Chapter 7
Functional Salivary Gland Regeneration

Miho Ogawa and Takashi Tsuji

Abstract Oral health and homeostasis are maintained by the functional interactions of many organs, including the salivary glands, teeth, and tongue. Salivary gland dysfunction leads to dry mouth diseases, such as dental caries, bacterial infection, swallowing dysfunction, and reduced quality of life. The current clinical therapies for dry mouth are temporary, and they cannot repair salivary gland dysfunction. Salivary gland regenerative therapy with tissue repair and whole salivary gland replacement is a novel organ regenerative therapy. To achieve the recovery of the salivary gland function, adult tissue stem cells may be used as a cell source for salivary gland tissue repair therapies. To attain the entire salivary gland replacement therapy, which represents the next-generation regenerative therapy, we developed a novel cell manipulation method that can regenerate the ectodermal organ germ. The bioengineered salivary gland germs successfully engrafted grew in the transplantation site, generating the correct structure. The bioengineered salivary glands were able to secrete saliva into the oral cavity and improve dry mouth symptoms. In this chapter, we describe the recent progress and developmental methods for salivary gland regeneration therapy.

Keywords Salivary gland regeneration • Salivary gland replacement regenerative therapy • Saliva • Bioengineered salivary gland • Organ germ method • Transplantation

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7.1 Introduction

The salivary gland is an exocrine organ that synthesizes and secretes saliva. There are three major pairs of glands: the parotids (PG), submandibular glands (SMG), and sublingual glands (SLG) (Fig. 7.1a). Additionally, there are many minor salivary glands. The SMG and PG secrete serous saliva, which mainly contains amylase proteins. SLG secrete mucous saliva, which contains glycoproteins, such as mucin proteins (Edgar et al. 2004; Tucker and Miletich 2010; Avery 2002). Saliva plays various roles, including food digestion, taste, swallowing, protection from dryness, and oral health maintenance and homeostasis. Thus, salivary gland dysfunction induces various clinical problems in oral health. Salivary gland dysfunction is attributed to acinar cell atrophy, which is caused by radiation therapy for patients with head and neck cancer, aging, and autoimmune diseases (such as Sjögren's syndrome), and can be a side effect of various medications. Acinar cell atrophy results in xerostomia (dry mouth syndrome) (Saleh et al. 2015; Vissink et al. 2010; Ship et al. 2002; Fox 2004).

Xerostomia causes various clinical oral problems, such as serious dental decay, oral bacterial infection, taste disorder, voice disorder, and swallowing disorder, which

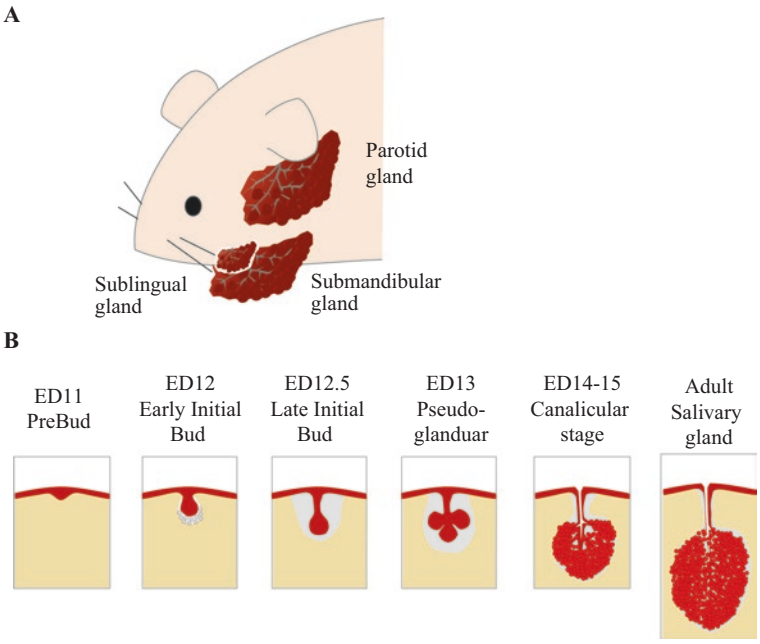

Fig. 7.1 Schematic representation of the salivary glands. (**a**) The three major salivary glands include the submandibular glands, sublingual glands, and parotid glands. (**b**) Development of submandibular glands which are produced from organ germ induced by the interaction of reciprocal epithelial and mesenchymal tissue (EDs 11–12). The epithelial tissue invaginates into the mesenchymal tissue and forms the epithelial stalk and terminal bulb (EDs 12–13), which form the duct and acinar cells (ED 14). The acinar cells mature and begin to synthesize and secrete secretory proteins (adult)

result in a general reduction in the quality of life (Atkinson et al. 2005). Current therapies for xerostomia include symptomatic treatments, the use of artificial saliva substitutes, and the administration of salivary gland stimulants and sialogogues, which enhance moisture retention in the oral cavity (Fox 2004; Nakamura et al. 2004). Parasympathetic stimulation drugs, such as pilocarpine and cevimeline, promote saliva secretion via the stimulation of residual acinar cells (Fox 2004). However, the effects of these therapies are temporary, and they cannot reproduce salivary gland dysfunction. Therefore, the development of alternative treatments that provide enduring effects or recover salivary gland function is expected (Kagami et al. 2008).

Recent regenerative therapy to restore organ function has been developed in many research fields, such as developmental biology, stem cell biology, and tissue engineering (Brockes and Kumar 2005; Langer and Vacanti 1999; Atala 2005; Madeira et al. 2015). Notably, transplantation therapy with tissue stem cells or cell sheet has been attempted for the repair of damaged tissues and organs in divergent diseases many years ago (Copelan 2006; Segers and Lee 2008). In salivary gland regeneration therapy, many research groups have reported various strategies including stem cell transplantation, gene modification, and tissue engineering to reproduce the damaged acinar tissue and restore saliva secretion (Yoo et al. 2014; Feng et al. 2009; O'Connell et al. 1999). Recently, ectodermal organ regeneration has been reported using bioengineered organ germ transplantation methods (see Chaps. 5, 6, and 8). In this chapter, we will discuss the recent findings and technologies for partial salivary gland tissue repair and whole salivary gland regeneration as a next-generation regenerative therapy that can recover function and prevent xerostomia.

7.2 Salivary Gland Development During Embryogenesis

The salivary gland is an exocrine organ arising from the salivary gland germ, which is generated by reciprocal interactions between the oral ectodermal epithelium and the neural crest-derived mesenchyme during embryogenesis (Tucker and Miletich 2010; Knosp et al. 2012; Patel et al. 2006; Knox and Hoffman 2008) (Fig. 7.1b). On embryonic day (ED) 11, the mesenchymal cells provide signals and induce oral epithelial thickening and invagination (Knosp et al .2012; Jaskoll and Melnick 2004). The expression of *Fgf10*, *Fgfr2b*, *Pitx1*, and *p63* is essential for initial salivary gland development. The epithelial bud grows and forms terminal bulbs and a stalk (initial bud), and then branching morphogenesis occurs, including cell proliferation, cleft formation, migration, and apoptosis, which proceed during EDs 12.5–14.5 (pseudoglandular) (Sakai 2009; Hsu and Yamada 2010; Harunaga et al. 2011). After ED 15.0, the salivary gland germ begins functional differentiation. The epithelial stalk differentiates into duct cells, including the excretory, striated, and intercalated ducts, and the terminal bulbs differentiate into acinar cells and mature (Denny and Denny 1999). There are three types of acinar cells: the serous, mucous, and seromucous cells. The seromucous cells secrete both serous and mucous saliva. In the excretory duct, adult tissue stem cells are maintained and supplied to the acinar and duct cells after the salivary gland tissue is injured (Man et al. 2011; Ihrler et al. 2002; Lombaert and Hoffman 2013).

7.3 Salivary Gland Tissue Repair Using Tissue-Derived Stem Cells

Adult tissue-derived stem cells have a general capacity for self-renewal and differentiation to repair injured tissue (Fig. 7.2). Salivary gland-derived stem cells have been isolated and characterized from the exocrine ducts of PG and SMG (Rotter et al. 2008; Lombaert et al. 2008; Jeong et al. 2013; Kawakami et al. 2013). The salivary gland-derived stem cells isolated from PG express mesenchymal stem cell (MSC) markers (CD44, CD49f, CD90, and CD105). These cells have the ability to differentiate into adipocytes, osteocytes, and chondrocytes and have the capacity to recover their function in radiation-damaged salivary glands (Rotter et al. 2008). The salivary gland-derived stem cells isolated from SMG express stem cell markers (c-kit and scal-1), and these cells can induce acinar and duct cells. Furthermore, these stem cells have the potential to differentiate into liver or pancreas tissues and to form salispheres in in vitro cultures. The salisphere can repair radiation-induced atrophied acinar cells by stem cell transplantation, restoring saliva flow (Lombaert et al. 2008). Additionally, bone marrow MSCs or extracts called "soups" have the potential to repair damaged tissues, increase the tissue regeneration ability of the surviving salivary gland tissue stem cells, and promote the regeneration of damaged acinar cells after radiation (Sumita et al. 2011; Tran et al. 2013). Tissue repair by adult tissue-derived stem cell transplantation therefore has the therapeutic potential to regenerate salivary glands.

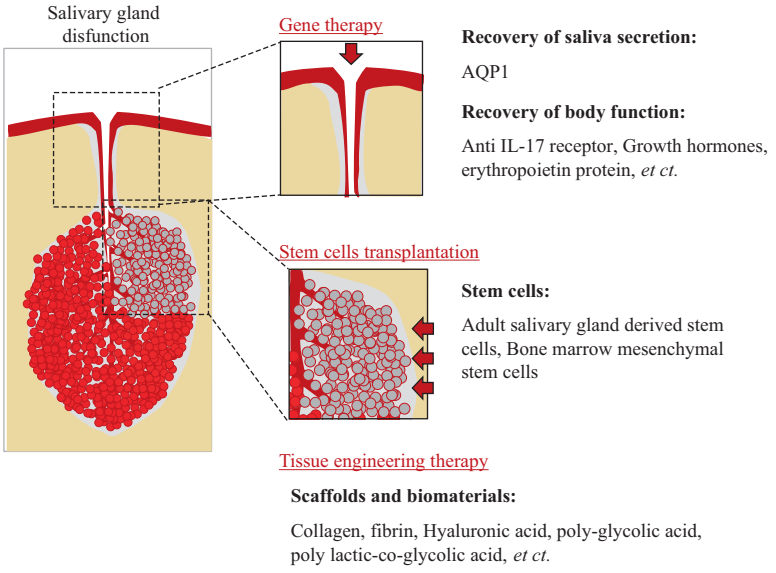

Fig. 7.2 Tissue repair by using stem cell transplantation and gene therapy. As a regenerative approach for salivary gland dysfunction or injury, stem cell transplantation, gene therapy, and tissue engineering therapy have been reported

7.4 Salivary Gland Tissue Repair Using Gene Therapy

Gene therapy is also a general technique for salivary gland regeneration (Rotter et al. 2008; Denny and Denny 1999; Horie et al. 1996; Sugito et al. 2004; Bücheler et al. 2002; Tran et al. 2005; Sun et al. 2006; Kishi et al. 2006) (Fig. 7.2). Because the salivary glands have ducts that open into the oral cavity and are close to the surface, it is very convenient to directly inject into the ductal epithelium with an adenovirus or adeno-associated virus that expresses a particular gene. Gene transfer of water channel aquaporin-1 (AQP1), which is important for transcellular water transport, can significantly restore saliva secretion in irradiated salivary glands (Delporte et al .1997). Additionally, interleukin-17 (IL-17) receptor antibodies, growth hormones, and erythropoietin protein have been altered using gene transfer in salivary gland tissue. Because the salivary glands have both exocrine and endocrine functions, substances are secreted into the bloodstream and can perform systemic functions (Kagami et al. 1996; He et al. 1998; Voutetakis et al. 2005). Gene therapy has progressed to phase I and is also expected to be a new strategy for the regeneration of salivary glands and other organs.

7.5 Salivary Gland Tissue Repair Using Tissue Engineering Therapy

The important aspects involved in the tissue engineering of salivary glands are cell-cell adhesion, cell-extracellular matrix (ECM) protein adhesion, and the biocompatible and biodegradable 3D scaffold used, which can maintain the adhesion (Aframian and Palmon 2008) (Fig. 7.2). ECM proteins, such as laminin and glycosaminoglycans, are important for salivary gland epithelial cell polarity and proliferation. A combination of scaffolds, including collagen gel, Matrigel, hyaluronic acid (HA), and polyglycolic acid, causes physical changes and promotes cell migration, polarity, and cell adhesion (Peters et al. 2014; Pradhan and Farach-Carson 2010; Lombaert et al. 2016). Therefore, it is important to select the suitable cells, ECM, and scaffold and to take into consideration the molecules involved in salivary gland development and branch morphogenesis.

7.6 Whole Salivary Gland Regeneration Using Organ Germ Methods

The ultimate goal of regenerative organs is the replacement of injured and dysfunctional organs with fully functional bioengineered organs. One concept for functional organ regeneration is to mimic the developmental process of organogenesis. Organ germ reconstruction using a cell aggregation method is a typical technique to

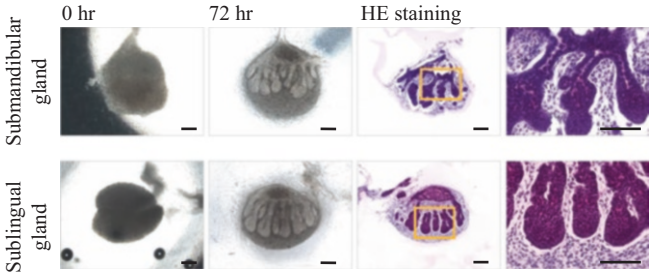

Fig. 7.3 Regeneration of salivary gland germ using organ germ methods. Phase-contrast images of the bioengineered submandibular and sublingual gland germ on 0 and 72 h of organ culture. The bioengineered salivary gland germ developed a blanching morphogenesis followed by stalk elongation and cleft formation within 72 h

reproduce self-organization and organ development. The salivary gland epithelial and mesenchymal cell mixture aggregates promote organ development and branching morphogenesis (Wei et al. 2007). Additionally, we demonstrated that a bioengineered organ germ, using organ germ methods, could regenerate ectodermal organs, such as teeth, hair follicles, and lacrimal glands (see Chaps. 5, 6, and 8). This method could be applied to achieve functional salivary gland regeneration (Ogawa et al. 2013). A bioengineered salivary gland germ was reconstructed using single epithelial and mesenchymal cells isolated from submandibular gland germs of ED 13.5 mice. The bioengineered submandibular gland germ successfully initiated salivary gland development, with branching morphogenesis followed by stalk and cleft formation in organ culture (Fig. 7.3). Bioengineered sublingual and parotid gland germs were also developed using organ germ methods, and they grew similarly to the submandibular gland.

7.6.1 Transplantation of the Bioengineered Salivary Gland Germs

Correct duct formation to connect the oral cavity and the bioengineered salivary gland germ is essential for correct acinar formation and saliva secretion. To achieve duct formation, bioengineered salivary gland germs were transplanted into the parotid gland ducts using an inter-epithelial tissue-connecting plastic method in a mouse model of salivary gland defects (Ogawa et al. 2013). Thirty days following transplantation, the bioengineered salivary gland and the host parotid duct were connected with nylon thread (Fig. 7.4a). The bioengineered submandibular gland regenerated serous acinar cells, and the sublingual gland regenerated mucous acinar cells. These bioengineered salivary glands had the correct organ structure, including localization of the water channel aquaporin-5 (AQP5), myoepithelial cells, and nerve fibers, which were similar to natural fibers (Fig. 7.4b).

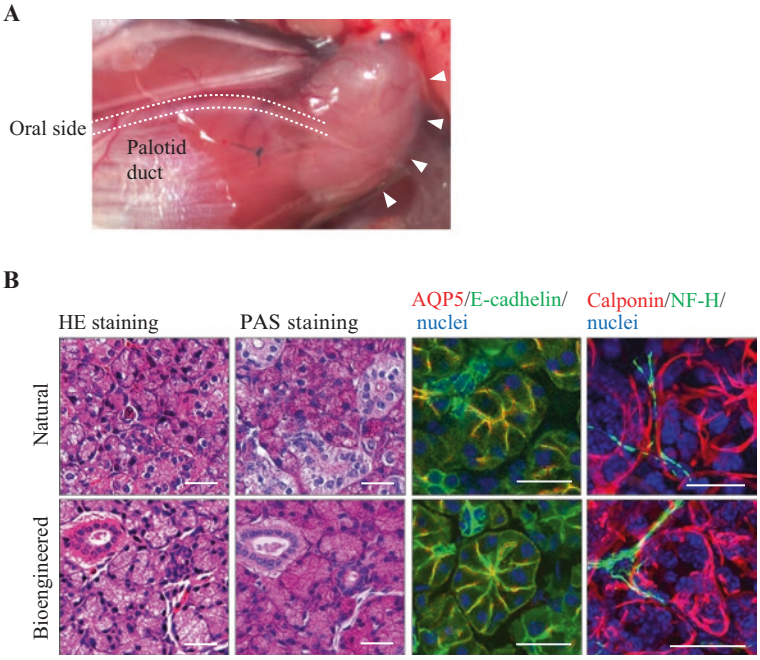

Fig. 7.4 Transplantation of bioengineered salivary gland germ. (**a**) Photographs of the bioengineered submandibular gland after 30-day transplantation. The bioengineered submandibular gland and parotid gland duct have established a correct connection. *Arrowhead* bioengineered submandibular gland. (**b**) Histological analysis of the natural (*upper columns*) and bioengineered (*lower columns*) submandibular gland. Images of HE staining (*left*) and periodic acid and Schiff (PAS) staining (*second from the left*). Immunohistochemical images of AQP5 (*red*) and E-cadherin (green; third from the *left*) and calponin (*red*) and NF-H (*green*; *right*) are shown. The bioengineered submandibular gland had a correct organ structure and regenerated serous acinar cells

7.6.2 Saliva Secretion from Bioengineered Salivary Glands

Restoration of the central nervous system is an important issue in organ regenerative therapy. Food, heat, and pain stimulation to the oral cavity induce saliva secretion via afferent and efferent nervous stimulation (Proctor and Carpenter 2014) (Fig. 7.5a). Additionally, saliva is essential for tasting; therefore, a saliva secretion was analyzed using gustatory tests, including sour (citrate), bitter (quinine hydrochloride), salty (NaCl), sweet (sucrose), and umami (glutamate) tastes (Matsuo 2000; Froehlich et al. 1987; Sasano et al. 2010; Ogawa et al. 2014). Citrate stimulation induced similar quantities of saliva secretion from the bioengineered salivary glands as natural salivary glands (Fig. 7.5b). All gustatory stimulation induced significant quantities of saliva secretion compared with non-stimulation, and the amount of saliva was dependent on the type of stimulus in the order of sour > bitter > umami > salty = sweet (Fig. 7.5c). These results indicate that saliva secretion from

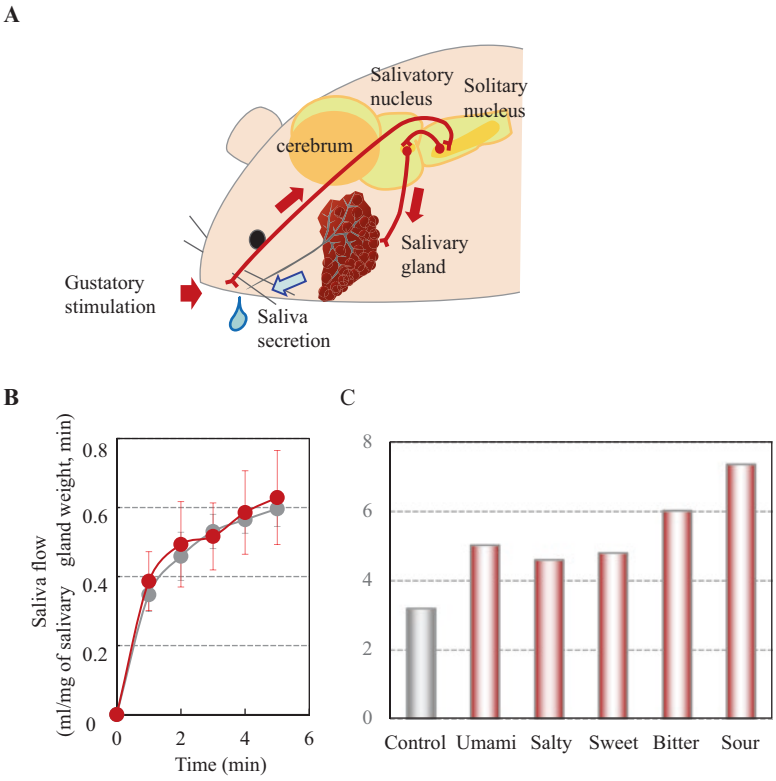

Fig. 7.5 Assessment of saliva secretion. (**a**) Schematic representation of saliva secretion via the central nervous system using gustatory stimulation. (**b**) The time course of the amount of saliva secretion associated with normal mice (*gray dots*) and bioengineered submandibular gland-engrafted mice (*red dots*) after the gustatory stimulation by citrate. The amount of secreted saliva was not significantly different. (**c**) The amount of saliva secretion after 5 min of stimulation was associated with water stimulation (*gray bar*) and gustatory stimulation, including umami (glutamic acid), salty (NaCl), sweet (sucrose), bitter (quinine hydrochloride), and sour (citrate) (*red bars*)

the bioengineered salivary glands occurred via proper nerve innervations and neurotransmission.

7.6.3 Protection from Bacterial Infection and Dry Mouth

Saliva contains numerous proteins and cytokines that are essential for the maintenance of oral health and homeostasis, including amylase, lysozyme, IgA, lactoferrin, myeloperoxidase, NGF, EGF, and parotin (Lamy et al. 2010, Cohen 1962). Saliva reduction induces various clinical problems, such as bacterial infection, dental caries, sleep disorders, and swallowing dysfunction. In mouse models of salivary

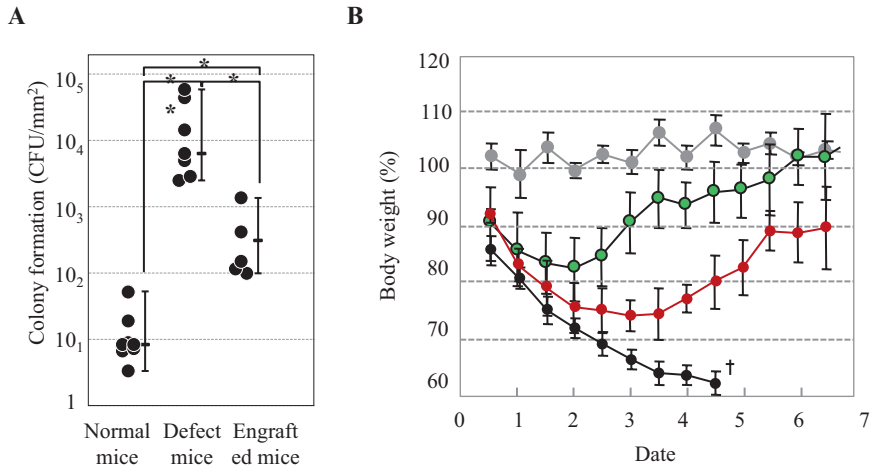

Fig. 7.6 Improvement of xerostomia by bioengineered salivary gland. (**a**) Assessment of bacterial propagation in the buccal mucosa of normal mice, salivary gland defect model mice, and bioengineered salivary gland-engrafted mice. *$P < 0.05$, **$P < 0.001$ by Student's *t*-test. (**b**) Measurement of body weight every 0.5 days after transplantation in normal mice (*gray dots*), salivary gland defect mice (*black dots*), salivary gland-engrafted mice (*red dots*), and salivary gland defect mice that were given high-viscosity water (*green dots*). All salivary gland defect mice died within 5 days (†) after the removal of all of the major salivary glands

gland defects, the volume of oral bacteria increased compared with normal mice. In contrast, it was significantly reduced in the bioengineered salivary gland-engrafted mice compared with salivary gland defect mice (Fig. 7.6a) (Ogawa et al. 2013). These results indicate that bioengineered saliva has a cleansing function that prevents bacterial growth and dryness of the oral cavity.

7.6.4 Swallowing Function Recovery

Among the salivary gland functions, swallowing is important for the absorption of nutrition and reduces the risk of aspiration, which can cause chronic lung disease (Sreebny and Schwartz 1997). Saliva promotes the formation of a bolus of food and results in swallowing reflex. In salivary gland defect mice, body weight decreased abnormally, and all of the mice died within 5 days, despite free access to food and water. However, high-viscosity water prevented the decrease in body weight and improved the survival rate in the salivary gland defect mice (Fig. 7.6b) (Ogawa et al. 2013).

High-viscosity water is usually used to support swallowing in dry mouth patients and geriatric nursing. Thus, the salivary gland defect mice may represent a useful animal model for studying difficulties in swallowing. In the bioengineered salivary gland-engrafted mice, their body weights increased 4 days after transplantation, and

they survived (Fig. 7.6b). These findings indicate that saliva secretion from bioengineered salivary glands can improve the swallowing function associated with oral health maintenance.

7.7 Future Perspectives for Salivary Gland Regenerative Therapy

Organ regenerative technology has advanced significantly, and many patients can expect to be treated with salivary gland regenerative therapy. To address the future clinical applications of salivary gland replacement therapy, it is essential to identify suitable cell sources. One candidate cell source is the patient's own cells because there is no immunological rejection. Recent stem cell studies have revealed the presence of adult tissue stem cells in the salivary gland. These adult tissue-derived stem cells, which express stem cell markers or MSC markers, can repair injured acinar cells by stem cell transplantation. However, the possibility of utilizing these stem cells has not been studied with regard to inducing similar salivary glands to those induced by epithelial-mesenchymal interactions. In contrast, pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, also have the capacity to become cell sources because these cells can differentiate into endodermal, ectodermal, and mesodermal cells (Wu and Hochedlinger 2011; Cohen and Melton 2011; Yan et al. 2010). The regeneration of some organs, such as the optic cup and pituitary gland, has been reported using ES cells or iPS cells. In the future, it is likely that methods for salivary gland regeneration using these pluripotent stem cells will be established.

In autoimmune diseases, atrophy of acinar cells and cell damage is caused by autoantigens. Because the transplanted regenerated acinar cells may also be affected by the autoimmune response, a genetic modification that decreases the expression of autoantigens against patient-derived stem cells must be performed to achieve future clinical applications of salivary gland replacement therapy in autoimmune disease. Current whole-organ regenerative therapy has the potential to become a future therapeutic technology for several diseases. Salivary gland replacement and regenerative therapy is expected to be realized by promoting fundamental technology development and the clinical application of regeneration research.

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Conflict of Interest M. Ogawa and T. Tsuji have no competing interests.

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