The DF is generated from the dental mesenchyme during the cap stage of tooth germ development in the embryo. Development of the DF progresses during the early- and late-bell stages of the tooth germ, but no morphological changes are observed. Differentiation of the DF begins during the tooth root-forming stage and the mature PDL is subsequently formed (lower panel). DF differentiation commences after the tooth root dentin is formed by reciprocal interaction between the HERS and dental papilla. During tooth root dentin formation, the HERS is fragmented into the Malassez epithelial rest and the DF is then capable of migrating onto the root dentin to be differentiated into cementoblasts, PDL and alveolar bone to connect the tooth root and jaw bone (adult tooth).

PDL: Periodontal ligament; DF: Dental follicle; HERS: Hertwig's epithelial root sheath.

extracted teeth of miniature pigs can regenerate and repair a surgically created periodontal defect [55]. This finding suggests that PDLSCs obtained from an easily accessible tissue resource and expanded *ex vivo* using wisdom teeth might represent a feasible therapeutic approach to the reconstruction of tissues destroyed by periodontal disease.

In addition to the clinical application of stem cell transplantation, cell sheet engineering therapies for periodontal tissue regeneration are now being developed for clinical application [56,57]. In this technology, temperature-responsive dishes are used to harvest the cell sheets through a simple decrease in the temperature, thus avoiding the use of proteolytic enzymes [58]. The use of this method allows PDL cell sheets to be easily harvested and transplanted into periodontal defects *in vivo* [56,57,59,60]. PDL cell sheets have the potential to induce periodontal regeneration, including the reformation of the PDL and cementum. The available data also suggest that this technique has the appropriate efficacy for periodontal regeneration in patients with periodontal disease.

3.2 Cytokine therapies

Some new treatments that accelerate the regeneration of periodontal tissue by local application of human recombinant cytokines have now been established. This approach stimulates the proliferation and differentiation of stem cells/progenitors from the PDL into hard tissue-forming cells. The local application of human recombinant cytokines such as platelet-derived growth factor (PDGF) and IGF-1 [16,61], BMP-2 [62,63], TGF-β [64], osteogenic protein (OP)-1 [65] and brain-derived neurotrophic factor (BDNF) [66] stimulates and promotes the regeneration of regional periodontal tissue in animal models. The potency of PDGF-BB plus β-tricalcium phosphate (β-TCP, an osteoconductive scaffold) in periodontal tissue regeneration in human has also been recently reported [67]. In addition, a clinical Phase I study of fibroblast growth factor (FGF)-2 has shown that it stimulates the regeneration of periodontal tissue lost due to periodontal disease and demonstrated the safety of this treatment [15]. The results of this trial were clinically interpreted as a demonstration of

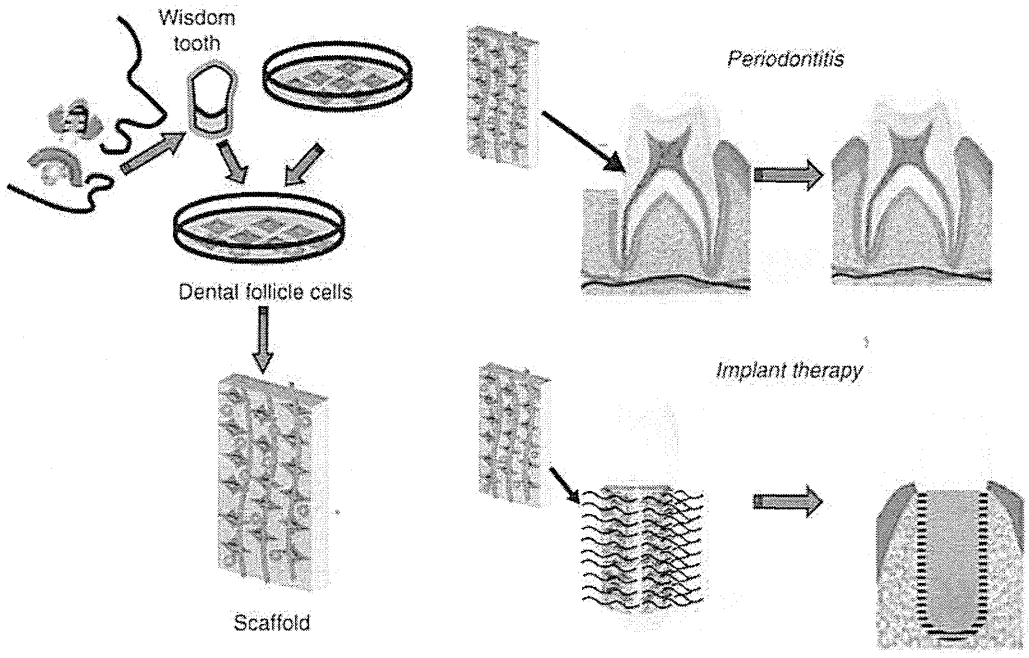

Figure 2. Potential approach to regeneration therapy for periodontal disease. By using cell sheet-engineering, stem cells obtained from the periodontal ligament (PDL) or dental follicle (DF) of wisdom tooth germ are harvested as temperature sensitive sheets for transplantation into periodontal tissue 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

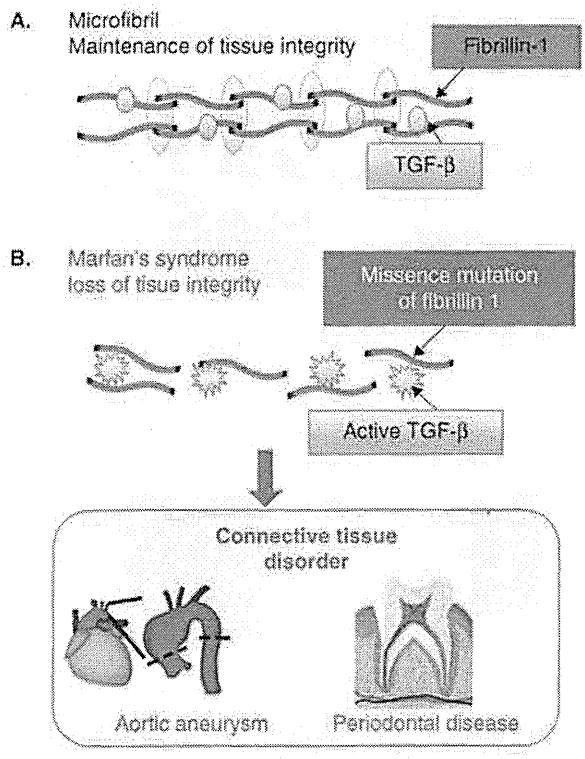

Figure 3. Schematic representation of the pathogenic mechanisms of MFS. A. Fibrillin-1 comprises insoluble extracellular matrix components in connective tissue microfibrils and provides limited elasticity to tissues through fibrillin-1 microfibril formation. **B.** Missense mutations in the *fibrillin-1* 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 ADAMTSL6β 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, ADAMSL6β is essential for the development and regeneration of the PDL [23]. ADAMTSL6β 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 ADAMTSL6β [83,84]. In an animal model of MFS microfibril disorder [85], ADAMSL6β expression can rescue fibrillin-1 microfibril formation through the promotion of fibrillin-1 microfibril assembly (Figure 4A). More importantly, the local administration of ADAMTSL6β 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 ADAMTSL6β 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 ADAMTSL6β 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 ADAMTSL6β during

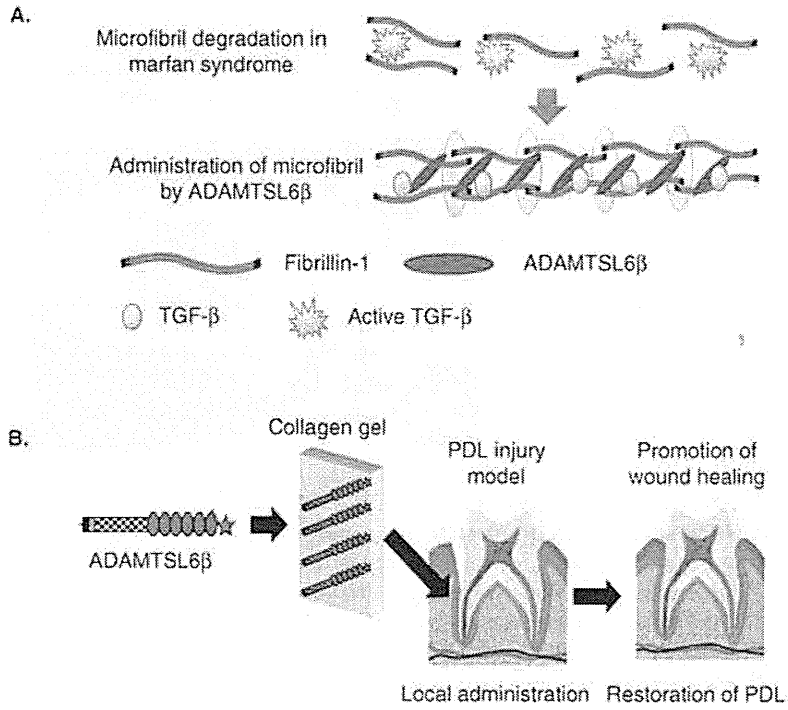

Figure 4. Microfibril administration by administration of ADAMTSL6β. A. Administration of microfibrils by ADAMTSL6β An ECM administration therapy that induces restoration of properly formed microfibrils via ADAMTSL6β is essential not only for improvement of the microfibril disorder, which is a predominant 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

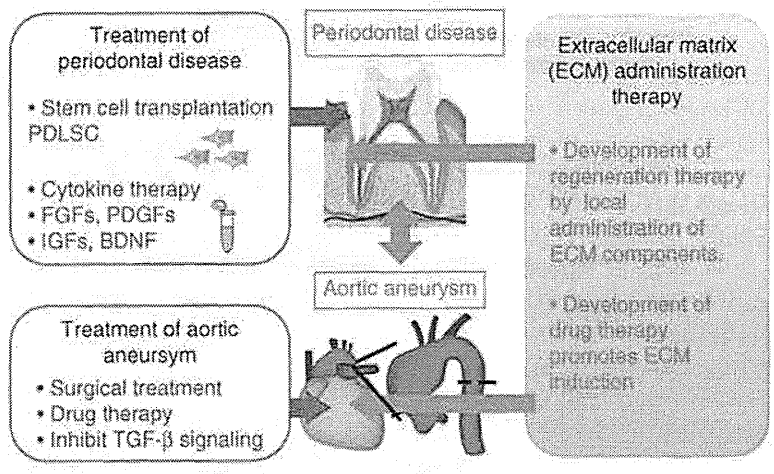

Figure 5. Extracellular matrix (ECM) administration therapy as a novel therapeutic strategy for MFS syndrome. Left panel: A variety of MFS therapies have been developed, including surgical therapy for aortic root aneurysms, traditional medical therapies and mechanisms to deregulate TGF-β activation and 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 ADAMTSL6β 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 ADAMTSL6β expression for the treatment of aortic aneurysms.

administration therapy through the promotion of microfibril assembly by ADAMTSL6β 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 ADAMTSL6β, 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 ADAMTSL6β improves fibrillin-1 microfibril assembly, indicating that the reinforcement of fibrillin-1 microfibrils by ADAMTSL6β 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 ADAMTSL6β 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 ADAMTSL6β 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 ADAMTSL6β has the capacity to facilitate drug discovery for treating periodontal diseases, and MFS-associated disorders.

Declaration of interest

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ADAMTSL6β 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 ADAMTSL6β.
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 ADAMTSL6β 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. ADAMTSL6β (A disintegrin-like metalloprotease domain with thrombospondin type I motifs-like 6β) 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 ADAMTSL6β plays an essential role in the development and regeneration of connective tissues. ADAMTSL6β 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 ADAMTSL6β 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 ADAMTSL6β to fibrillin-1 microfibril formation. These findings also suggest a new therapeutic strategy for the treatment of MFS through ADAMTSL6β-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 is in 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 ADAMTSL6α and -6β 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, ADAMTSL6β 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 ADAMTSL6β as a novel MFS therapy by promoting fibrillin-1 microfibril assembly and regulating TGF-β activation.

In our current study, we report that ADAMSL6β 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 ADAMSL6β 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 ADAMTSL6β 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 ADAMTSL6β is essential for fibrillin-1 microfibril formation and suggest a novel therapeutic approach to the treatment of MFS through the promotion of ADAMTSL6β-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-μm 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.

ADAMTSL6β cDNA—As described previously (17), *ADAMTSL6β* or *Adamtsl6β* was cloned into p3XFLAG-

CMV-14 to generate p3XFLAG-CMV-*ADAMTSL6β*. The coding sequence of the cDNA was confirmed to be identical to the published sequence. The *ADAMTSL6β* 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 *ADAMTSL6β* was constructed via the recombination of pDONR221-containing *ADAMTSL6β* segments into CSII-CMV-RfA using a LR reaction to generate CSII-CMV-*ADAMTSL6β*. CSII-CMV-RfA was kindly provided by Dr. Hiroyuki Miyoshi (Riken, Tsukuba, Japan). Lentiviruses were produced essentially as described previously (23). Next, a 500-μl 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 μg/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 Adamtsl6β miRNAs to target sense (5′-TGCTGAATAACAGGTAGCTGACAAACGTTTTGGCCACTGACTGACGTTTGTCATACCTGTTATT-3′) and antisense (5′-CCTGAATAACAGGTATGACAAACGTCAGTCAGTGGCCAAAACGTTTGTCAGCTACCTGTTATTC-3′) transcripts were used to generate lentiviruses for the knockdown of Adamtsl6β. A control miRNA was purchased from Invitrogen.

Infection of Developing Tooth Germ with Adenovirus—To investigate the effects of Adamtsl6β 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 Adamtsl6β—The expression and purification of recombinant Adamtsl6β was performed using 293F cells (Invitrogen) and nickel-agarose (Qiagen, Hilden, Germany) as described previously (17). Briefly, pSecTag2A containing an *Adamtsl6β* 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 Adamtsl6β. 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 μg/ml ascorbic acid, and 100 units/ml streptomycin and penicillin in a humidified atmosphere of 5% CO_2 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 Adamtsl6β, C-terminal histidine-tagged mouse Adamtsl6β was prepared as described previously (17). Adamtsl6β protein (10, 5, 2.5, 1.25, or 0.625 μg) 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-μg aliquots of total RNA in a 20-μl reaction containing 10× reaction buffer, 1 mM dNTP mixture, 1 unit/μl RNase inhibitor, 0.25 unit/μl reverse transcriptase (M-MLV reverse transcriptase; Invitrogen), and 0.125 μM 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′-ACCATATGCTATATTCTTCGATAACAAT-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′-CCTAGGCCCCTCCTGTTATTATGG-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 Adamtsl6β and TGF-β1 proteins were performed as described previously by Nakajima *et al.* (28). Briefly, purified recombinant mouse Adamtsl6β (5 μg) was incubated with 0.1 μg of recombinant TGF-β1 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 μl 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 Adamtsl6β upon the TGF-β1-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 Adamtsl6β. After 12 h, the

cells were treated with TGF-β1 (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 Adamtsl6β adenovirus for 8 h. After incubation for 24 h, mesenchymal cells overexpressing Adamtsl6β 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-μm 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 *Adamtsl6β* miRNAi were used to generate *Adamtsl6β* miRNAi-transgenic bioengineered tooth germ by incubation for 10 min at 37 °C, placement on cell culture inserts (0.4-μm 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 ADAMTSL6β Using a PDL Injury Model—To create gels for injection, 2 μl of recombinant Adamtsl6β (10 μg/μl) (see supplemental Methods) and 1.5 μl of PKH67 green fluorescent cell linker (Sigma-Aldrich) were suspended in a 9-μl 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 Adamtsl6β 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.

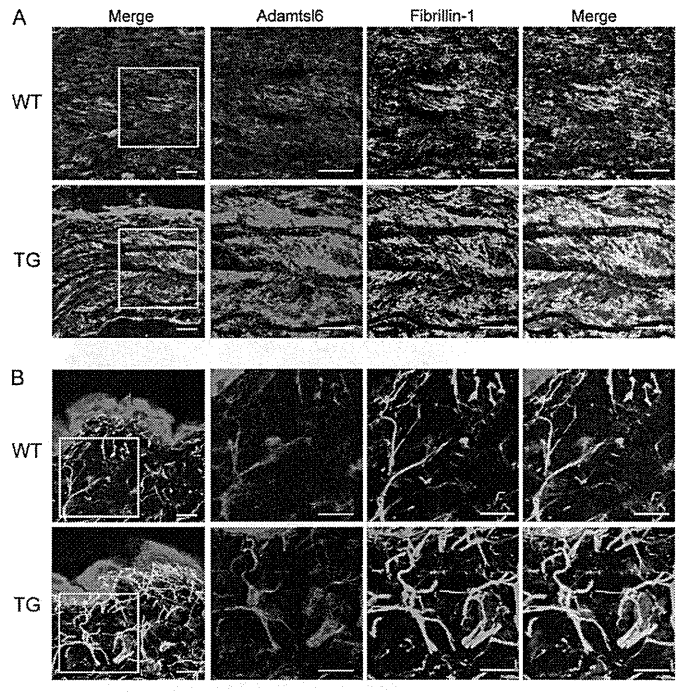

FIGURE 1. **Immunohistochemical analysis of *Tsl6β-TG* mice.** Immunofluorescence detection of ADAMTSL-6 proteins and fibrillin-1. Frontal cryosections were prepared from the skin (*A*) or aortas (*B*) of wild type (*top*) or *Tsl6β*-TG (*bottom*) littermates and subjected to double immunostaining with 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 *Tsl6β TG* mice compared with WT mice. The *merged images* illustrate that these fibrils are colocalized in the skin and aorta.

RESULTS

ADAMTSL6β Regulates Microfibril Assembly in Various Connective Tissues—To investigate whether *Adamtsl6β* plays a critical role in microfibril assembly in connective tissues, we generated *Adamtsl6β*-transgenic mice (*Tsl6β*-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 *Tsl6β*-TG mice. Immunohistochemical analysis revealed that Adamtsl6-positive microfibril assembly was barely detectable in WT mice but strongly induced in the aorta of *Tsl6β*-TG mice (Fig. 1*A*). 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 *Tsl6β*-TG mice. This confirmed that Adamtsl6 induces fibrillin-1 microfibril assembly in connective tissue, such as the aorta and skin (Fig. 1*B*).

ADAMTSL6β Is Involved in Microfibril Formation during PDL Development and Wound Healing—To investigate whether Adamtsl6β 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