### *Investigation of effects of treatment with SP cells*

Salivary gland tissue collected from green fluorescent protein (GFP) transgenic mice was digested with collagenase and hyaluronidase to remove interstitial tissue, and epithelial clusters were isolated using a filter mesh. The isolated epithelial clusters were then treated with trypsin to disperse the cells, stained with Hoechst 33342, and subjected to FACS with UV laser irradiation and measurement at two wavelengths (450 nm and 675 nm). As a result, SP cells were detected as a characteristic cell population with low fluorescence intensity which accounted for about 0.5% to 1.0% of salivary gland cells. Next, Hoechst 33342-negative SP cells and Hoechst 33342-positive non-SP (main population, MP) cells were collected using FACS [7]. The collected cells were then transplanted into salivary glands of mice with irradiation-induced salivary gland dysfunction (15 Gy local irradiation). Saliva production associated with treatment with pilocarpine, a muscarinic acetylcholine receptor agonist, was then measured serially to determine the effects of SP cell transplantation. The results showed the restoration of secretion volume at 1 month after transplantation. In addition, examination of the removed tissues by fluorescence microscopy indicated the sparse distribution of GFP-positive cells. However, because the observed number of GFP-positive cells was low, the transplanted SP cells were unlikely to have directly contributed to the restoration of secretory ability, suggesting that soluble factor(s) secreted from SP cells may be involved in the secretory mechanism of residual acinar cells.

### *Functional analysis of SP cell-specific expression gene*

Hoechst 33342-negative SP cells and Hoechst 33342-positive non-SP (main population, MP) cells were collected using FACS, and RNAs were extracted from the collected SP and MP cells using a PicoPureRNA isolation kit (Arcturus). In addition, RNA amplification was performed by a T7 polymerase-based method using a RiboAmp RNA amplification kit (Arcturus). The amplified RNAs derived from SP and MP cells were then used to synthesize cDNAs, which were fluorescence-labeled with Cy3 or Cy5 and subjected to competitive hybridization on NIA 15K mouse cDNA array (Version 2) to compare their gene expression profiles based on the detected signals. This method identified multiple genes specifically expressed in SP cells, among which we selected clusterin for functional analysis. Specifically, clusterin gene was introduced into STO cells, a mouse embryonic fibroblast cell line, by lipofection to prepare a cell line that stably expresses clusterin following drug selection. We next investigated the possible function of clusterin in reducing damage caused by reactive oxygen species (ROS), on the basis that irradiation-induced cell damage is mediated by ROS. Specifically, we counted viable cells stained using trypan blue 24 hours after stimulation of clusterin-expressing and control cells with different concentrations of hydrogen peroxide solution. The results showed significantly higher cell viability among clusterin-expressing cells than control cells, and a decrease in ROS production in the cells.

We then investigated the effects of treatment with SP cells collected from clusterin gene knockout mice to verify the involvement of clusterin in the treatment effects of SP cells. Although autoimmune myocarditis has been reported in clusterin knockout mice, we saw no histological change in 12-week-old mice at least, and no difference in SP cell fraction compared to control mice [8]. However, pilocarpine-stimulated salivation was not restored in mice with irradiation-induced salivary gland dysfunction even after transplantation of SP cells of the above-mentioned knockout mice. These findings indicated that clusterin makes a critical contribution to treatment effects in SP cell transplantation.

### *Verification of treatment effects of clusterin using a mouse model with salivary gland dysfunction*

To determine whether clusterin directly contributes to the reversal of cellular dysfunction of the salivary gland, clusterin-expressing recombinant lentivirus (Lenti-Clu, 5 x $10^6$ TU) was injected into one submandibular gland of mice with irradiation-induced salivary gland dysfunction 4 days after irradiation, and GFP-expressing lentivirus (Lenti-GFP, 5 x $10^6$ TU) was injected into the other [9]. Gene transfection efficiency and time-dependent change in saliva volume were then measured to assess restoration of secretory ability. The results indicated that Lenti-GFP transfection led to GFP positivity in approximately 16% of cells. In contrast, Lenti-Clu-injected mice showed an improvement in pilocarpine-stimulated salivation at 4, 8, and 16 weeks after virus injection compared to Lenti-GFP-injected mice. These results suggested that clusterin, which is expressed in SP cells, is involved in the functional restoration of glandular secretion.

These results revealed that SP cells or clusterin, a specific factor expressed in SP cells, is effective in the treatment of irradiation-induced salivary gland dysfunction. We are planning to examine the possible clinical application of these factors in the future.

## 2. *Role of Tooth Regeneration in Anti-Aging Medicine*

### *Introduction*

Teeth possess not only a masticatory function (*i.e.*, "chewing") but also act as sensory receptors, sending masticatory stimulation to centers in the brain. Caries and tooth loss secondary to periodontal disease, the incidence of which are increasing in the elderly, are known to cause significant problems with masticatory function and to affect systemic condition. Thus, the development of dental regenerative medicine that can essentially restore the physiology of natural teeth will be useful in preventing a decline in oral function and in promoting Anti-Aging. Here, we discuss the current status of R&D in dental regenerative therapy against tooth loss, as well as its potential role in Anti-Aging Medicine.

### *Tooth regeneration by a bioengineered organ germ method*

Previously, functional complementary therapies with artificial devices such as dentures, dental bridges, and dental implants have been used as dental support for tooth loss. Although these complementary therapies are effective in the restoration of masticatory function, they cannot restore the innate physiological aspects of teeth, such as tooth movement associated with aging and response to masticatory stimulation.

Thus, a more biological treatment approach to tooth regeneration has been sought.

Teeth develop through continuous interaction between odontogenic epithelial cells and odontogenic mesenchymal cells which together constitute the embryonic tooth germ [10]. For this reason, technologies that enable the regeneration of tooth germ from epithelial and mesenchymal cells through three-dimensional cell manipulation techniques have been developed with the goal of regenerating third teeth, in addition to primary and permanent teeth. To date, however, the ability to produce highly effective and normal tooth development has not been reached [11]. In 2007, we developed the "bioengineered organ germ method," in which epithelial and mesenchymal cells derived from the tooth germ are compartmentally arranged at high cellular density (***Fig. 1***, top) [12]. When ectopically transplanted *in vivo*, the bioengineered tooth bud cells develop with structurally normal regenerated teeth as well as periodontal tissue (periodontal ligament, cementum, alveolar bone), indicating the potential use of bioengineered tooth bud cells in tooth regenerative therapy [12].

## *Regeneration of functional teeth*

Successful tooth regenerative therapy requires not only the histological normality of the regenerated tooth but also its eruption/occlusion within the recipient's intraoral environment, and the full spectrum of normal tooth physiology, such as functional regeneration of the periodontal ligament and response to external noxious stimuli. When bioengineered tooth bud cells were transplanted into sites of tooth loss in adult mice, the bioteeth erupted and grew, and occlusion of the regenerated teeth was established with hardness comparable to that of natural teeth (***Fig. 1***, top). Further, the tissue structure of the regenerated teeth was similar to that of natural teeth, and a fully matured periodontal ligament structure was also observed, including alveolar bone. The periodontal ligament of the regenerated teeth was shown to retain the physiological capacity to remodel surrounding alveolar bone in response to experimental orthodontic force and to move teeth in a similar manner to natural teeth. In addition, similarly to natural teeth, the dental pulp and periodontal ligament of the regenerated teeth had multiple peripheral nerves, including sympathetic and sensory nerves. Upon application of mechanical stress through dental pulp exposure or dental makeover, upregulation of c-Fos expression in response to intraoral noxious stimuli in some neurons in the spinal trigeminal nucleus was observed for both regenerated and natural teeth, revealing restoration of physiological response to external noxious stimuli in the regenerated teeth. These results demonstrated that not only masticatory function but also the full spectrum of normal tooth physiology could be restored in a tooth regenerated by means of transplantation of bioengineered tooth bud cells, and clearly indicate the clinical applicability of the approach to regenerative therapy against tooth loss [13].

## *Tooth regeneration using bioengineered tooth units*

Elderly people often have severe progressive periodontal disease presenting with extensive destruction of periodontal tissue essential for mastication, and tooth loss has been known to lead to the absorption of surrounding alveolar bone resulting in severe bone defects [14]. For tooth regeneration in the elderly, an approach based on the regeneration of teeth with finished components (*e.g.*, dental prostheses) together with periodontal tissues for immediate functional recovery after implantation would be more appropriate than transplantation of bioengineered tooth germ. On this basis, we developed the "bioengineered tooth unit," which includes tooth and periodontal tissue (*i.e.*, functional unit of tooth), with the aim of developing tooth regeneration technology that allows for immediate functioning.

Because the culture of three-dimensional organs *ex vivo* is not currently possible, we demonstrated our concept by constructing bioengineered tooth units suitable for transplantation by transplanting bioengineered tooth bud

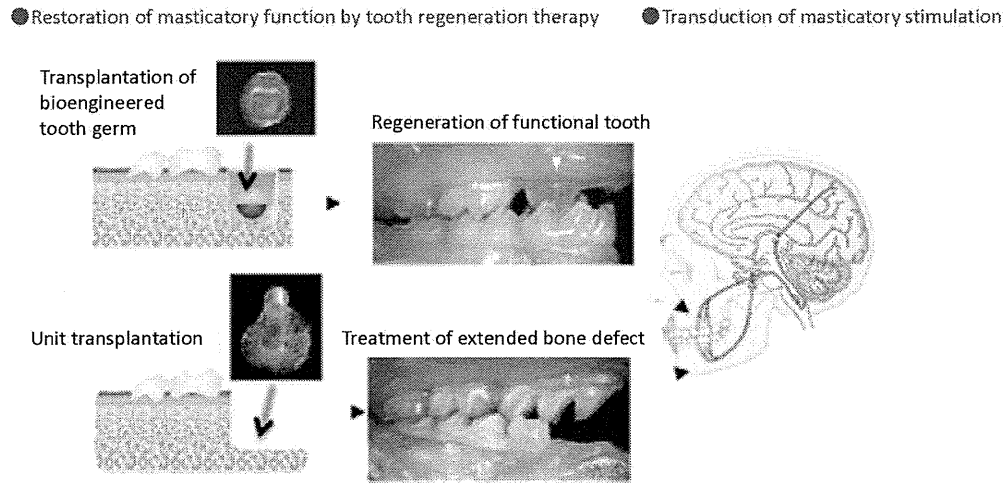


***Fig. 1.*** **Development of tooth regenerative therapy aimed at Anti-Aging.**
Regeneration of functional teeth using bioengineered tooth bud cells and treatment of extended bone defects by bioengineered tooth unit transplantation are expected to aid progress in anti-aging regenerative medicine technologies which enable the transduction of masticatory stimulation to be restored. (Scale bar: 200 μm)

cells into kidney capsules. When the bioengineered tooth unit was implanted into sites of tooth loss to secure normal occlusion, osseointegration was seen between the alveolar bone of the bioengineered tooth unit and that of the recipient, as was the restoration of a functional periodontal ligament and responsiveness to external noxious stimuli (***Fig. 1***, bottom). In addition, when the bioengineered tooth unit was implanted into an extensive bone defect model in mice, osseointegration was seen between the alveolar bone of the bioengineered tooth unit and jaw bone of the recipient, indicating the ability of alveolar bone to regenerate vertically. These results indicate the potential of transplantation of bioengineered tooth units in regenerative therapy to generate an immediately functioning tooth and in patients who experience tooth loss with major bone defect [15].

### *Summary*

Against the background of the aging society, dental therapy requires the development of dental therapeutic techniques that allow for the promotion of Anti-Aging. We consider regenerative therapy aimed at achieving a functional tooth is a desirable option. Our previous studies demonstrated that transplantation therapy against tooth loss using either bioengineered tooth bud cells or a bioengineered tooth unit can regenerate teeth that have similar physiological functions to natural teeth (***Fig. 1***). Realization of these therapies requires the identification of patient-derived stem cell seeds for use in regeneration of tooth germ [16,17], development of technique to prepare bioengineered tooth bud cells using iPS cells [18], and development of size control techniques to achieve suitable tooth size for the transplantation site [19]. Solving these problems would result in the realization of tooth regenerative therapy as an Anti-Aging dental therapy.

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## *3. Development of a New Periodontal Tissue Regeneration Method Aimed at Anti-Aging Use*

### *Introduction*

Periodontal tissue, which consists of the gingiva, periodontal ligament (PDL), cementum, and alveolar bone, is a collective term for the tissues that surround the tooth and play a role in supporting its function. Periodontal disease (periodontal disorder), which destroys these periodontal tissues, is a chronic inflammatory disorder which ultimately leads to tooth loss. Periodontal disease affects approximately 80% of adults and is the most common cause of tooth loss in the aged. Thus, the regeneration of periodontal tissue that has been lost due to causes such as periodontal disease is a major goal of dental care. Recently, associations between periodontal disease and diabetes (a lifestyle-related disease), cardiovascular disease (*i.e.*, heart disease, arteriosclerosis), and systemic disorders (*e.g.*, aspiration pneumonia) have been suggested [20], indicating that periodontal disease represents an aging factor that is associated not only with oral health but also general health. With the rapid transition into the era of an aging society, the regeneration of periodontal tissue destroyed by periodontal disease is a critically important research area in Anti-Aging Medicine, because of its systemic Anti-Aging effects, rather than simply the restoration of oral function, such as chewing.

The PDL, a periodontal tissue, is a fibrous connective tissue with a thickness of approximately 200 μm that surrounds the tooth root and connects the tooth with the supporting alveolar bone. It lies between the alveolar bone and cementum, and fixes the tooth to the jaw bone [21]. The whole periodontal ligament is made up of a mixture of various types of cells, including fibroblasts, osteoblasts, cementoblasts, osteoclasts, undifferentiated mesenchymal cells, and epithelial cells derived from epithelial cell rests of Malassez. Of these, PDL-derived fibroblasts play an important part in the remodeling of PDL fibers [20]. However, when periodontal disease occurs, periodontal pockets are formed and the lysis/disappearance of PDL fibers occurs, leading to the destruction of periodontal tissue and finally to tooth loss.

### *Regeneration of periodontal tissue using periodontal ligament-derived cells*

Thanks to recent progress in dental medicine, the pathology and mechanism of periodontal disease have become increasingly clear. Previous studies have strongly suggested that the presence of newly formed PDL is important for the regeneration of periodontal tissue [21]. As such, researchers have investigated the regeneration of periodontal tissue using autologous transplantation of fibroblasts derived from PDL and grown *in vitro* [22]. Van Dijl *et al.* reported that the regeneration of periodontium-like hard tissues (newly formed cementum-like hard tissues) was possible using the autologous transplantation of PDL-derived cells into experimentally-created periodontal tissue defects in an experimental animal (beagle dog) [23]. In addition, Dogan *et al.* reported that periodontal tissues (newly formed cementum and new bone) could be regenerated using blood clots as carriers of cultivated PDL-derived cells [24], and Nakahara *et al.* reported that differentiation of newly formed cementum was enhanced using collagen sponge as a cell culture substrate to facilitate close contact between cultivated cells and the tooth root surface [25]. These reports indicate that

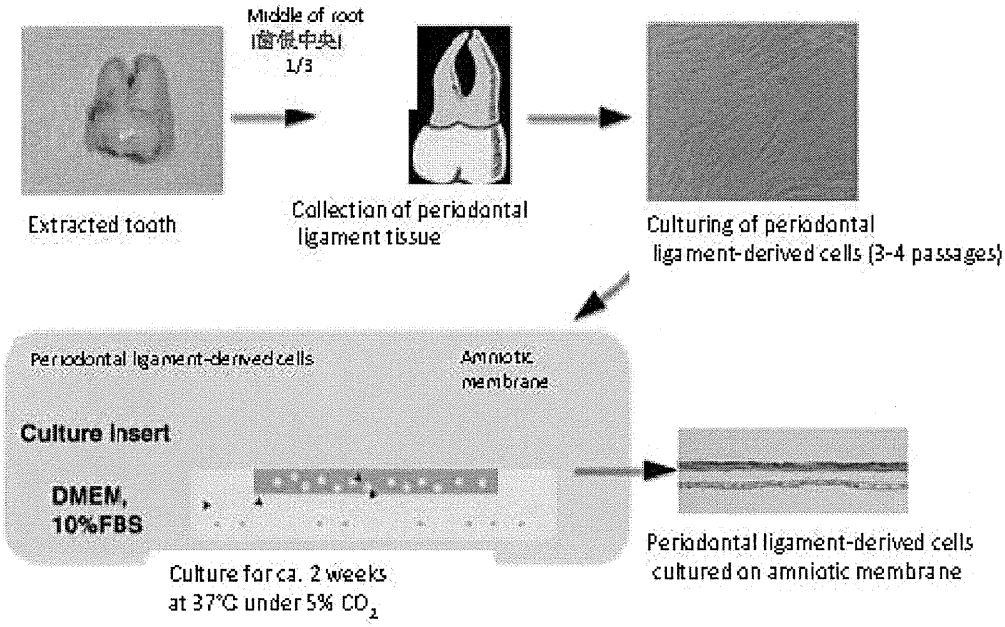


*Fig. 2.* **Examples of reconstruction of oral mucosal defects in dental oral surgery using oral mucosal epithelial sheet cultured on amniotic membrane.**

periodontal tissue can be regenerated using PDL-derived cells, and that a carrier (substrate) is important for the transplantation of cultivated cells. We have performed studies using amniotic membrane (AM), a biomaterial that has attracted interest as cell culture substrate in various medical fields [26-32], and from these developed the idea of using this as a substrate for PDL-derived cells.

## *Development of a new periodontal tissue regeneration method using amniotic membrane*

The AM is a thin membrane that covers the outermost surface of the placenta and consists of parenchymal tissue with a specific thickness. The tissue is normally discarded after parturition, can be collected from the placenta almost aseptically, and can be obtained without ethical or technical problems. It has unique characteristics, including anti-inflammatory and infection-reducing effects [33,34], and has been utilized as a biomaterial in various surgical therapies for purposes such as the prevention of adhesion/scarring in skin transplantation/abdominal surgery, healing acceleration as a skin burn wound dressing, and ocular surface reconstruction in ophthalmology [35-39]. In addition to its use as a transplantation material, it has also attracted attention for its high usefulness and effectiveness as a culture substrate [39]. We have previously shown the effectiveness of new amniotic membrane-based regenerative therapy to oral healthcare through successful preparation of a cultivated oral mucosal epithelial cell sheet on AM and the establishment and clinical application of an autotransplantation technique for various types of oral mucosal defects in dental oral surgery (***Fig. 2***) [28,30,31]. Recently, we applied this AM-based cell-culture system to culture PDL-derived cells for regenerative therapy for periodontal tissue. Below, we present progress to date and future prospects of our investigation for the development of cell sheets aimed at regeneration of periodontal tissue [29,32].

## *Preparation of periodontal ligament-derived cell sheets cultured on amniotic membrane*

We have previously confirmed that PDL-derived cells can be successfully cultured to form a sheet using an AM-based cell-culture system [26,27]. In addition, we have reported that PDL-derived cell sheets cultured on AM could potentially regenerate periodontal tissue, based on the observation that periodontal tissues (*i.e.*, newly formed cementum and new bone) were regenerated by autologous transplantation of these sheets into periodontal tissue defects in an experimental animal (beagle dog) [29]. Growth factor, cell type, and substrate are important aspects of transplantation and regenerative therapies [40] which are expected to act in combination in the regeneration of tissues, including in periodontal tissue defects. Among them, a variety of culture substrates have been investigated for PDL-derived cells [41], but an ideal substrate for periodontal tissue regeneration has not yet been developed. In addition, PDL-derived cells on substrate have not been evaluated, and a wide review of the literature reveals that the proliferation and differentiation abilities of PDL-derived cells on AM is poorly understood. Considering that the preparation of cultured human PDL-derived cell sheets will have clinical applications, we also performed an immunohistochemical study of the cell kinetics of PDL-derived cells on AM.

Human AM collected from placenta obtained during cesarean section was used. PDL tissues were collected from tooth roots after tooth extraction, etc., as appropriate. The collected PDL tissues were subjected to primary culture, and cells derived from them were used after 3-4 passages. The cells were seeded onto AM and cultured for approximately 2 weeks (***Fig. 3***), then subject to immunostaining for Ki-67 (cell proliferation marker), vimentin (mesenchymal marker), desmoplakin (desmosomal marker), and ZO-1 (tight junction marker). In addition, to investigate adhesion between the AM and these cultured cells (*i.e.*, cell-substrate adhesion), immunostaining for laminin 5/10 and collagen IV/VII (components of basement membrane) and scanning electron microscopic (SEM) observation were performed.

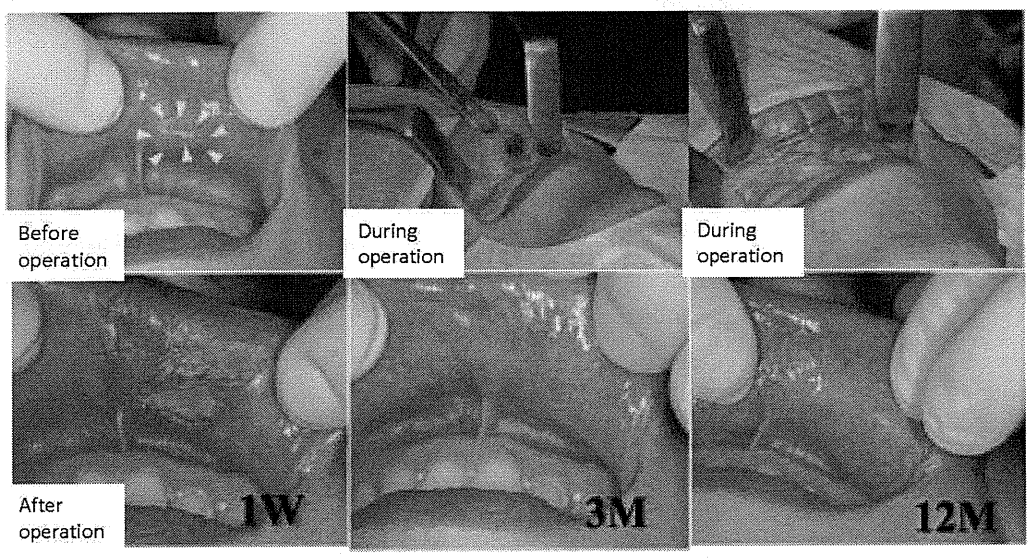


*Fig. 3.* **Culturing of periodontal ligament-derived cells on amniotic membrane.**

Tissue collection from extracted teeth and the use of AM for experimental purposes were conducted after obtaining informed consent from patients following sufficient explanation. Experimental use of PDL tissues, PDL-derived cells, and AM was approved by the Medical Ethical Review Board of Kyoto Prefectural University of Medicine (RBMR-R-21).

Results showed that the PDL-derived cells formed a monolayer on the AM after approximately 2 weeks of culture. Immunofluorescence showed the localization of Ki-67- and vimentin-positive cells and expression of desmoplakin and ZO-1. These cells were considered capable of proliferation and potentially maintaining their PDL-like properties even on AM. In addition, strong cell-cell adhesion structures, namely desmosomes and tight junctions, were shown to be present between cells [32]. Laminin 5/10 and collagen IV/VII were expressed at the basal region of the PDL-derived cells (*i.e.*, cell-AM boundary), and SEM images showed that the cells had differentiated and proliferated on AM with lateral conjugation and adhesion to the AM, indicating strong adhesion between PDL -derived cells and AM.

### *Summary*

These results confirm the proliferation of PDL-derived cells on AM and the presence of strong cell-cell adhesion structures and basal membranes. AM was shown to be a potentially suitable culture substrate, and PDL-derived cells were considered to form a sheet on AM, and not to be in the form of disparate individual cells. PDL-derived cell sheets cultured on AM can be considered to represent a novel material for a new periodontal tissue regeneration method, provided its ability to regenerate periodontal tissue is confirmed and some AM-specific effects are demonstrated.

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## *4. Current Status and Future Prospects of Corneal Regenerative Therapy using Oral Tissue*

### *Introduction*

Human ocular surfaces consist of corneal epithelium and conjunctival epithelium. These are uniquely differentiated surface ectoderm-derived mucosal membranes which maintain homeostasis of the ocular surface in cooperation with lacrimal fluid. The tissue structure can be divided into three cellular layers: an outermost corneal epithelium layer, a corneal stroma layer, and an inner corneal endothelium layer. The corneal epithelium consists of stratified squamous epithelia with a thickness of approximately 50 μm which provides physical/biological protection from the external environment to the ophthalmus. Thanks to progress in a variety of fundamental research programs, corneal epithelial stem cells have been found to exist in the basal layer of the corneal limbus, which is positioned at the periphery of the cornea [42,43]. When the corneal limbus (*i.e.*, corneal epithelial stem cell) is lost for various reasons, biological reactions occur in which the surrounding conjunctival epithelia cover the corneal surface with accompanying inflammation or vascularization, etc., thereby resulting in significant visual disorder. Diseases associated with abnormalities of the corneal epithelial stem cell like the example above are called "refractory ocular surface disease," and have been extensively investigated in both fundamental and clinical studies to elucidate the condition and develop treatment methods.

## *Development of ocular surface reconstruction*

To date, surgical reconstruction after refractory ocular surface disease has usually consisted of corneal epithelial cell transplantation (keratoepithelioplasty, corneal limbal transplantation) using donor tissue [44,45] and cultivated corneal epithelial cell transplantation [46-49]. However, because these involve allotransplantation, heavy long-term use of immunosuppressive agents is required after operation. The problems of postoperative rejection, infection, and decreased quality of life in these patients indicate the need for a safer and more effective transplantation technique. Because many refractory ocular surface diseases are binocular diseases, autologous corneal epithelium cannot be used, making it important to select a cell source that has no risk of postoperative rejection. We investigated the possibility of ocular surface reconstruction using autologous oral mucosal epithelium, with the aim of developing a novel surgical technique that uses mucosal epithelium other than ocular surface mucosal epithelium (*Fig. 4*).

## *Development of a cultivated oral mucosal epithelial sheet using amniotic membrane*

### *Cultivated oral mucosal epithelial sheet*

Regeneration of a living tissue *in vitro* requires the establishment of an extracellular environment that facilitates the differentiation and proliferation of cells (*i.e.*, scaffold for cells). Particularly in the case of refractory ocular surface diseases, normalization of the substrate, including the extracellular matrix, is considered essential, in addition to reconstruction of the epithelium. Amniotic membrane, a biomaterial, is a thin membrane over a thick basal membrane devoid of vasculature that covers the fetus and placenta within the uterus. It has been reported to have a variety of biological effects, including the suppression of scarring and inflammation, suppression of neovascularization, and acceleration of wound healing [51-53].

First, we initiated the development of a cultivated oral mucosal epithelial sheet using amniotic membrane. Our research team first investigated the suitability of amniotic membrane with the epithelium scraped off as a culture substrate for oral mucosal epithelium in an animal study in rabbits [39]. An oral mucosal cell suspension was prepared from oral mucosa collected from white rabbits, and cultured on amniotic membrane for about 3 weeks. During the culture process, cocultivation with 3T3 fibroblasts by culture insert was performed using air-lifting for differentiation induction of epithelial cells. As a result, oral mucosal epithelial cells cultured on amniotic membrane adhered to and grew on the amniotic membrane substrate and reached confluence after 1 week. On culture for 2-3 weeks, they were found to stratify, forming 5-6 layers of cells, and to have a morphology comparable to that of the basal cells, wing cells, and superficial cells of normal corneal epithelium. Morphological investigation by electron microscopy revealed that the cultivated oral mucosal epithelial sheet has desmosomes, hemidesmosomes, and tight junctions, all of which are involved in cell adhesion between epithelial cells. Numerous microvilli were observed on the cell surface, showing properties of mucosal epithelium. Immunostaining for keratin, an epithelial cytoskeletal protein, showed the expression of keratin 4/13, a mucosal-specific keratin, but not keratin 1/10, which are epidermis-specific keratinizing type keratins. In addition, among keratinizing-type keratins 3/12, immunostaining was observed only for keratin 3. While normal oral mucosal epithelium is a unique mucosal membrane in the body that expresses keratin 3, our cultivated oral mucosal epithelial sheet was found to have the cytoskeleton characteristics of non-keratinized mucosa and cornea.

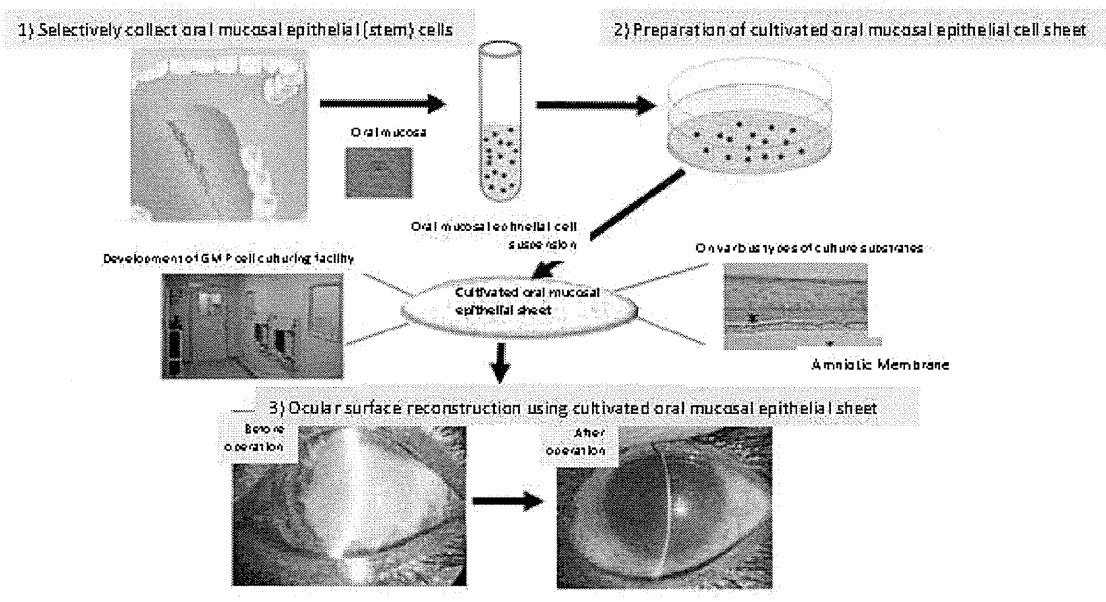


*Fig. 4.* **Diagram showing the concept of cultivated oral mucosal epithelial transplantation for refractory ocular surface disease.**

### *Ocular surface reconstruction using autologous cultivated oral mucosal epithelial sheet*

After examining the biological characteristics of the resulting cultivated oral mucosal epithelial sheet, it was autologously transplanted into the ocular surface of rabbits [39]. A rabbit ocular surface disease model was created by superficial keratectomy. At 48 hours after transplantation, the mucosal epithelial sheet was confirmed by fluorescein staining to have remained transparent and on the ocular surface without defects. At 10 days after transplantation, it was observed to have remained on the ocular surface and to have extended outward compared to its position at 48 hours. In addition, histological examination of all corneal layers at this time point showed that the cultivated oral mucosal epithelial sheet had engrafted onto the ocular surface without stromal edema or cell infiltration, and with excellent biocompatibility with the ocular surface. These results indicate that our cultivated oral mucosal epithelial sheet has characteristics of corneal epithelium-like differentiation and stratified non-keratinized mucosal epithelium in terms of its histological and cell biology characteristics. In addition, oral mucosal epithelial sheet cultured on amniotic membrane was shown to engraft and survive even on the ocular surface, a unique environment in the body, suggesting its possible use as an alternative to corneal epithelium which maintains transparency after operation.

### *Clinical study of cultivated oral mucosal epithelial sheet transplantation*

Based on the above basic data obtained from animal studies, a clinical study of autologous cultivated oral mucosal epithelial transplantation against refractory ocular surface disease was initiated in 2002 after approval by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine [54,55]. Of 17 eyes of 19 patients who underwent transplantation for corneal reconstruction at the Department of Ophthalmology, Kyoto Prefectural University of Medicine, up to January 2007 with long-term follow up for 3 years or more, approximately 53% showed a visual improvement of 1 grade or more at 3 years after operation. Postoperative complications included prolonged corneal epithelium disorder observed in approximately 37% during the follow-up period. During long-term follow-up, some patients showed ongoing reconstruction of the ocular surface with the transplanted cultivated oral mucosal epithelial sheet at 71 months after operation, revealing that oral mucosal epithelial cells, representing ectopic mucosal epithelial cells, can engraft and function on the ocular surface when applied using this surgical procedure. Considering that refractory ocular surface diseases have not been approved as an indication for corneal transplantation, the efficacy of cultivated oral mucosal epithelial sheet transplantation using autologous tissue was clinically adequate.

### *Future prospects*

In the history of corneal transplantation, recent progress in regenerative medicine/regenerative therapy research has produced significant innovation. Cell transplantation therapy from the *in vitro* to *in vivo* environments has produced a paradigm shift to corneal transplantation techniques in which replacement is limited to the defect site. The major challenges at present are the conduct of a comprehensive clinical examination of the long-term results of previous cultivated epithelium transplantation procedures, and ensuring the safety and improving the quality of the cultivated epithelial sheets. Research tasks required to meet these challenges include the identification of stem cells in the cultivated epithelial sheet and the establishment of a culture environment, including niche. In addition, problems such as serum and feeder cells, which are used in the preparation process of the cultivated epithelial sheet, have to be resolved. Furthermore, in 2006, the Ministry of Health, Labour and Welfare (MHLW) implemented its "Guidelines for clinical research using human stem cells", which mandates review by the MHLW in addition to review by an academic ethics committee when clinical research using tissue stem cells is conducted. Further development of culture techniques will need to conform with these guidelines. In any case, our responsibility is the development of safer and more evidence-based regenerative therapy.

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## *Conflict of interest statement:*

The authors declare no financial or other conflicts of interest in the writing of this paper.

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# マルファン症候群における歯根膜治癒不全の回復機構

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マルファン症候群とは，機械的圧力の負担の大きい大動脈や，骨，肺，歯根膜で，体の弾力を調節する微細線維の形成不全により大動脈瘤，骨格の異常成長，肺気胸，水晶体脱臼，歯周病などの重篤な疾患を発症する結合組織疾患である。近年，微細線維の成分であるADAMTSL6βが微細線維形成の誘導能を有することが見られ，さらにマルファン症候群における微細線維形成不全を回復させることを明らかにした。このことからADAMTSL6βはマルファン症候群の微細線維形成不全による疾患の治療に有効である可能性が示された。

本稿では，ADAMTSL6βのマルファン症候群における微細線維形成不全の回復効果について，歯根膜をモデルに解説する。

**Calcium metabolism associated with oral diseases.**

## *Molecular mechanisms for the improvement of wound healing ability of periodontal ligament in Marfan's syndrome.*

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***Masahiro Saito, Takashi Tsuji***

Marfan's 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 microfibril-associated extracellular matrix protein that has been implicated in fibrillin-1 microfibril assembly is able to improve microfibril insufficiency in MFS mice model. These findings suggest a new therapeutic strategy for the treatment of MFS through ADAMTSL6β-mediated fibrillin-1 microfibril assembly. We here review effect on ADAMTSL6β to the improvement of microfibril insufficiency in periodontal tissue as a model.

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## はじめに～背景～

マルファン症候群は微細線維と呼ばれる細胞外マトリックスの異常が原因で体全体の結合組織が脆弱化し，弾力性が減少してしまい，大動脈，肺，皮膚，関節，骨，歯根膜といった機械的圧力の負担の大きい組織が機能に異常をもたらしてしまう結合組織疾患である[1]。そのため，大動脈瘤，肺気胸，関節の異常可動，骨の異常成長，歯周病といったさまざまな病気を起こす。このマルファン症候群は遺伝病であるにも関わらず，５千人に１人と高い割合で発病し，国内でも２万５千人近くの患者がいると考えられている[2]。マルファン症候群は，症状によりⅠ型，Ⅱ型に分類されており，Ⅰ型は微細線維の主成分であるfibrillin-1遺伝子のミスセンス変異が原因で症状がはっきりとみられるタイプで，Ⅱ型では眼に特徴的な症状が出ており，それ以外の症状が見られる場合もある[3][4]。Ⅰ型を発病するものが最も多く，その原因となるfibrillin-1はcalcium-binding Epidermal Growth Factor-like (caEGF) motifの繰り返し構造を有する細胞外マトリックス因子であり，組織の強度の維持に重要な弾性機能の役割を果たすばかりでなく，**TGF-β**と結合することでその機能を調節する作用も持っている[5]。Fibrillin-1遺伝子のcaEGF

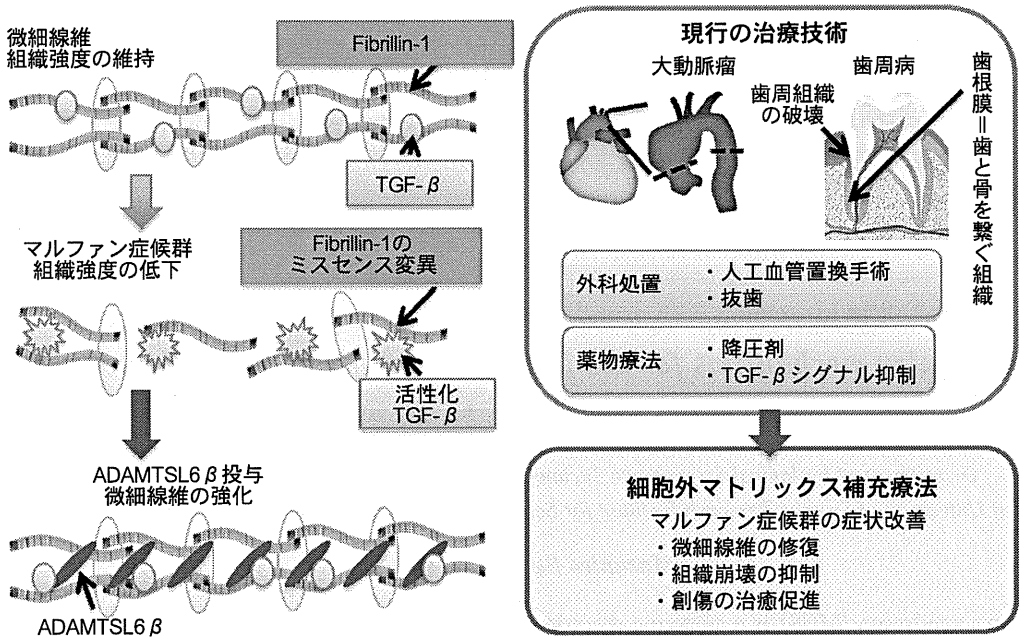


**図１　マルファン症候群と細胞外マトリックス補充療法による治療戦略図**

左側：マルファン症候群では微細線維形成不全が起こり，弾性力が低下するばかりかTGF-βが活性化して組織の崩壊が進行する。

右上図：マルファン症候群では大動脈瘤，歯周病などの疾患を発症する。これらの疾患に対し，外科処置あるいは薬物療法で対応されてきたが，微細線維崩壊を改善する治療は開発されていない。

右下図：細胞外マトリックス補充療法：微細線維成分であるADAMTSL6βを用いて微細線維を強化し，マルファン症候群の症状改善を図る。

（筆者作成）

**TGF-β**：transforming growth factor-β（トランスフォーミング増殖因子。結合組織の構成成分や分解酵素の産生を促す。骨においては，骨芽細胞の分化および成熟に対しては，抑制的に作用する。）

motifの変異がマルファン症候群で多く検出されており，その結果として微細線維の形成不全を起こすため，体の強度が著しく低下するばかりでなく，TGF-βが活性化し，周囲の細胞に作用してマトリックスメタロプロテアーゼ（MMP）-9の産生が高まり，組織の崩壊が促進する[6]（**図1**左上側）。そのためマルファン症候群でみられる特徴的な症状が発症する（**図1**右上側）。

マルファン症候群の治療は，脆弱化した大動脈や心臓の負担を下げるため，降圧薬を用いた薬物療法，また，大動脈解離が起こりそうなケースでは，人工血管で置き換える外科手術で対応され，また，歯周病においては心内膜炎の原因になることから，そのほとんどが抜歯処置で治療されてきた[2)7)8)]。最近ではロサルタンがTGF-βシグナル抑制効果を有することが報告され，同薬剤を用いて組織崩壊を抑える大動脈瘤の予防治療も行われるようになった（**図1**右側）。このようにマルファン症候群に対する外科手術およびTGF-βを抑制する薬物療法で，その症状を予防できるようになったが[9)10)]，マルファン症候群の主原因である微細線維の機能低下を回復させる，新たな治療技術の開発が大きな課題として考えられてきた。

## 微細線維再生を誘導するADAMTSL6βの発見

これまで微細線維形成機構は，fibrillin-1がタンデムに自己結合し線維構造を形成し，また結合部分で球状構造を形成することで弾性機能を発揮していると考えられてきた，しかし，fibrillin-1による微細線維の形成機構のほとんどは不明であった（**図1**左側上）。近年になり，fibronectinとfibrillin-1の結合が微細線維の形成に必須であることが報告され，fibrillin-1と結合するタンパク質が微細線維形成を調節している可能性が示され

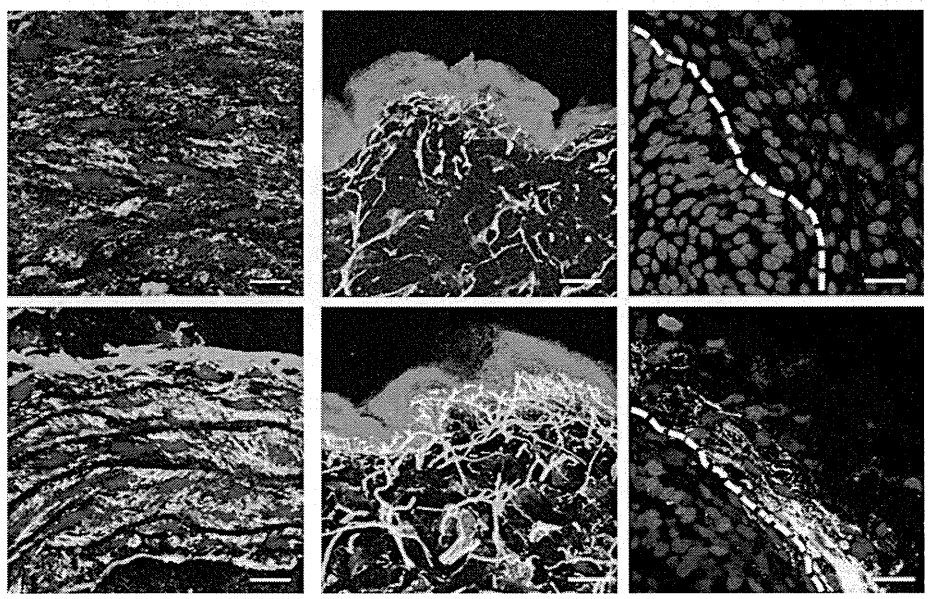


**図2　ADAMTSL6βは結合組織の微細線維を増加する**

ADAMTSL6βを過剰発現させ（TSL6β-TG）WT（正常マウス）と大動脈，皮膚および歯根膜における微細線維形成能を比較した。TSL6β-TGでは微細線維（黄緑の線状構造物）が増えていることが観察される。Bar：50μm

（文献15より）

**MMP**：matrix metalloproteinase（マトリックスメタロプロテアーゼまたはマトリックスメタロプロティナーゼ）

た[11)12)]。一方，大阪大学蛋白質研究所の関口清俊教授のグループでは，新規タンパク質を網羅的に探索し，線維形成能力を有するタンパク質のスクリーニングが行われた[13)]。その結果，2010年にADAMTSL6βが微細線維と結合するタンパク質として発見され，そしてこのタンパク質も微細線維の形成を促進できることが示された[14)]。そこで筆者らの研究グループでは，ADAMTSL6βを用いてマルファン病における微細線維形成不全を解決する治療技術を開発できるかを調べるために，

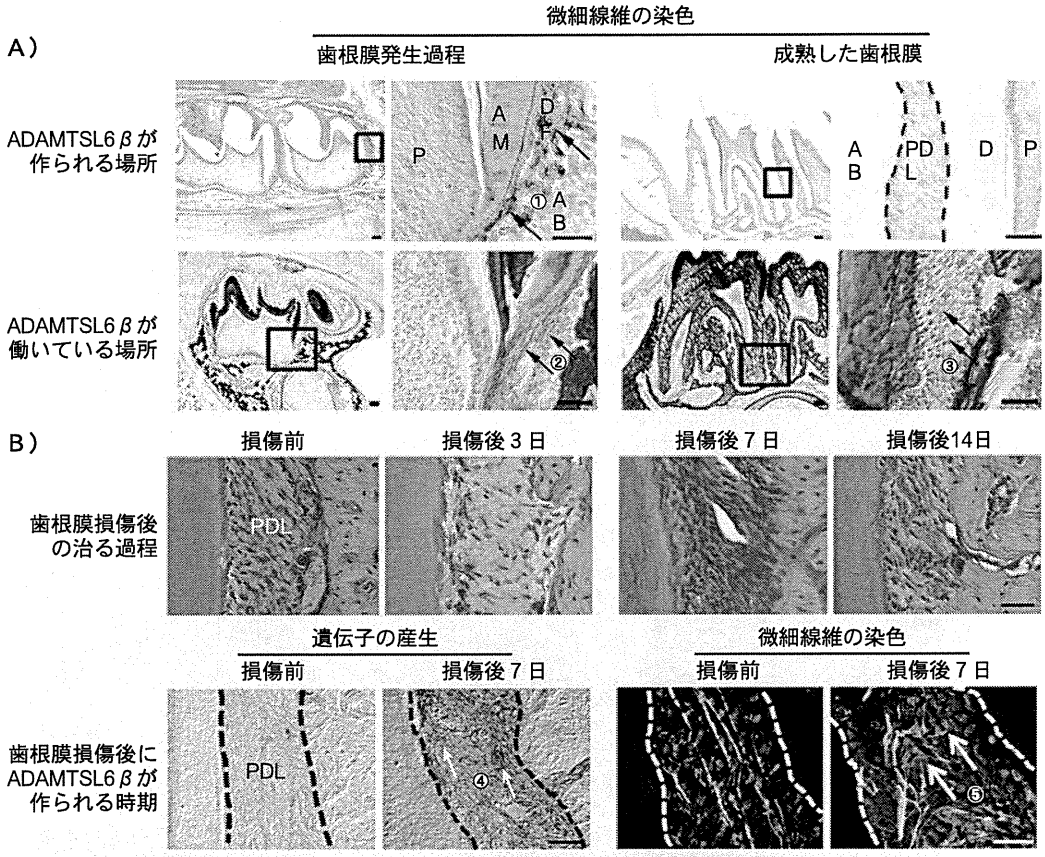


**図3　ADAMTSL6βは歯根膜の微細線維の修復に関わる**

A）歯根膜の発生過程におけるADAMTSL6βの産生を示す。歯根膜発生過程におけるADAMTSL6βの産生の働く場所（下段，矢印②，③）と遺伝子の産生される場所（上段，矢印①）。ボックスの拡大図をそれぞれの右側に示す。

B）歯根膜治癒過程におけるADAMTSL6βの産生を示す。

上段：歯を再植し歯根膜損傷後の治る過程を示す。3日目では破壊されている像が観察されるが，7日以降で元に戻るのが観察される。

下段：歯根膜修復過程でADAMTSL6β遺伝子が産生されている像を示す（矢印④）。微細線維の形成量もが歯根膜修復過程で高まる（矢印⑤）。

AB：歯槽骨
D：象牙質
PDL：歯根膜
AM：エナメル芽細胞
DF：歯小嚢
バーの長さ：50μm

（文献15より）

ADAMTSL6βが血管および皮膚の微細線維を増やせるかどうかを調べた。全身でADAMTSL6βが過剰に発現するトランスジェニックマウス(TSL6β-TG)を用いて調べたところ，血管，皮膚では正常の動物(WT)と比較して明らかに微細線維が増えていることが観察された。また歯根膜にADAMTSL6βをアデノウイルス発現系で過剰発現させると，微細線維形成促進効果が見られた(**図2**)[15]。この研究成果より，ADAMTSL6βは体内で微細線維を増やす作用を持つことが分かり，マルファン症候群の治療に応用できる可能性が示された。

## ADAMTSL6βは歯根膜の傷の治癒に関与する

次に筆者らは，ADAMTSL6βの微細線維形成不全の改善能力を調べるため，破壊された微細線維を修復する能力を持っているかを調べた。筆者らはこの目的に大動脈，皮膚とくらべ微細線維を

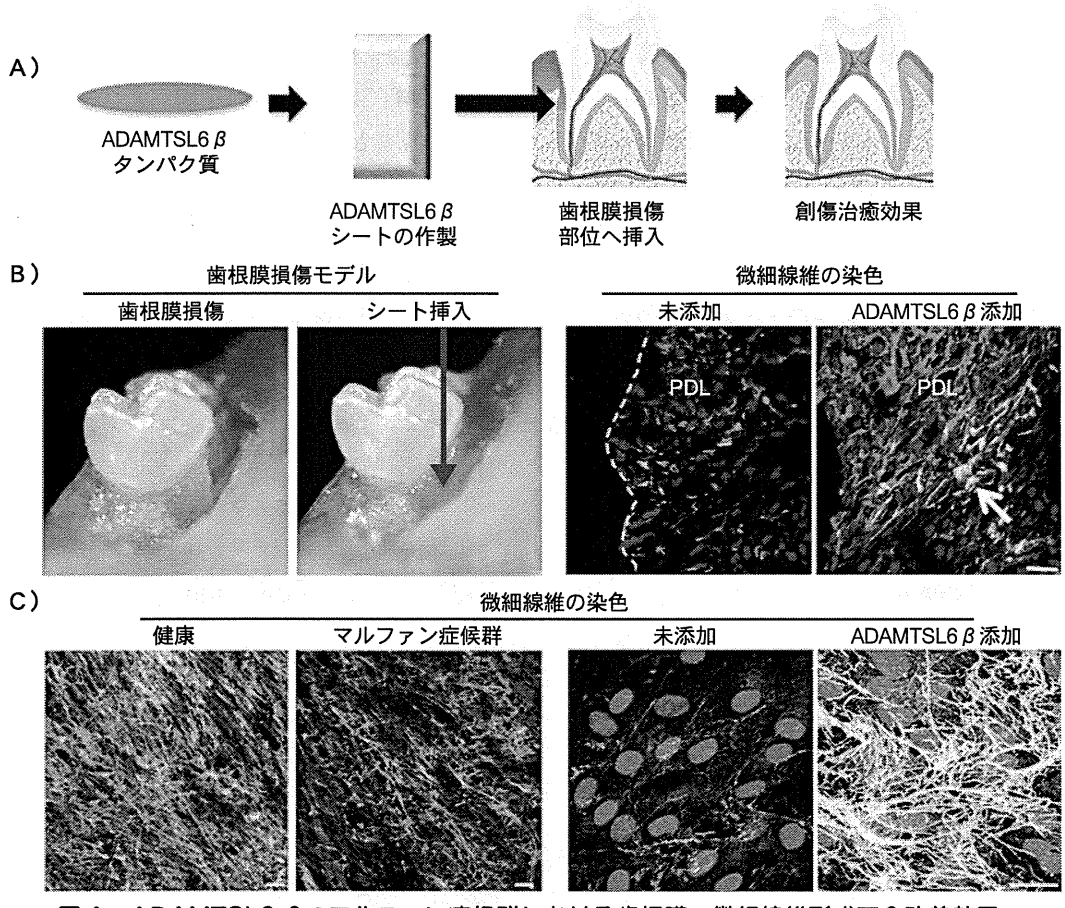


**図4　ADAMTSL6βのマルファン症候群における歯根膜の微細線維形成不全改善効果**

A) ADAMTSL6βシートを用いた歯根膜への投与方法の開発
B) 左側：ADAMTSL6βシート挿入の手順，右側：ADAMTSL6βのシート挿入後微細線維の回復像（矢印）
C) マルファン患者由来歯根膜細胞を用いた微細線維改善能力の解析
　左側：マルファン症候群患者の歯根膜細胞と健康な人の微細線維形成能の比較
　右側：ADAMTSL6βによるマルファン症候群患者の歯根膜細胞の微細線維の回復像
バーの長さ：50μm

(文献15より)

豊富に含む歯根膜をモデルとして解析した。歯根膜とは歯を支える歯周組織の中でも歯と骨を繋げる靱帯とよく似た組織で，主に咬む力を緩衝するために働いている[16)17)]。また歯根膜は歯周病により非可逆性の崩壊を受けるため，歯周病治療では歯根膜の再生が重要課題となる。マルファン症候群においても広範囲に及ぶ骨の破壊を伴う重篤な歯周病に罹患することが報告されており，歯根膜の微細線維の機能低下が歯周病の悪化に関わる可能性も示唆されている[8)]。ADAMTSL6βが歯根膜の微細線維形成に関わるかを調べるために，歯根膜が作られる発生過程を解析した。歯根膜発生過程で微細線維の形成される様子を観察すると，まだ歯小嚢と呼ばれる歯根形成過程に形成される未熟な歯根膜で豊富に作られていることが分かり，その場所にADAMTSL6βが存在することが観察された（**図3A** 矢印②）。しかし成熟した歯根膜では，微細線維の形成量の低下と共にその量も減少した（**図3A** 矢印③）。次にADAMTSL6βがいつ産生されるのかを見るため，その遺伝子が出ているところを観察すると，歯根膜発生過程の歯小嚢で遺伝子の産生が観察され，しかし成熟した歯根膜では産生されないことが判明した（**図3A** 矢印①）。このことからADAMTSL6βは歯根膜の微細線維が作られる過程で働く物質であることが考えられた。次にADAMTSL6βが歯根膜の微細線維の修復に関わるかを調べる目的に，歯根膜の創傷治癒過程でも同じことが起こるかを観察した。そのため，抜歯により歯根膜を断裂し，その後の治癒過程を観察する歯根膜の損傷モデルでADAMTSL6βの動態を調べた。歯根膜の治癒過程を見ると損傷後3日では組織の破壊が観察されるが，損傷後7日以降で治癒することが観察された（**図3B**，上段）。この過程でADAMTSL6βの産生を調べると，歯根膜が治る時期で遺伝子の産生が高まり（**図3B** 矢印④），また同時期に微細線

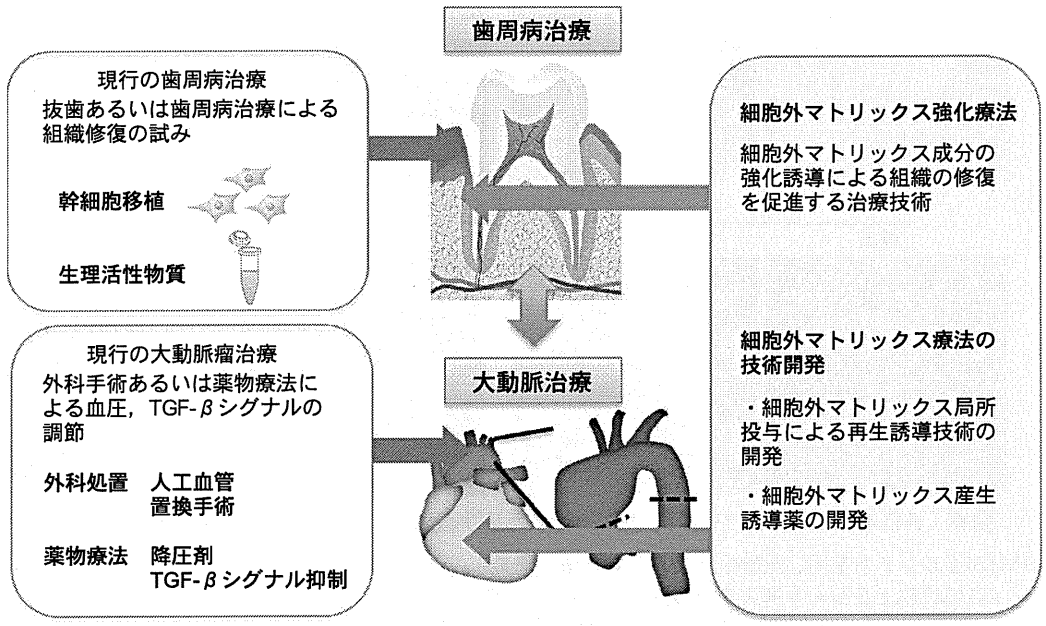


**図5　細胞外マトリックス補充療法による新規マルファン症候群の治療技術の開発**

細胞外マトリックス強化療法の技術開発は，マルファン症候群の現行の歯周病，大動脈瘤の治療技術で補えなかった症状を改善する新規治療技術として発展する可能性が期待される。

（筆者作成）

維の産生も高まりその修復が行われていることも確認できた（**図 3B** 矢印 ⑤）。これらの結果より，ADAMTSL6βは歯根膜の微細線維が修復する時に働いている可能性が示された[15]。

## ADAMTSL6βはマルファン症候群患者由来細胞の微細線維形成不全を改善する

ADAMTSL6βのマルファン症候群に対する治療効果を解析するためには，モデル動物を用いて治癒効果を検証する必要性が考えられる。この目的を達成するため，私たちはマルファン症候群モデル動物を用いて解析を行った。このモデル動物の歯根膜の微細線維形成不全を回復するため，コラーゲンにADAMTSL6 βを含ませたシート状のゲルを作製し（ADAMTSL6βシート：**図 4A**），これを歯根膜に挿入して症状を改善できるかを解析した（**図 4B**，左側）。その結果，ADAMTSL6βシートの効果により，微細線維の形成不全を改善させるばかりでなく，傷の治りを促進させることが分かった。（**図 4B**，右側）

次にADAMTSL6βがマルファン症候群患者の微細線維の形成不全を改善できるかを解析するため，マルファン症候群患者から提供された歯根膜細胞を用いて解析した。この細胞は健康な人の歯根膜細胞と比較して微細線維の形成不全が見られる特徴を有している[8]。（**図 4C**，左側）。この細胞にADAMTSL6βを加え，微細線維の形成を回復出来るかを調べたところ，微細線維形成不全が改善が確認された（**図 4C**，右側）。この結果より，ADAMTSL6βは，マルファン症候群の微細線維形成不全を改善できることが示された[15]。

## おわりに

マルファン症候群の治療は人工血管置換手術の進歩で飛躍的に改善され，降圧薬およびTGF-βシグナルを抑制する薬物療法と組み合わせることで大動脈瘤の予防効果できることも報告されてきた。ADAMTSL6βにより微細線維形成不全を回復可能であることが判明したことで，細胞外マトリックスの再編成により組織強度を高める「細胞外マトリックス補充療法」の概念がマルファン症候群の新たな治療技術になる可能性が示された。本研究成果により，歯周病のように直接投与可能な部位は組み換えタンパク質の局所投与が有効であるが，大動脈瘤に対処するためにはADAMTSL6βの遺伝子発現を誘導する薬剤の開発の必要性が示唆された。このように「細胞外マトリックス補充療法」は，現行のマルファン症候群の歯周病および大動脈の治療で補えなかった微細線維の強化を導き，これらの病気を予防する新規治療技術として発展する可能性が期待される（**図 5**）。

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研究室

# 蘇る臓器，再生医療の実現化への挑戦

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## はじめに

私たちの研究室では，再生医療を中心とする「革新的な医療」の創出につながる技術開発を目指した研究活動を行っています。そのために，生命科学の領域のなかでも，「器官再生」と「糖タンパク質工学」の分野を重点研究プロジェクトとした研究活動を推進しています（図1）。器官再生プロジェクトでは，人工臓器を作製し，病気で働かなくなった臓器を取り換える臓器置換再生医療，また臓器を生きた状態で長期間維持する臓器培養の技術開発を行っています。このプロジェクトで，歯および毛の再生医療，また臓器培養装置の開発研究を推進しています。糖タンパク質工学プロジェクトでは糖タンパク質医薬品の開発による革新的バイオ医薬品の創造を目指しています。私たちの研究室では，これらの研究活動を通じて，「学術への貢献」「社会への貢献」「人材の成長への貢献」の理念のもとに大学での教育に取り組んでいます。

本稿では上述の研究活動の中でも，歯と毛をモデルとした臓器置換再生医療の研究成果に関して紹介させて頂きます。

## 人工臓器の作製と臓器置換再生医療

一般的に臓器の再生と発生は同じ機構で制御されています。例えば，イモリは目の水晶体を取り除いても再生することが知られています。この場合は虹彩の中に存在する幹細胞が失われた水晶体部分へ移動し，水晶体の発生を再現して再生します。再生医療とは，幹細胞を利用して，病気により働かなくなった臓器を機能回復させる治療技術のことです。再生医療は，ドナー不足などの多くの問題を抱えている「臓器移植」を必要とする病気に苦しむ人を助ける新たな治療技術として期待されています。

ほとんどの臓器は，その発生過程で上皮と間葉由来の幹細胞の集合体から成る器官原基と呼ばれる種のような組織から形成されます。私たちは，この器官原基に着目し，細胞操作により人工的に器官原基を形成し臓器を再生させる「器官原基法」の開発を試みました。この「器官原基法」は，次世代の再生医療技術として，生体外で人工的に再生した臓器と置換する臓器置換再生医療への発展が期待されています。

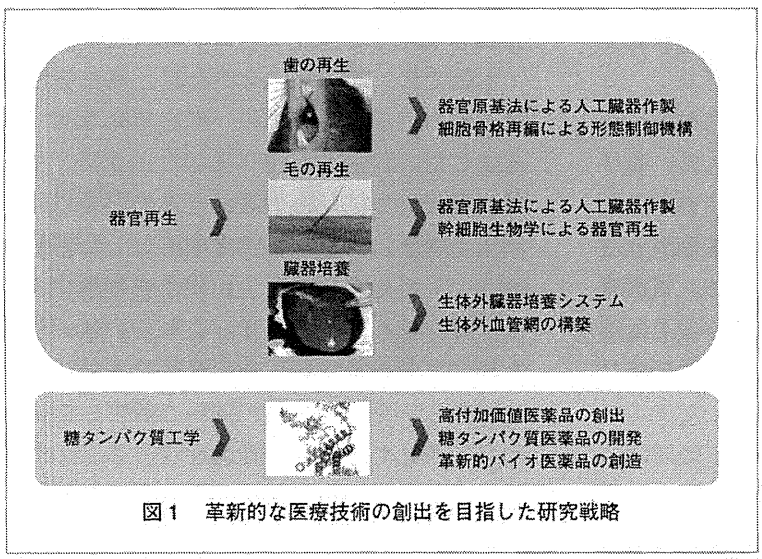


図1 革新的な医療技術の創出を目指した研究戦略

## 臓器置換再生医療のモデルとしての歯と毛

臓器置換再生医療の実現化を目指すため，私たちの研究室では歯と毛をモデルに人工臓器である“再生歯”ならびに“再生毛”作製の技術を開発しました。図2に，器官原基法を用いて歯と毛を再生させた実施例を示しています。歯の場合，器官原基は歯胚と呼ばれ，上皮と間葉から構成され，これら二つの細胞がお互いに作用しながら歯を形成します。そこで私たちはマウス胎児より歯胚を取り出し，酵素処理によって歯胚の上皮と間葉の単一化細胞を得た後に，コラーゲンゲル内でこれらの細胞を高細胞密度で区画化して再構築することにより，歯胚を再構成（再生歯胚）しました。この再生歯胚をマウス顎骨に移植すると，再生歯として萌出することが分かりました。再生歯の萌出過程を観察すると，歯胚の発生成長を再現していることが確認され，また自然の歯と同じく周囲の骨と靭帯を介して強固に結合していることが分かりました。さらに緑色蛍光タンパク質（GFP）で標識した再生歯胚を移植するとGFP陽性の再生歯を形成したことから，再生歯は移植した再生歯胚により形成されたことが証明されました。

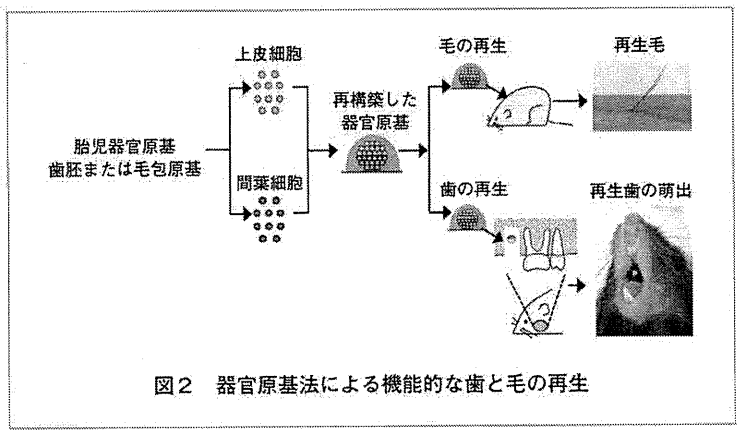


図2　器官原基法による機能的な歯と毛の再生

同様の技術を用いて毛の器官原基である毛包原基を用いて再生毛を作製しました。電子顕微鏡で再生毛を分析すると，自然の毛と同様に中心に毛髄，周囲に毛皮質が確認されました。また再生毛は自然の毛と同様に成長後に抜け，新たに生え変わる毛周期を繰り返すことが観察されるばかりでなく，その周囲には「立毛筋」と呼ばれる筋肉が付いており，自然の毛と同様に寒冷刺激で再生した毛も立つことが判明しました。さらに色素細胞を含めて再生毛包を培養すると黒い毛になることも分かりました。

このように歯と毛をモデルとした研究成果により，器官原基法を用いれば機能臓器は再生できることが示されました。今後の臓器置換再生医療が実現化するための課題としては，器官原基法による三次元的な細胞操作に加えて，人為的な血管網の作製を含む，器官原基を育成する培養技術開発が必要になります。そのため，わたしたちの研究室では，生体から臓器を摘出し，生体外で培養する技術開発に取り組んでいます。この技術開発は，将来の器官原基育成とともに，移植医療のための臓器を長期間維持する移植医療の基盤技術として発展することが期待されます。

## おわりに

本稿では，器官再生を中心に，私たちが取り組んでいる研究内容の一部を紹介させて頂きました。冒頭でも述べましたように，私たちの研究室では，再生医療技術の開発以外に糖鎖生物学，細胞生物学を中心とした基礎的研究で，再生に必要な生命現象を理解する研究活動も進めております。これらの研究活動を通じて学術的に価値ある成果をあげ，社会に貢献する長期的展望に立った幅広い知識と視野，そして研究開発能力が養うことのできる，次の世代を担う学生へと成長するような人材育成を目指しています。

生涯研修コード 22 99

SCIENCE サイエンス

KEEP 20 TEETH TILL YOUR 80

# 次世代の歯科治療システムとしての歯科再生治療

## ～組織修復再生治療と臓器置換 再生治療としての歯の再生～

1）　2）　3）

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●日歯ホームページメンバーズルーム内「オンデマンド配信サービス」および「Eシステム（会員用研修教材）」に掲載する本論文の写真・図表（の一部）はカラー扱いとなりますのでご参照ください。

要　約

口腔機能は，健康や生活の質の維持，向上に重要な役割を果たしている。齲蝕や歯周病などの疾患に対する治療として，人工物による代替治療だけではなく，天然歯が有する生理機能を本質的に回復させる歯科再生治療の研究が進められており，21世紀の新たな治療システムとしての歯科治療へと発展することが期待されている。本稿では，歯科再生医療に関わる技術開発の現状について，組織修復治療と歯をまるごと再生する治療とに分類して解説する。

キーワード
歯科再生治療／再生歯胚／幹細胞

## 1．歯科再生治療のコンセプト

咀嚼や発音などの口腔機能は，健康や生活の質の維持，向上に重要な役割を果たしている。歯と歯周組織は，上皮・間葉相互作用によって誘導される歯胚から発生し，象牙質，エナメル質，セメント質や歯槽骨などの硬組織と，歯髄や歯根膜といった軟組織からなる特徴的な構造を有している[1,2]。そのため多様な疾患が起こりやすく，齲蝕や歯周疾患による歯の実質欠損や喪失は，発音や咀嚼，咬合などの口腔機能に重大な問題を引き起こし，全身の健康状態に影響を及ぼすことが知られている[1)]。

これらの疾患に対する歯科治療として，入れ歯やブリッジ，歯科用インプラントなどを用いた歯の機能代替治療が行われてきた[3)]。これらの人工物による代替治療は機能回復において有効であるとされているものの，骨リモデリングを介した歯の移動能や，侵害刺激