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Rho-associated kinase inhibitor에 의한 생쥐 타액선 세포의 생체 외 배양 조건 개선

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서울대학교 대학원
치의과학과 구강내과·진단학 전공
한 충 성

Abstract

Rho-associated kinase inhibitor improves the *in vitro* culture condition of isolated mouse salivary gland cells

Choongseong Han, D.D.S., M.Sc

Program in Oral Medicine and Oral Diagnosis

Department of Dental Science, Graduate School

Seoul National University

Directed by Associate Professor **Hee-Kyung Park**, D.D.S., M.S.D., Ph.D

Hyposalivation because of curative radiation therapy in patients with head and neck cancer is a major concern. At present, there is no effective treatment for hyposalivation, highlighting the importance of cell therapy as a new therapeutic approach. To provide functional cells for cell replacement therapy, it is important to overcome the limitations of current *in vitro* culture methods for isolated salivary gland cells. Here, an improved culture condition method for the cultivation of isolated salivary gland cells was suggested in this study. The

dissociated submandibular salivary gland cells of mice were seeded and treated with Rho-associated kinase (ROCK) inhibitor (Y-27632), which resulted in an increase in their cell adhesion, viability, migration, and proliferation. In particular, ROCK inhibitor (ROCKi) treatment maintained the expression of α-amylase in the primary cultured salivary gland cells for a long time as compared with untreated cells. The expression of C-Met, a ductal cell marker, was increased in cells treated with ROCKi. This modified culture condition may serve as an easy and convenient tool for culturing primary salivary gland cells for their application in hyposalivation therapy.

Key words : ROCK inhibitor, Y-27632, Hyposalivation, Submandibular salivary gland, Ductal cells, Primary cell culture

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Contents

List of Figures

1. Introduction

Hyposalivation is the most predictable side-effect of radiation therapy in patients with head and neck cancer, attributable to the irreversible damage caused to salivary glands. Prolonged reduction in the saliva by hyposalivation may cause several problems, including xerostomia, halitosis, dental caries, mucosal infections, burning mouth syndrome, and dysphagia (Vissink A and Burlage FR, 2003; Vissink A and Jansma J, 2003). Although several patients undergoing irradiation therapy exhibit hyposalivation or salivary gland atrophy, there are no satisfactory treatments to combat these issues. Therefore, clinical and experimental approaches that may replace or regenerate the impaired salivary gland tissue are desirable (Aframian DJ and Palmon A, 2008; Jensen DH et al., 2014). The application of primary cultured salivary gland cells in regenerative medicines for hyposalivation treatment requires modifications in the current cell culture conditions, as acinar cells that produce and secrete saliva in the salivary gland are rapidly degenerated and lose their secretory functions during the first 24-48 h *in vitro* cultures (Wigley CB and Franks LM, 1976). Although several reports show that the modulation of the extra-cellular matrix or basal membrane gel may improve the culture conditions and functional properties of isolated salivary gland cells *in vitro* (Oliver C, 1987; Durban EM, 1990; Fujita-Yoshigaki J, 2005; Maria OM, 2011), isolation and culture of salivary gland epithelial cells is still difficult.

The enzyme Rho-associated kinase (ROCK) mediates various important cellular functions such as cell shape, viability, secretion, proliferation, and gene expression (Liao JK et al., 2007). Modulation of ROCK expression in cultures affects the cellular functions of various cell types. ROCK inhibition suppresses proliferation and down-regulates migration of vascular smooth muscle cells (Kiian I et al., 2003; Loirand G et al., 2006). On the other hand, a ROCK inhibitor (ROCKi) has been shown to remarkably improve the proliferation and migration of osteoblasts on hydrophobic surfaces (Tian YS et al., 2009; Yang S et al., 2011). In human embryonic stem cells (hESCs), ROCK inhibition resulted in anti-apoptotic effects and treatment with the ROCKi significantly increased the survival rate of hESCs (Watanabe K et al., 2007). In osteoblastic cells, ROCK inhibition may reverse the common problems of osteoblast culture, including those related with low adhesion, proliferation, and migration of osteoblasts on hydrophobic surfaces (Tian YS et al., 2009). Furthermore, ROCK inhibition significantly enhances the migration of human corneal endothelial cells but fails to induce proliferation of human corneal endothelial cells (Pipparelli A et al., 2013). As seen in various reports, the modulation of ROCK activity showed various effects on cell cultures; however, the effect of ROCK modulation on salivary gland cells is still unknown. Therefore, in this study, the effects of ROCKi on the survival, proliferation, migration, and functions of primary cultured salivary gland cells were evaluated.

2. Review of Literature

2.1 Overview of Salivary gland

2.1.1 salivary gland structure

The saliva exists in the whole oral cavity in order to cover the teeth and oral mucosa by secreting from the three parts of major salivary glands, the parotid, submandibular, and sublingual glands, and from minor salivary glands spread throughout labial, buccal, palatal, retromolar and lingual regions (Nauntofte B et al., 1999). It has multiple functions, including the protection of teeth and oropharyngeal mucosa, articulation of speech, mastication, swallowing, digestion of foods, and maintenance of a balanced microbiota (Pedersen AM et al., 2002; de Almeida Pdel V et al., 2008; Ekström J et al., 2011; Pedersen AML et al., 2012; Ekström J et al., 2012; Ligtenberg AJM et al., 2014, Marsh PD et al., 2016; Kilian M et al., 2016).

The salivary glands harbor parenchymal and stromal components. The parenchymal compartment is consisted of acini (secretory end pieces), which secrete the primary saliva, connected to ductal system (intercalated, striated, and excretory) of salivary glands. The produced saliva is modified in the ductal system. Each acinus contains either serous or mucous cells, or mucous cells capped by serous demilunes (only found in the submandibular gland), arranged around the central

lumen. The salivary glands are histologically classified according to their structural composition and secretions (Young JA et al., 1987; Edgar WM et al., 1992; Garrett JR et al., 1998; Anderson LC et al., 1999; Humphries SP et al., 2001; Proctor GB et al., 2007). The diameter and the length of the ductal system vary depending on the type of glands. The major salivary glands have long and branched ducts, whereas the parotid and submandibular glands contain all ductal systems (intercalated, striated and excretory). On the other hand, the sublingual and minor glands lack striated ducts (Hand AR et al., 2008). Myoepithelial cells are stellate-shaped and contractile cells that surround the acini and intercalated ducts variably in the different glands. They are located between the basal lamina and the cytoplasmic membrane of acinar or ductal cells. The connective tissue that surrounds the major salivary glands forms septa, which divide the gland into lobes and lobules. These septa contain large blood vessels, nerves and ducts. The acini, intercalated ducts, striated ducts, small blood vessels and nerve cells are located within the lobules. In the minor salivary glands, the connective tissue merges imperceptibly with the surrounding connective tissue. The parenchyma has a rich supply of blood vessels that can form a capillary plexus, particularly adjacent to the ducts (Garrett JR et al., 1979).

2.1.2 Salivary gland secretion factors

The function of salivary gland is under the control of various factors and stimuli, which can change the volume, flow, and composition of saliva in general. It is suggested that normal salivary secretion is influenced by circadian clock mechanism, which may play a role in different salivary gland pathologies as well (Papagerakis S et al., 2014). The level of hydration in the body also affects salivary secretion. Previous report showed that the flow rates of parotid saliva significantly decreased in both young and old healthy adults after a 24 h-period of fasting fluid and food (Ship JA et al., 1997), and conditions of acute dehydration are also related to the reduction of salivary flow rates (Walsh NP et al., 2004; Fortes MB et al., 2012). Gland size is another factor associated with saliva flow rates (Ericson S et al., 1971; Inoue H et al., 2006). With regard to ageing, numerous studies have investigated whether the ageing is associated with decreasing salivary flow rates, since both major and minor salivary glands undergo age-related structural anddegenerative changes, such as loss of secretory acini and stromal alterations.

2.1.3 Function of salivary gland

Saliva has multiple pivotal functions for the maintenance of oral health.

It lubricates and cleanses the teeth and oral mucosa, and it maintains neutral pH through its buffering capacity. The saliva also prevents tooth demineralization, exerts antimicrobial actions, and aids in taste and bolus formation. Furthermore, the saliva initiates enzymatic digestion of starch as well as the saliva is imperative for mastication and swallowing and articulation of speech (Pedersen AM et al., 2002; de Almeida Pdel V et al., 2008; Ekström J et al., 2011; Pedersen AML et al., 2012; Ekström J et al., 2012; Ligtenberg AJM et al., 2014, Marsh PD et al., 2016). It also plays a pivotal role in the formation of the acquired enamel pellicle and the mucosal pellicle, which have a protective function that determines the initial adhesion and colonization of microorganisms and the composition of the resident oral microbiota (Kilian M et al., 2016).

2.1.4 Dysfunction of salivary gland

Dysfunction of salivary gland is any quantitative and/or qualitative defects in the output of saliva. It also can be a reduction in salivary secretion (hypofunction) or an increase in salivary secretion (hyperfunction). The latter, called sialorrhea (drooling or excessive salivation) is a relatively uncommon symptom in adults (Potulska A et al., 2005). Salivary gland dysfunction is often associated with xerostomia, which is a persistent sensation of dry mouth. Generally,

xerostomia occurs when the unstimulated whole saliva flow rate falls by 40-50% of its normal value in patients, indicating that more than one major salivary gland must be affected in the patients (Dawes C et al., 1987). However, the xerostomia may also occur without any evidence of salivary gland hypofunction (Fox PC et al., 1987). Thus, it may be a result from changes in salivary composition or function, particularly in lubricating mucins. Xerostomia is a common disease that affects at least 10% of an adult population (Thomson WM et al., 2006; Sreebny LM et al., 2010). In general, women and older people show lower salivary flow rates than in men and younger people, and they result in a higher prevalence of xerostomia than in men and younger people (Locker D et al., 1993; Nederfors T et al., 1997; Astor FC et al., 1999; Smidt D et al., 2011; Villa A et al., 2015). Regardless of the etiology, patients with hyposalivation frequently complain of dryness in oral cavty during the daytime. Persistent and severe hyposalivation commonly cause mucosal changes, and then these changes increase activities of caries with the lesion site on cervical, incisal and cuspal tooth surfaces as well as oral fungal infections (Edgar WM et al., 1992; Nauntofte B et al., 1999; Rudney JD et al., 2000; Pedersen AML et al., 2005; Fox PC et al., 2008; Pedersen AML et al., 2012; Villa A et al., 2015; Lynge Pedersen AM et al., 2015). Disturbed taste sensation, impaired lubrication and dysphagia may lead to changes in behavior like avoiding certain foods. Gradually, changes

in dietary intake possibly result in nutrient deficiency and atrophy of the masticatory muscles, and then decreased masticatory ability (Loesche WJ et al., 1995; Jensen SB et al., 2003; Bardow A et al., 2000; Dusek M et al., 1996). Consequently, salivary gland hypofunction and its related symptoms and clinical consequences frequently have negative effects on social quality of life (Pedersen AM et al., 1999; Gerdin EW et al., 2005; Enoki K et al., 2014).

2.1.5 Causes of hyposalivation

Intake of medications, especially of antidepressants, anxiolytics, opiates, antihypertensives, diuretics and antihistamines, is the most common reason that leads to salivary gland hypofunction and xerostomia. Medications can affect the salivary secretory mechanisms in various ways. The adverse effects of medication on salivary secretion are reversible and salivary gland function will usually be able to recover after withdrawal of the pharmacotherapy (Aliko A et al., 2015; Villa A et al., 2016).

Various diseases and medical conditions also can cause salivary gland dysfunction, including hypofunction and alteration of salivary composition. Some systemic diseases like Sjögren's syndrome, granulomatous diseases, graft-versus-host disease, cystic fibrosis, human

immunodeficiency virus infection, thyroid disease, late-stage liver disease, and uncontrolled diabetes permanently affect the salivary gland tissue and function, while salivary gland infections, sialoliths, dehydration, depression and anxiety have transiently and negatively affect salivary functions. Furthermore, hormonal disturbances, inflammatory gastrointestinal diseases, and malnutrition can also indirectly affect salivary secretion, (vonBültzingslöwen I et al., 2007; Jensen SB et al., 2010; Pedersen AML, 2015).

Radiotherapy to the head and neck region of patients harboring cancer commonly causes xerostomia and hyposalivation. In this case, the development of hyposalivation depends on the accumulative dose of radiation and the affected volume of salivary gland from the radiation. Although the turnover rate of the salivary gland tissue into disease status is slow (approx. 60 days), salivary dysfunction already occurs within the first week of radiation treatment, and the salivary secretion is continuously decreasing during 1-3 months after radiation therapy. Treatment of high doses (over 60 Gray (Gy)) usually results in irreversible salivary gland hypofunction and xerostomia, while the damage from mild doses (range from 30 to 50 Gy) may be reversible (Jensen SB et al., 2010). Specifically, radiotherapy directly damages the acinar cells, and the initial damage is mainly in the serous acini, but also can affect the surrounding blood vessels and nerve cells. However, the propagation of xerostomia in patients receiving

chemotherapy may usually restore the damage within 6 months to 1 year after treatment, and then more than the half of the patients shows normal salivary gland. Thus, it is not clear whether combinational therapy with radiotherapy and chemotherapy affects the risk on the development of salivary disease.

2.1.6 Current treatment of hyposalivation

Currently, there are several therapeutic strategies for treating patients of hyposalivation from different causes, however, the strategies are still not strong enough to recommend a particular treatment, either pharmacological or not. Several treatments used in patients with xerostomia temporarily improve their symptoms, in terms of some extent, salivary flow, but without medium or long term control in all cases. When the hyposalivation is caused by a irradiation of the parenchyma, pilocarpine is the most-prescribed parasympathomimetic medicine for the patients with the best results with topical and localized treatment (Taweechaisupapong S et al., 2006; Kim JH et al., 2014). In clinical trials, bethanechol and cevimeline have also been reported the successful improvements on the levels of salivary flow and symptoms in irradiated patient (Jham BC et al., 2007; Leung KC et al., 2008). On the other hand, recently emerging biological therapies have not produced satisfactory results for Sjögren's syndrome. For

example, rituximab, a monoclonal antibody with proven its efficacy in aslymphoma or rheumatoid arthritis, has not always resulted the better efficacy than that of placebo (Devauchelle-Pensec V et al., 2007). Furthermore, the use of malic acid with fluoride and xylitol, both spray and tablets, just relieves the symptoms of the patients (da Silva Marques DN et al., 2011; Gómez-Moreno G et al., 2013).

The use of alternative stimulating agents including oral electro-stimulation and acupuncture could be another good therapeutic option for the treatment of xerostomia and hyposalivation, because these alternative therapy have no side effects in most cases (Strietzel FP et al., 2011; Simcock R et al., 2013). On the contrary, several trials including intraoral devices for storing artificial saliva, mouthwash with triclosan, iron administration, oral supplementation with omega-3 and vitamin E have shown ineffective (Robinson PG et al., 2005; Singh M et al., 2010; Saleh J et al., 2015). In short, application of pilocarpine, a sialogogue drug, to the patients has been resulted the best efficacies in patients with irradiation-induced xerostomia and Sjogren's syndrome. In patients suffering from drug-related dry mouth, the use of malic acid along with other elements results some positive evidence in terms of the efficacy, but these treatment counteract the harmful effects on dental enamel. In general, the lubrication of the oral mucosa including mouth rinse with artificial saliva reduces symptoms, even though the effects are short-lived until now (Gil-Montoya JA et

al., 2016).

2.2 Cell transplantation for therapeutic approaches

2.2.1 Existence of salivary gland stem cells

The salivary glands are exocrine organs that produces and secretes saliva. Ductal ligation of rat submandibular salivary glands led to degeneration of saliva, but saliva flow rate returned to normal levels and acinar cells were reborn after deligation, suggesting the remarkable regenerative capacity of the salivary gland (Osailan SM et al., 2006; Cotroneo E et al., 2010). The salivary glands of rodents and human are basically composed of two saliva-producing cells (mucous and serous acinar cells), myoepithelial cells that facilitate saliva expulsion. Another compartment, a ductal system, modifies saliva composition and secretes the saliva into the oral cavity. The whole union of cells is keeping in close physical proximity to each other by supportive tissues (Pavlov IP et al., 1906; Proctor GB et al., 2007). The impact of radiation therapy on function of salivary glands is critical. Radiation therapy of the salivary gland induces severe early loss (phases 1 and 2, 0–10 days and 10–60 days after irradiation, respectively) in saliva production, suggesting that the salivary gland is radiosensitive (Coppes RP et al., 2001). The later phases of radiation-induced hyposalivation

(phases 3 and 4, from 60 to 120 and 120 to 240 days after irradiation, respectively), where in functionally mature acinar cells become senescence, the cells are not replenished anymore. This suggests that the radiation-induced sterilization of a salivary gland stem/progenitor cell population in the salivary glands.

Stem or progenitor cells have capacities of self-renewal and differentiation to replenish damaged cells in our body. Because the presence of stem cells have been identified in many tissues including human organs, the number of remaining undamaged salivary gland stem or progenitor cells will determine the regenerative capacity of the gland after irradiation. In fact, compensatory responses of damaged tissue in non-irradiated regions (presumable site for salivary gland stem/progenitor cells) have been demonstrated after radiation, indicating the potential of surviving salivary gland stem or progenitor cells to regenerate the tissue.

2.2.2 Marker expression of stem cell in salivary gland

Genetic labeling studies demonstrated that salivary gland stem cells with slow turnover capacity are located in the duct and able to regenerate the different cell types in the rat salivary gland (Denny PC et al., 2005). Branching morphogenesis study identified the salivary

gland stem cells that expressed keratin (KRT), which are dependent on nerve stimulation for their growth and survival (Knox SM et al., 2010). Lineage tracing studies using KRT5 promoter revealed that the cells expressing both KRT5 and KRT14 in the developing oral epithelium were able to contribute to acinar, ductal, and myoepithelial cells in the salivary gland (Lombaert IM et al., 2008). Additional two other KRTs, KRT15 and KRT19 have also been suggested as markers of stem cells in the salivary gland. KRT15 is expressed, together with KRT5, in the duct of the developing salivary gland (Knox SM et al., 2010). The expression of KRT19 is confined to the developing ducts and depletion of KRT19 in mice resulted in abnormal morphogenesis of the duct in the developing salivary gland (Nedvetsky PI et al., 2014). These studies suggests that cells expressing KRT19 are key players for efficient ductal development in the salivary gland.

Multiple receptor families of different growth factors have been suggested as a marker of stem cells in the salivary gland. A receptor tyrosine kinase, KIT was first identified as a major marker of hematopoietic stem cells and found to be expressed in a subset of KRT14+ salivary gland stem cells (Lombaert IM et al., 2013). Although the expression pattern of KIT is similar to that of KRT14, a subset of KIT+ cells do not express KRT14 in pre-acinar cells, suggesting distinct populations of progenitors exist in the salivary gland. Other markers of salivary gland stem cells are leucine-rich

repeat containing G protein-coupled receptors (LGRs). They are components of the WNT signaling pathway. Similar to KRT5, LGR5 is expressed mainly in the primary duct of the developing salivary gland and also expressed in multipotent salivary gland mesenchymal stem cells (Lu L et al., 2015).

A panel of transcription factors are thought to be involved in the regeneration and stem cells of the salivary gland. ASCL3 (also referred as Salivary Glands 1, Sgn1) is prominently expressed in excretory duct cells in the adult mouse salivary gland (Yoshida, S et al., 2001). The expression of Ascl3 (begins at E14) is also observed in the ductal regions of the developing salivary gland, where salivary gland stem cells reside, and ASCL3+ cells is able to differentiate into ductal and acinar cells during development (Bullard T et al., 2008). SOX2 is expressed throughout the oral epithelium and has a critical role in the generation of acini, and lack of Sox2 expression before gland ontogenesis perturbed the generation of Sox10+ acinar cells (Emmerson, E et al., 2018). Furthermore, ablation of Sox9 under the control of the Krt14 promoter inhibited acinar morphogenesis and impaired specification of distal putative progenitors, suggesting an essential role of Sox9 in morphogenic processes and cell fate in the salivary gland (Chang D et al., 2013). Transformation-related protein 63 (Trp63/P63) is also postulated as a marker for the salivary gland. The expression of p63 isoform was observed in basal epithelial and myoepithelial cells in

the salivary gland (Bilal H et al., 2003), and ablation of p63 caused depletion of all squamous epithelia and their derivatives, including the salivary glands (Yang A et al., 1999).

2.2.3 Clinical applications for salivary gland by stem cells

Based on markers described above, a number of studies have attempted to isolate salivary gland stem cells from human salivary gland for clinical application (Emmerson E et al., 2018). Feng et al. were able to generate human salispheres that expressed KIT by optimizing culture conditions used for murine salivary cells (Feng J et al., 2009). The human salivary gland-derived salispheres were able to produce multiple branches under 3D culture conditions with matrix (Okumura et al., 2008). A different study demonstrated that without marker-based cell sorting, adherent cells in cultures of the salivary gland tissues showed properties of mesenchymal stem cells (MSCs), and reconstructed acinar tissues, and alleviated hyposalivation in irradiated animals (Jeong J et al., 2013). Furthermore, other studies enriched for salivary gland stem cells by using clonal expansion and showed that the enriched population had multipotency of the salivary gland stem cells (Rotter N et al., 2008). However, more detailed characterization for the salivary gland stem cells obtained from different sources and different methods as well as careful evaluation of therapeutic efficacy upon

transplantation should be followed for safe and efficient clinical application in the treatment of hyposalivation.

Moreover, partial salivary gland loss-of-function can within certain situations be spontaneously recovered post intensity-modulated radiation therapy (IMRT) in certain situations (Li Y et al., 2007). This lead to the hypothesis that endogenous salivary gland cells can participate in organ repair, and thus that cell transplants could potentially be beneficial in regeneration of severe loss of function.

Many studies serve to emphasize the importance of a functional residual salivary gland stem or progenitor cell population in the salivary gland for hyposalivation recovery. Pre-radiation therapy isolation of salivary gland stem or progenitor cells followed by post-radiation could increase the regenerative potential of the salivary gland and potentially completely restore tissue homeostasis following radiation therapy. To develop a cell-based therapy for hyposalivation, determining the most potent salivary gland stem or progenitor cell population, and further characterizing of these cells, and having ability to manipulate putative salivary gland stem or progenitor cells *in vitro* is paramount.

2.2.4 Therapeutic use of primary culture with salivary gland

cells

The first proof-of-concept study for transplanting autologous salivary gland cells to repair salivary function was carried out in a rodent model by transplantation of epithelial cells expressing KIT (c-Kit, CD117). In this study, about 100 – 300 KIT+ cells were enough to generate new acinar and ductal structures in damaged salivary glands and this treatment significantly improved organ function after radiation (Lombaert IM et al., 2008). This research demonstrated that KIT+ cells are possible mouse salivary gland stem cells, and then the transplantation of the KIT+ cells could restore the functions of salivary glands in mouse model. From several studies using transplantation of murine KIT+ sub-populations, it was demonstrated that KIT+ cells (KIT+ CD24+, KIT+ CD49f+, KIT+ CD24+ CD49f+, KIT+ CD24+ SCA+), has different levels of stem cell or progenitor activity, with KIT+ CD24+ (CD49f+/SCA+) cells reported to be the most potent (Nanduri LS et al., 2013; Xiao N et al., 2014). These cells are likely located within the major ducts of the central salivary gland region where the highest stem and progenitor cell number resides (van Luijk P et al., 2015). Thus, KIT+ cells could be potentially used for future cell therapy applications, because presence of KIT+ cells was proven in human salivary glands (Lombaert IM et al., 2013). and can be isolated and cultured ex vivo (Feng J et al., 2009). A very recent ground-breaking study has further supported the clinical use of enriched

KIT+ sub-populations. In this study, only 500 KIT+ salivary gland cells per gland were capable of rescuing hyposalivation in an *in vivo* mouse model (Pringle S et al., 2016).

In many studies, it has shown that restoration of function of irreversibly damaged mouse salivary glands after injection of *in vitro* cultured cells presumably containing salivary gland stem cells is observed. These findings suggest the prospectiveness of clinical autologous salivary gland stem cell transplantation after radiotherapy. Another report demonstrated that injection of *in vitro* cultured cells into glands resulted in the remarkable morphological and functional restoring abilities over prolonged time intervals. (Lombaert IM et al., 2008). Furthermore, repopulation of the original ductal compartment and differentiation of transplanted cells into acinar cells were observed as well in the same study (Lombaert IM et al., 2008).

Most interestingly, repairing of salivary gland is not only driven by transplanted cells, but also by promoting the regenerative capacity of remaining endogenous stem or progenitor cells (Pringle S et al., 2016). Radiation can induce stem or progenitor cell awakening *in vivo* (Lombaert IM et al., 2008; Hai B et al., 2012), and thus these stem cell population can be activated with appropriate stimuli. For example, any type of transplanted cells could enhance local endogenous repair when the appropriate stimuli are produced locally and a quiescence stem or progenitor cells are present nearby. However, clinically, this

approach may be limited to autologous cell therapy because salivary gland cells from aging patients contain fewer stem and progenitor cells (Feng J et al., 2009; Maimets M et al., 2015). This implies that more stem and progenitor cells (than those obtained in the pre-radiation therapy biopsy) may be required for organ repair. Recent efforts to increase the number of KIT1 cells ex vivo using growth factors (Patel VN et al., 2014) or Aldehyde dehydrogenase-3 (ALDH3) activator (Banh A et al., 2011) may be useful, however, the requirement of absolute cell number for functional regeneration in human remains unclear yet. These considerations may necessarily lead to find out more improved techniques *in vitro* culture system for enhancing the number of cells cultured.

2.2.5 Limitations of salivary gland cell cultures

The development of a viable artificial salivary gland is a novel option in bringing relief to many patients afflicted with hyposalivation. Ideally, autologous primary cells should be used clinically (Hoekstra et al., 2002). In cases of severely damaged or absent salivary glands, however, these individuals would need to depend on an artificial salivary gland grown from donor cells. Nevertheless, cells in primary cultures display slow growth, de-differentiation, and a finite lifespan (Redman et al., 1988; Yeh et al., 1991; Quissell et al., 1994a and

1994b).

Isabelle L et al have been reported that the difficulties in expansion of salivary gland stem cells *in vitro* for future clinical use (Isabelle L et al., 2017). Alternatively, transplantation of hematopoietic stem cell have been tried over the past 50 years, but the *in vitro* culture of hematopoietic stem cells is still challenging as well. (Wilson A et al., 2006). Although a report has been shown that salivary gland cells can be cultured *in vitro* and produce saliva (Kishi T et al., 2007), this report could not suggest the culture capacity for application into a transplantable preclinical model without contamination of other potentially damage-inflicting cells. The recently demonstrated models may appear readily transplantable to the clinic and lead in the near future to clinical use of salivary gland tissue stem cells. Isabelle L et al propose the possibility that transplantation of these cells will result in amelioration of the significantly reduced quality of life of surviving cancer patients (Isabelle L et al., 2017). Furthermore, These approaches are the potential proof for the potential use of stem cell transplantation to functionally rescue solid organ deficiency and have now demonstrated that *in vitro* culture of processed salivary gland tissue is possible (Lombaert IM et al., 2008). Some studies have used a monolayer culture technique, where adherent, proliferative colonies of

presumed salivary gland cells, stem cells or progenitor cells were cultured from rat salivary glands, and after 7 days of culture with added epidermal growth factor and hepatocyte growth factor demonstrated expression of ductal (cytokeratins 18 and 19 and c-Met), acinar (amylase andaquaporin-5), and myoepithelial (vimentin and a-smooth muscle actin) differentiation marker proteins. Consequently, growing attention has been given to the improvement of salivary cell culture conditions in developing an artificial gland.

2.3. ROCKi and its application

2.3.1. Roles of ROCK and ROCKi in biological systems

ROCKi, Y-27632, is an inhibitor of ROCK and was discovered as a downstream target of the small GTP-binding protein Rhos (Osailan SM et al., 2006). Previous studies showed that once Rho was activated by its activators, including sphingosine-1 phosphate and lysophosphatidic acid, Rho guanine nucleotide exchange factor (GEF) was stimulated and ROCK eventually modulates a wide spectrum of cellular events involving the actin cytoskeleton (Cotroneo E et al., 2010). It has been reported that ROCKi modulates cellular growth, migration, adhesion, apoptosis and metabolism by regulating the cytoskeletal assembly (Nedvetsky PI et al., 2014). Since ROCK mediates actomyosin

contraction, and activates apoptotic signaling cascades, ROCKi has been used in different biomedical purposes using cultured cells and tissues, and various animal models with multiple human diseases (Lombaert IM et al., 2013; Lu L et al., 2015).

Particularly, one of ROCKi, Y-27632, is quite stable in aqueous solution for a long time, it is widely used for *in vitro* culturing of cells or tissues at approximately 10 μM. By promoting focal adhesions and actomyosin contraction, it has been known that ROCKs regulate not only the migration, but also polarity of cells, suggesting the involvement of ROCKs in tumor metastasis (Bourguignon LY et al., 1999). Indeed, a previous study showed that overexpression of activated ROCK promoted tumor invasion (Yoshioka K et al., 1999). Therefore, Y-27632 was previously used *in vitro* study that used different cancer cells or cell lines such as NIH 3T3 fibroblasts, Swiss 3T3 fibroblasts, and HeLa cells to investigate the role of Rho proteins in stress fiber formation, focal adhesion, and transformation. Y-27632 treatment in rat hepatoma cells and breast cancer cells reduced tumor-cell dissemination, suggesting its potential use in cancer therapy (Itoh K et al., 1999).

In addition to cell lines, it was previously reported that Y-27632 increased the survival rate of human keratinocytes cultured *in vitro* (Chapman S et al., 2010). ROCKs also modulate the phagocytic activity of macrophages and the permeability of endothelial cells by

regulating actin cytoskeletal protrusions and tight and adherent junctions (Wojciak-Stothard B., 2001; Wojciak-Stothard et al., 2002). Additionally, ROCKs are involved in differentiation program of adipogenesis and myogenesis, and thus Y-27632 treatment in mice defecting RhoGAP restores normal adipogenesis (Sordella R et al., 2003).

2.3.2. Apoptosis and ROCKi

A large body of recent evidence demonstrated that ROCK is involved in apoptotic and survival pathways in different types of cells. As described earlier, ROCK is involved in many morphological processes of apoptosis, including cell contraction, fragmentations of nucleus, membrane blebbing, and formation of apoptotic bodies (Orlando K et al., 2006). Previous studies showed that ROCKi treatments reduced apoptosis but others demonstrated that it promoted apoptosis (Svoboda, K et al., 2004). Overall, it is evident that ROCK plays key roles in both apoptosis and survival of cells while the exact signaling pathways involved in ROCK remain to be elucidated.

When adherent cells detached from ECM, cell membrane retracts and a series of morphological changes was occurred upon the onset of cell death, called apoptosis (Danial N et al., 2004). In the apoptotic

cascade, active caspase 3 cleaves and activates ROCK and the active form of the ROCK causes the membrane blebbing after cell detachment from ECM before nucleus condensation and fragmentation into apoptotic bodies (Coleman ML et al., 2001). Therefore, treatment with Y-27632 in apoptotic cells probably inhibit membrane blebbing and the related events in apoptotic program. However, other apoptotic events including caspase activation and release of cytochrome c from mitochondria still occurred in the presence of ROCKs (Coleman ML et al., 2001), suggesting that inhibiting ROCK by Y-27632 is not sufficient to allow cells escape cell death, but reduce a part of events in the apoptotic process. Thus the exact role of ROCK or ROCKi in the apoptotic program remains to be investigated.

2.3.3. ROCKi and *in vitro* expansion of cells

Human pluripotent stem cells (hPSCs) (human induced pluripotent stem, hiPSCs; and hESCs) have the potential to be an important source of a diverse cell type for basic research, clinical cell replacement therapies, and drug discovery and screening. However, culture and genetic manipulation of hPSCs are technically much difficult than mouse pluripotent stem cells due to their slow growth rate and anoikis, a programmed cell death of anchorage-dependent cells (dissociation-induced apoptosis) (Amit M et al., 2000).

Mouse pluripotent stem cells are able to grow as multiple-layered compact colonies when they were dissociated into single-cell suspension and plated in culture dishes as individual single cells. However, hPSCs grow as flat monolayer colonies and most of the cells undergo apoptosis when the colony is disrupted by dissociation into single-cell suspensions (Watanabe K et al., 2007). The cell dissociation is the most common and inevitable procedure to manipulate cells *in vitro* for different purposes, including cell passaging, gene transfection, cyropreservation, purification using FACS (fluorescent activated cell sorting), and differentiation. Unfortunately, survival rate of single hPSC suspension was quite low due to anoikis (single-cell dissociation-induced apoptosis) of hPSCs. Therefore scientists have been performed only partial dissociation of hPSC colonies to make small cell clumps consisting of several cells, not as single cells, to maintain their survival (Leung et al., 1995). However, this attempts resulted in formation of heterogeneous sizes of clumps and colonies, contributing to the reduction in reproducibility of data obtained from studies. Therefore the dissociation-induced cell death was a major hurdle to overcome for efficient manipulation and maintenance of hPSC lines for stem cell research.

Recently, it was reported that ROCKi dramatically increased the survival of single-cell dissociated hPSCs (Watanabe K et al., 2007), suggesting that ROCKi promoted the survival of single-cell dissociated

hPSCs by perturbing apoptotic progress or increasing cell-cell interactions. ROCKi has now been widely used in the growth, freezing, gene transfer, and other manipulations of hPSCs and other cell types (Li X et al., 2008). For example, Y-27632 was used to increase cell recovery of sorted hPSCs after FACS (Emre et al., 2010). In addition, Y-27632 was useful for generating hiPSCs by enhancing survival of colonies after the gene delivery of reprogramming factors into cells (Park I et al., 2008). Y-27632 in culture mediums significantly enhanced the efficiency of hPSC colony formation after freezing and thawing (Li X et al., 2008). It has been reported that ROCKi was effective in culture of hPSCs in serum- and feeder-free culture conditions to avoid xenogeneic contaminations (Pakzad et al., 2010).

Furthermore, Y-27632 also affects on differentiation of hPSCs, promoting formation of neural progenitors of specific regions of the brain (Osakada et al., 2009) and enhancing neurite outgrowth in neural stem cells by upregulating YAP expression (Jia XF et al., 2016). A recent study demonstrated that Y-27632 suppressed the expression of senescence-related proteins and significantly promoted cellular proliferation of progenitor cells and mesenchymal cells of the salivary gland *in vitro* (Nakamura K et al., 2013; Lee J et al., 2015). Therefore, ROCKi, Y-27632, has been broadly used in different research areas in the cell culture studies, including expanding, freezing, differentiation, and gene delivery.

3. Materials and methods

3.1 Preparation of primary submandibular salivary gland cells from mouse and *in vitro* culture

Submandibular gland tissues were harvested from 8-week-old C57/BL6 mice (Raon bio co. Ltd., Korea). The tissues were dissociated from cervical fascia and connective tissues under a dissecting microscope and gently collected in phosphate-buffered saline (PBS; Welgene, Daegu, Korea). Freshly dissociated tissues were washed twice with Dulbecco's modified Eagle's medium and Ham's F-12 mixture (DMEM/F12 medium, 1:1, Gibco, Grand Island, NY, USA) supplemented with 100 U/mL penicillin G and 100 mg streptomycin (Gibco), minced with ophthalmic curved scissors, and incubated with DMEM/F12 medium containing 4 mg/mL collagenase type IV (Sigma-Aldrich, St Louis, Mo) at 37°C for 30 min. Dissociated cells were centrifuged at 1,000 rpm for 1 min and washed twice with DMEM/F12 medium. The collected cell pellet was resuspended in DMEM/F12 medium and filtered through a 100 μm cell strainer (BD Falcon, Bedford, MA, USA). The purified cells were collected by centrifugation at 1,000 rpm for 5 min and the cell pellet was resuspended in the culture medium. The cells were plated at a density of 5 X 10^4cells/well in four-well plate in DMEM (Gibco) medium supplemented with 100 U/mL penicillin G and 100 mg streptomycin,

20 ng/mL epidermal growth factor (Sigma-Aldrich), 20ng/mL basic fibroblast growth factor (PeproTech, RockyHill, NJ, USA), 1/100N2 supplement (Gibco), 10 mg insulin-transferrin-selenium (Gibco), and 1 mM dexamethasone (Sigma-Aldrich) with/without 10 mM ROCKi, Y-27632 (Calbiochem, Merck, Rockland, MA, USA) and incubated for 24, 48, 72, and 96 h in 5% CO_2at37°C. The plate was pretreated with 20 mg Matrigel (BD Falcon) for 2 h before use.

3.2 Measurement of the anti-apoptotic effect of Y-27632 on submandibular salivary gland cells

Analysis of the survival rate of submandibular salivary gland cells was performed in two different conditions. Two days after cell seeding, cells were treated with fresh medium with or without 10 mM Y-27632 at different time points (24, 48, 72, and 96 h). The analysis of cell proliferation was performed by determining the kinetic values at each time point using a microscope (Nikon, Tokyo, Japan)

3.3 Cell proliferation assay

To confirm the proliferation of primary mouse salivary gland cells during *in vitro* culture at each time point (24, 48, 72, and 96 h), cells

were incubated with 10 mM 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen, Grand Island, NY, USA) for 24h and their proliferation evaluated using Click-iT®EdU imaging Kit (Invitrogen), as per the manufacturer's instructions. The nucleus was stained with 5mg/mL 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and cells were visualized under confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, USA).

3.4 *In vitro* live-dead assay

Cell viability assay was performed to study the effects of Y-27632 on the *in vitro* culture of primary mousesalivary gland cells using acetoxymethyl diacetyl ester of calcein-AM (Sigma-Aldrich) and propidium iodide (PI, Sigma-Aldrich). Cells were incubated with or without 10 mM Y-27632 in culture media for 24, 48, 72, and 96 h, followed by their treatment with 2 mM calcein-AM and 10 mM PI to differentiate between live and dead cells. The nucleus was stained with 10 mM Hoechst 33342 (Invitrogen) and cells were visualized under a confocal laser scanning microscope.

3.5 *In vitro* scratch assay

After seeding in a six-well plate, cells were cultured in the medium supplemented with Y-27632, as mentioned above. Upon confluence, cells were washed twice with PBS and scratched using a 200 µL micropipette tip. The cells were washed with PBS and incubated in the medium with or without Y-27632. The results were observed every 5 min until 24 h using a time-lapse live cell movie analyzer, JuLi^{TM}Br (NanoEnTek Inc, Seoul, Korea) in three independent experiments, then, calculated the percentage of confluence and cell migration rate by the JuLi^{TM}Br program.

3.6 Immunofluorescence staining

Cells were fixed with cold 4% paraformaldehyde (Sigma-Aldrich) in PBS. After blocking and permeabilization in 0.3% Triton-X 100 (Sigma-Aldrich) and 10% donkey serum (Sigma-Aldrich) in 0.1% bovine serum albumin (Sigma-Aldrich)/PBS, the cells were overnight incubated at 4°C with primary antibodies, followed by 1 h incubation at room temperature with secondary antibodies. The primary antibodies used were anti-c-amylase (1:50, Santa Cruz Biotechnology) and anti-C-Met (1:200, Santa Cruz Biotechnology), while secondary antibodies included AlexaFluor 488 anti-mouse, 594 anti-mouse, and 594 anti-rabbit (1:400, Invitrogen). Apotome-Axiovert 200M fluorescence microscope (Carl Zeiss) and confocal laser scanning

microscope (Carl Zeiss) were used to visualize cells after counter staining with 5 mg/mL DAPI.

3.7 Western blotting

Cells were harvested in a radio-immuno-precipitation assay (RIPA) lysis buffer (Upstate, Charlottesville, VA, USA) supplemented with a cocktail tablet of protease inhibitors (Roche, Mannheim, Germany). Equal amounts of proteins (50 µg/lane) were electroporated on NuPage™ 4-12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen). The membranes were overnight incubated with various primary antibodies at4°C, followed by their treatment with secondary antibodies for 1.5 h at room temperature. The primary antibodies used were anti-C-Met (1:200, Santa Cruz Biotechnology) and anti-β-actin (1:50, Sigma-Aldrich), while the secondary antibodies used included donkey anti-mouse IgG-horseradish peroxidase (HRP; 1:10000, Santa Cruz Biotechnology), donkey anti-rabbit IgG-HRP (1:10000, Santa Cruz Biotechnology), and donkey anti-goat IgG-HRP (1:10000, Santa Cruz Biotechnology). The proteins were visualized with the enhanced chemiluminescence (ECL), Supersignal® West Pico Chemiluminescence substrate (Thermo, Prod #34077).

3.8 Statistical analysis

All data were expressed as means ± SD from at least three independent experiments. Statistical differences of the two experimental groups (ROCKi+ and ROCKi-) were analyzed using Student's *t*-tests and *P* values less than 0.05 was considered statistically significant.

4. Results

4.1 ROCKi treatment enhances the growth and survival of isolated submandibular gland cells

To test the effect of ROCK inhibition on the growth of salivary gland cells, the submandibular gland tissue was dissociated from mice and the isolated cells were cultured in the presence or absence of Y-27632. After 24 h, cells were attached to the surface of the plates both with or without Y-27632. However, the cell attachment was better in the presence of Y-27632; most attached cells showed three-dimensional aggregates in both conditions (Fig. 1a). After 48 h, a significant increase in the number of attached cells was observed in Y-27632 treatment group and the cells started to spread out from the aggregates. Furthermore, the growth of cells dramatically increased following treatment with Y-27632 for 72 h and confluent cells were observed at 96 h (Fig. 1a and c). In the absence of Y-27632, most cells maintained their aggregated states at 48 h and exhibited slow growth compared to Y-27632 treated cells (Fig. 1c); several acellular areas were observed at 96 h (Fig. 1a).

The live/dead cell population of cultured cells following their treatment with Y-27632 using fluorescence-based calcein-AM (green) and PI (red) staining was assessed. Although PI-positive dead cells (red) were observed at all time points, the number of dead cells was significantly reduced in the presence of Y-27632 (Fig. 1b and d). Therefore, these data indicate that ROCK inhibition enhances the survival and growth of primary cultured salivary gland cells.

4.2 ROCK inhibition increases the proliferation of salivary gland cells

Following the observation of the increased survival and growth of salivary gland cells with ROCK inhibition, the proliferation of cultured cells with or without Y-27632 by EdU incorporation assay at 24, 48, 72, and 96 h was assessed and the number of cells during the culture period was counted. The cell number greatly increased after 48 h culture with or without Y-27632 treatment; however, a six-fold increase in the cell number was observed following treatment with Y-27632 from 48 to 72 h. On the contrary, only a three-fold increase in cell number was reported in the absence of Y-27632 at the same time point (Fig. 1c). Consistent with these observations, a significant increase in the number of proliferating cells was observed in the Y-27632 treatment group than in cells without inhibitor treatment (Fig. 2a and b). Furthermore, EdU-positive proliferating cells exponentially increased in Y-27632 treatment group after 72 h and the numbers of EdU+ proliferating cells were about three-fold increased in Y-27632 treated cells compared to control culture after 96 h (Fig. 2a and b). These data indicate that ROCK inhibition in the salivary gland cells may facilitate cell growth, as evident from the increase in cell proliferation.

4.3 ROCK inhibition increases the motility of salivary gland cells

The effect of ROCK inhibition on the migration of salivary gland cells was assessed with the *in vitro* scratch assay. Images were obtained with JuLi^{TM}Br live cell analyzer from 5 min to 24 h following treatment of cells with Y-27632 (Fig. 3). The time-lapse images at five time points (0, 4, 8, 10, and 23 h) after scratching showed that Y-27632 treatment increased the migration of salivary gland cells and the cell scratched over 90% confluency after 10 h (Fig. 3a). In the absence of Y-27632, the scratched area was slowly closed and the cell scratched confluency at 23 h (Fig. 3a). To confirm this morphological observation, a comparative analysis of the percentage confluency at different time points with an automated program was performed. Cells treated with Y-27632 reached confluency within averagely 13.5 h of treatment, whereas those without Y-27632 treatment took 23.5 h to reach confluency (Fig. 3b). Furthermore, the cell migration rate in the culture was assessed, then, Y-27632 treatment significantly increased cell migration rate of salivery gland cells (Fig. 3c). Thus, ROCK inhibition not only enhance the survival, growth, and proliferation of salivary gland cells but also increase their motility.

4.4 ROCK inhibition induces the expression of salivary gland cell-specific markers

As hyposalivation causes reduction in the salivary flow rate, it is important to maintain the secretory functions of the *in vitro* cultured salivary gland cells. Thus, the expression of markers for acinar and ductal cells that play pivotal roles in the secretory functions of the salivary gland was assessed. The activities of antibodies against acinar cells (α-amylase) and ductal cells (C-Met) in the mouse salivary gland tissue were evaluated (Fig. 4a). Staining results showed that α-amylase was well stained in the cluster-structured acinar cells in the mouse tissue, while C-Met was stained well in the ductal structure of the mouse tissue (Fig. 4a).

To determine whether ROCK inhibition may induce the secretory function of the salivary gland under *in vitro* condition, salivary gland cells were cultured and analyzed the expression of markers at 72 and 96 h (Fig. 4b-c). In the absence of Y-27632 treatment, the cultured cells showed the expression of α-amylase at 72 h but lost α-amylase expression at 96 h (Fig. 4b and c) and no C-Met expression was reported (Fig. 4b and c). On the contrary, Y-27632 treatment maintained the expression of α-amylase even at 96 h (Fig. 4b) and induced the expression of C-Met in salivary gland cells (Fig. 4b). The increase in the expression of ductal cell marker, C-Met, following

ROCK inhibition was also demonstrated by western blot analysis (Fig. 4c). Therefore, ROCK inhibition in the salivary gland cells may result in the maintenance of the secretory function of the cells.

5. Discussion

Hyposalivation as a consequence of curative radiotherapy in patients with head and neck cancer may reduce the function of salivary glands. As there is no effective therapy for hyposalivation, transplantation of primary cultured salivary gland cells has been considered as a therapeutic approach (Nelson J et al., 2013). However, acinar cells of the salivary gland easily lose their functions in *in vitro* cultures (Fujita-Yoshigaki J et al., 2009). Furthermore, these cells take a long time to reach confluency for their use in transplantation with the current methods of primary cell culture.

The enzyme ROCK mediates various cellular functions related to cell morphology, motility, and survival. In this experiments, it was observed that the treatment of salivary gland cells with ROCKi resulted in an increase in the adhesion of cells onto the cell culture plate surface and enhanced their survival, growth, and proliferation. From these observations, it is suggested that the treatment of primary mouse submandibular gland cells with ROCKi may protect them from cell death during the initial attachment stage and in prolonged cultures. In addition, ROCK inhibition may offer advantages related to cell proliferation and growth in cultures. It was demonstrated that ROCK

inhibition in salivary gland cells may increase the migration of cells. Cell motility is an important factor for cell therapy. In the clinical application of MSCs, homing of the injected cells into the target tissue is a significant issue (De Becker A and Riet IV, 2016). Therefore, It is hypothesized that injection of cultured salivary gland cells in the presence of a ROCKi may provide better therapeutic applications through increased homing capacity of the cells.

In the presence of Y-27632, the cultured salivary gland cells exhibited prolonged expression of markers of acinar and ductal cells of the salivary gland. Primary salivary gland cells rapidly lose their secretory functions *in vitro* (Wigley CB and Franks LM, 1976). Furthermore, parotid acinar cells may change to duct-like cells during *in vitro* epithelial-mesenchymal transition (Fujita-Yoshigaki J et al., 2009). However, it was found that the expression of α-amylase was retained in cells cultured in the presence of Y-27632. Furthermore, the inhibitor treatment also increased the expression of C-Met in salivary gland cells. Thus, ROCK inhibition in salivary gland cells may suppress the transition of acinar cells to ductal cells and amplify the number of ductal and acinar cells in the salivary cell culture.

6. Conclusion

In summary, this study demonstrates that ROCK inhibition may offer several advantages during the culture of primary salivary gland cells, such as increased cell survival, adhesion, growth and proliferation, migration, and cell type-specific marker expression. Therefore, it is concluded that ROCK inhibition in primary salivary gland cells may be used as a effective strategy for their easy and convenient application in clinical settings.

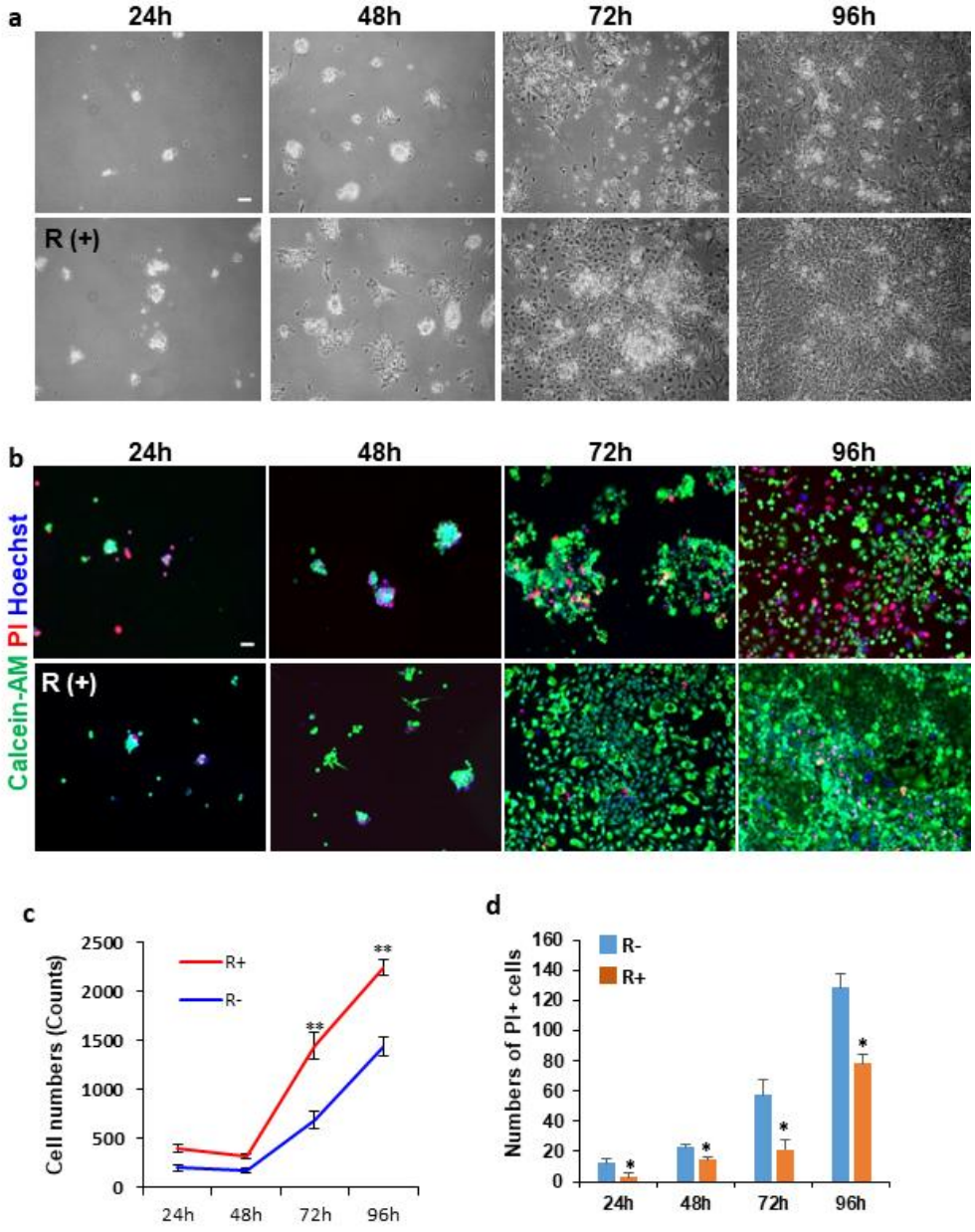

Fig. 1 Survival and growth of salivary gland cells by ROCKi.

Fig. 1 Survival and growth of salivary gland cells by ROCKi. (**a**) Representative morphological changes in isolated salivary gland cells during culture periods with or without Y-27632 treatment. (**b**) Calcein-AM/PI staining (live and dead cell assay) for isolated salivary gland cells during culture periods with or without Y-27632 treatment. R(+): cultured cells with Y-27632, R(−): cultured cells without Y-27632. (**c**) Number of isolated salivary gland cells during culture periods with or without Y-27632 treatment. (**d**) Numbers of PI-positive dying cells during culture periods with or without Y-27632 treatment. Scale bar = 200 μm. $*P < 0.05$, $**P < 0.01$ *versus* R (-).

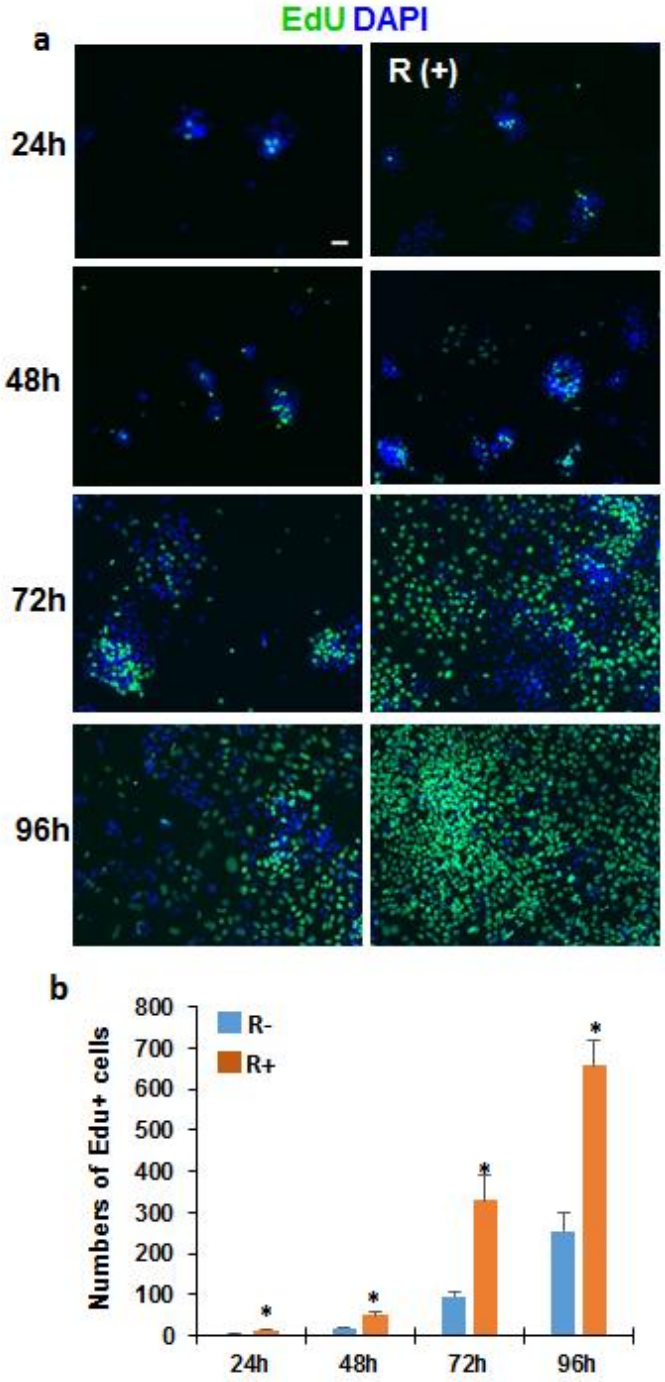

Fig. 2 Proliferation of salivary gland cells by ROCKi.

Fig. 2 Proliferation of salivary gland cells by ROCKi. (**a**) EdU staining of isolated salivary gland cells cultured with or without Y-27632 treatment. (**b**) Quantification of numbers of EdU-positive cells in (a). R(+): cultured cells with Y-27632 (n = 10 at each time point), R(−): cultured cells without Y-27632 (n = 10 at each time point). Scale bar = 200 μm. * $P < 0.05$ *versus* R(-).

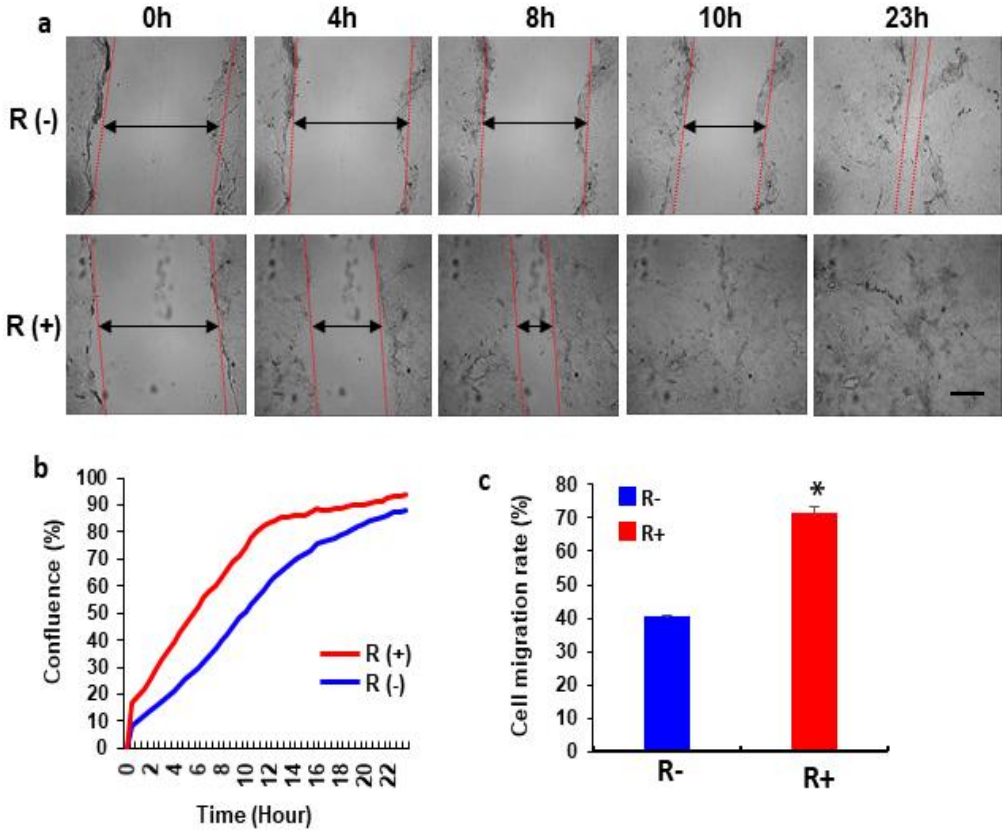

Fig. 3 Migration of salivary gland cells by ROCKi.

Fig. 3 Migration of salivary gland cells by ROCKi. (**a**) Representative images from the scratch assay of isolated salivary gland cells with or without Y-27632 treatment (**b** and **c**). Comparison of average confluency rate and cell migration rate of isolated salivary gland cells, calculated with JuLi^{TM}Br. R(+): cultured cells with Y-27632, R(−): cultured cells without Y-27632 treatment in three independent experiments. *$P < 0.05$ *versus* R(-). Scale bar = 200 μm.

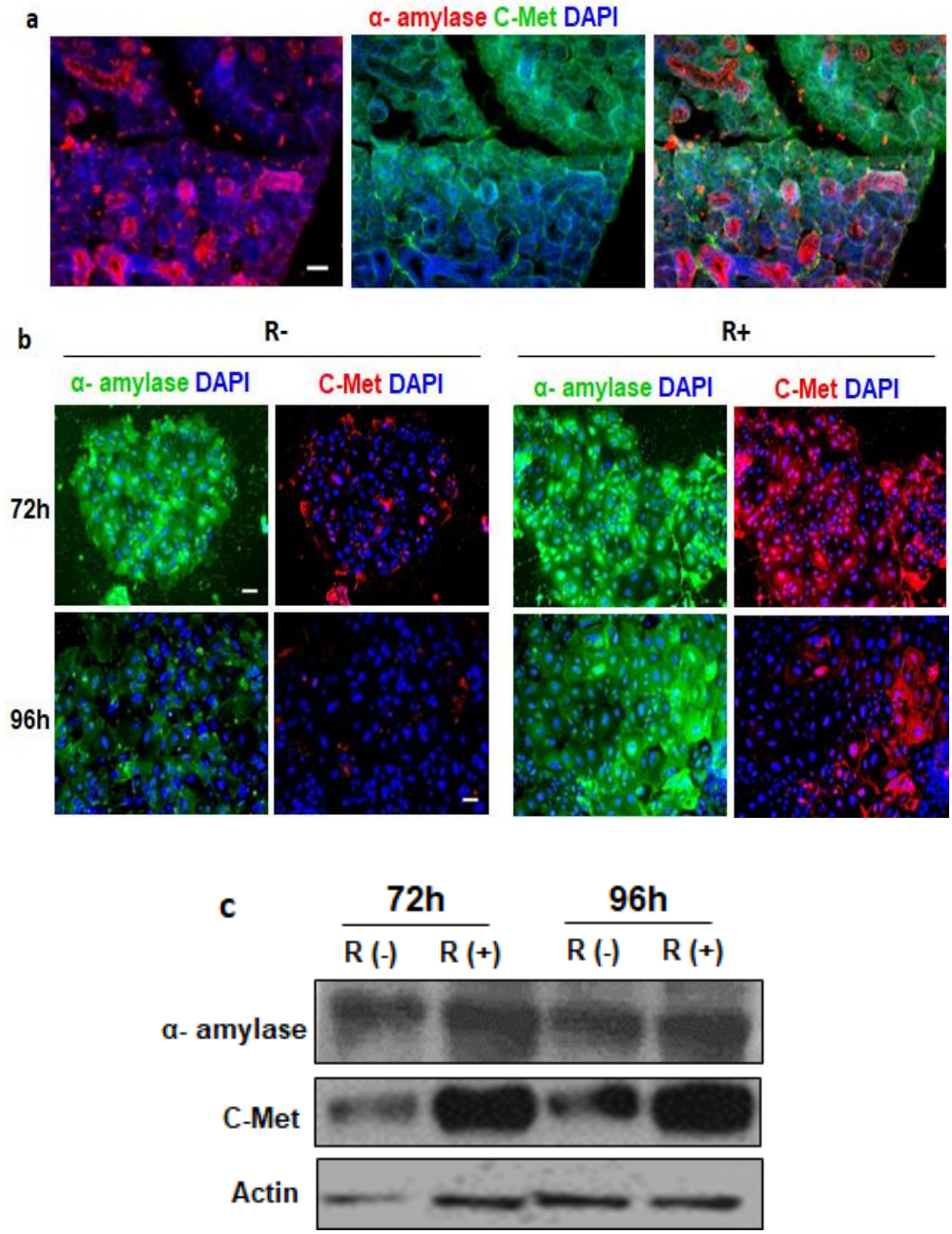

Fig. 4 Enhanced expression of salivary gland cell markers by ROCKi.

Fig. 4 Enhanced expression of salivary gland cell markers by ROCKi. (**a**) Immunostaining of α-amylase and C-Met in rat salivary tissue. (**b**) Immunostaining of α-amylase and C-Met in isolated salivary gland cells with or without Y-27632 treatment. (**c**) Western blot analysis of C-Met expression in isolated salivary gland cells with or without Y-27632 treatment. R(+): cultured cells with Y-27632, R(−): cultured cells without Y-27632 treatment. Scale bar = 50 μm.

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국문초록

방사선 치료를 받는 두경부 종양 환자들은 타액선 손상에 의한 타액분비저하로 부작용을 겪고 있다. 그러나 타액선 세포의 생체 외 배양시 낮은 생존율과 제한된 증식력은 세포 치료를 위한 충분한 양의 타액선 세포를 획득하는데 걸림돌로 작용하고 있다. Rho-associated kinase (ROCK)는 인간 줄기세포의 사멸을 저해함과 동시에 생존율을 증가시키는 것으로 알려져 있으나, 타액선 세포의 생체 외 배양에 미치는 영향은 크게 알려진 바 없다.

본 연구의 목적은 ROCK 억제제인 저분자 화합물, Y-27632이 타액선 세포의 생체 외 배양에 미치는 영향을 조사 분석함으로써 타액선 세포배양의 기술적 한계를 극복하기 위하여 실시되었다. 생존율, 사멸율, 증식율을 calcein-AM(생존세포 염색)/ propidium iodide(사멸세포 염색), 5-ethynyl-2′-deoxyuridine (EdU)로 염색하여 검사하였다. 세포증식과 이동능력 측정을 위해 in vitro scratch 분석을 시행하였고, 타액선 구성 세포의 표지 인자에 대한 세포면역형광염색과 Western blot 을 수행하였다. 연구 결과 Y-27632 처리에 의한 ROCK의 활성 억제가 타액선 세포의 체외 증식을 위한 초기 배양용기 부착 및 생착율, 배양 과정에서의 세포 성장과 이동 능력 뿐만 아니라, 생체 외 배양기간 동안 타액선 선포세포와 관세포의 특성유지를 모두 향상 시킬 수 있음이 확인되었다.

따라서 본 연구는 기존의 타액선 체세포의 배양에 따르는 기술적 한계를 극복하여 추후 타액선 세포치료에 적용할 수 있는 효과적인 방법을 제시하였다.

주요어 : ROCK 억제제, Y-27632, 타액분비저하, 악하선, 관세포, 초대세포 배양

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