Original Article

Int J Oral Biol 46:81-84, 2021
pISSN: 1226-7155 • eISSN: 2287-6618
https://doi.org/10.11620/IJOB.2021.46.2.81

International Journal of Oral Biology
IJOB
Check for updates

Expression patterns of Rho-associated protein kinase signaling pathway-related genes in mouse submandibular glands

Ki-Chul Kim and Sangho Roh*

Cellular Reprogramming and Embryo Biotechnology Laboratory, Dental Research Institute, Seoul National University School of Dentistry, Seoul 08826, Republic of Korea

Salivary glands are exocrine glands that secrete saliva into the oral cavity, and secreted saliva plays essential roles in oral health. Therefore, maintaining the salivary glands in an intact state is required for proper production and secretion of saliva. To investigate a specific signaling pathway that might affect the maintenance of mouse submandibular gland (SMGs), RNA sequencing was performed. In SMGs, downregulated expression patterns of Rho-associated protein kinase (ROCK) signaling pathway-related genes, including *Rhoa*, *Rhob*, *Rhoc*, *Rock1*, and *Rock2*, were observed. Gene expression profiling analyses of these genes indicate that the ROCK signaling pathway is a potential signal for SMG maintenance.

Keywords: Submandibular glands, RNA sequencing, ROCK signaling pathway

Introduction

Salivary glands are exocrine glands composed of three major glands (submandibular, parotid, and sublingual glands) that produce and secrete saliva into the oral cavity. Secreted saliva is essential to oral and intestinal health and functions such as food digestion, speech, taste perception, mastication, swallowing, and protection from bacteria [1,2]. Bicarbonate in saliva maintain pH of saliva at 6.5–7.4 and protect teeth from cariogenic pathogens [3]. Glycoproteins included in saliva bind with bacterial receptors and agglutinate oral microorganisms to eliminate overload of bacteria [4]. Saliva secreted from each of the major glands have distinguished constituent due to differences in secretory cell type [5]. Tenacious saliva from parotid glands by serous acini, mucin-rich saliva from sublingual glands by mucous acini, and mixed saliva from submandibular glands (SMGs) by both acinar cell types [6].

Xerostomia, a subjective feeling of dry mouth that may or may not be accompanied by hyposalivation, can be caused by radiotherapy for head and neck cancer, Sjögren's syndrome, or aging [1,2]. Irreversible loss of secretory acinar cells is the major cause of dry mouth [7,8]. Therefore, replacement of the acinar cells is a potential treatment method for dry mouth [9].

Activation of Rho-associated protein kinase (ROCK) signaling pathway is related to the development of various diseases such as asthma, erectile dysfunction, glaucoma, cancer, and kidney failure indicating the importance of ROCK signaling pathway in homeostasis of various organs [10–14]. In rat SMGs, small Rho GTPases, including RhoA and RhoB and RhoC, was detected in newly regenerated acinar cells [15]. Inhibition of ROCK using Y-27632 increased proacinar cells in salivary gland organoids [16] and delays senescence of salivary

Received May 21, 2021; **Revised** June 1, 2021; **Accepted** June 16, 2021
***Correspondence to:** Sangho Roh, E-mail: sangho@snu.ac.kr https://orcid.org/0000-0001-8082-6459

gland stem cells [17]. Based on previous studies, we hypothesized that ROCK signaling pathway may affect salivary gland maintenance and investigated the expression pattern of ROCK signaling pathway-related genes using RNA-sequencing (RNA-seq).

Materials and Methods

1. Animals

Five-week-old female mice (C57BL6×DBA2 F1-hybrid) were used. All animal experiments were carried out following the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (Approval number: SNU-180403-4).

2. Isolation of mouse SMGs

Salivary glands isolated from mice were washed twice with phosphate buffered saline (PBS). Using micro forceps, parotid and sublingual glands were removed. SMGs were then dissected by razor blade for total RNA extraction.

3. Primary isolation and culture of mouse skin fibroblasts

Underarm area was used to obtain skin fibroblasts. Incision site was sterilized with 70% ethanol and shaved with a scalpel. Approximately 1 cm^2 of skin was excised and placed in 60 mm culture dishes with PBS. Remaining subcutaneous fat on skin tissues was removed. Skin tissues were cut into 1 mm^2 pieces and transferred into 100 mm culture dishes. Cells from skin

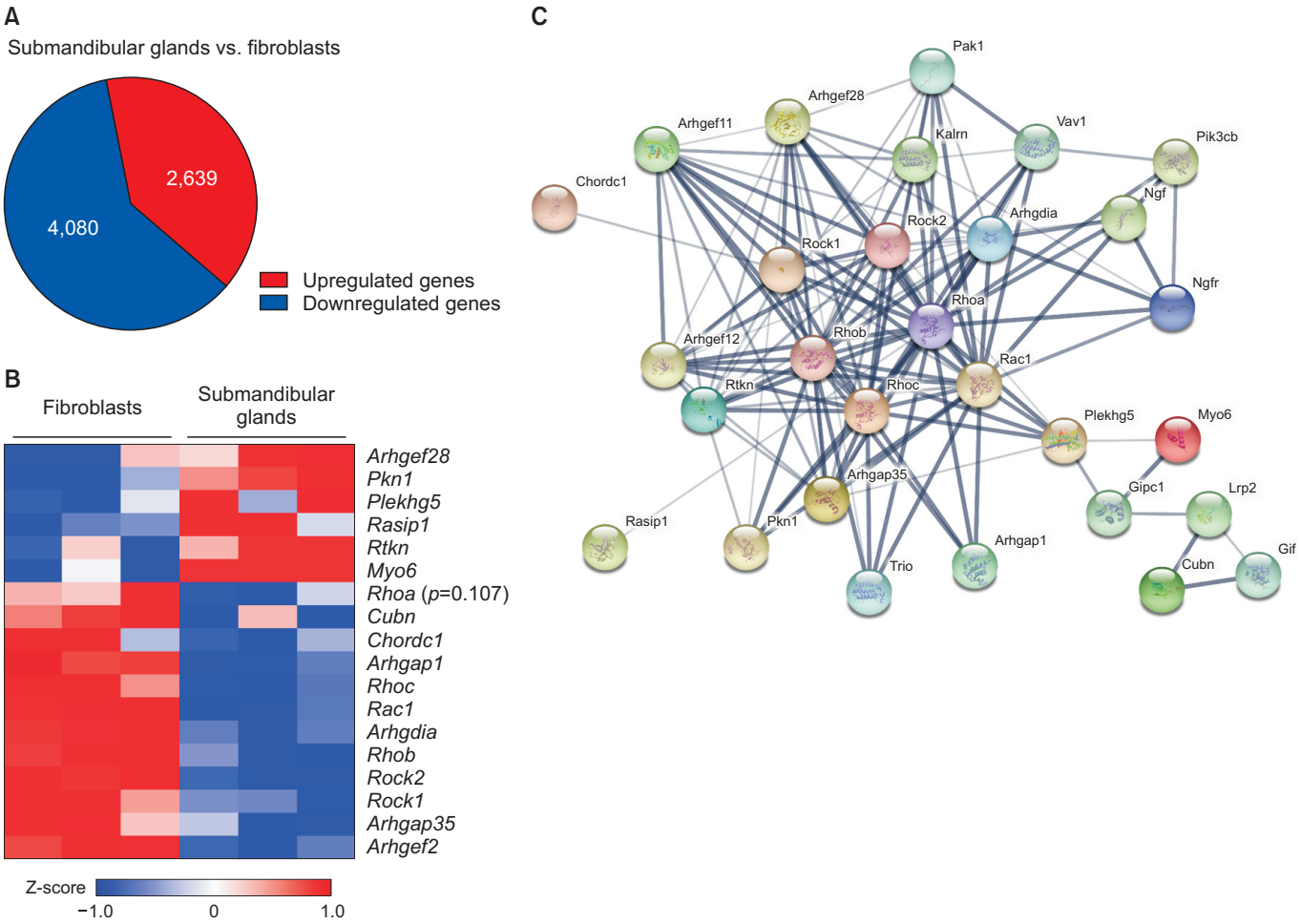

Fig. 1. Comparison of gene expression between mouse submandibular glands (SMGs) and skin fibroblasts. (A) Venn diagram indicating the up-regulated and down-regulated genes in mouse SMGs compared with mouse skin fibroblasts. (B) The expression data of 18 genes in fibroblasts and SMGs that satisfying the criteria are presented as heat map. (C) Extended protein-protein interaction network derived from genes of (B) was visualized using STRING (https://string-db.org/).

tissues were then grown in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone Laboratories) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

4. RNA extraction

Total RNA was extracted using a PureLink™ RNA Mini Kit (Invitrogen, Camarillo, CA, USA) following manufacturer's protocol.

5. RNA-seq expression profiling and analysis

Total RNA of mouse SMGs and skin fibroblasts was prepared and submitted to ebiogen (Seoul, Korea) for the construction of the library. RNA-seq data were analyzed using ExDEGA software ver. 1.6.5 (ebiogen). A heat map was generated with normalized gene expression data of salivary glands and mouse fibroblasts using MeV software ver. 4.9.0 (http://mev.tm4.org). ROCK signaling pathway-related gene candidates were obtained and used to visualize the protein-protein interaction network (PPIN) with STRING ver. 11 [18].

Results

1. Downregulated expression patterns of ROCK signaling pathway-related genes in the SMGs

To analyze the epithelial tissue-specific gene expression patterns of SMGs, RNA-seq was conducted and compared with the pattern of mouse skin fibroblasts which have mesenchymal characteristics. Genes with a 1.3-fold difference in expression and a *p*-value of less than 0.1 were selected. A total of 6,719 genes were selected based on these criteria as differentially expressed genes. Among these genes, 2,639 genes were upregulated and 4,080 genes were downregulated in SMGs (Fig. 1A). Expression profiling was conducted for genes that are directly or indirectly associated with the ROCK signaling pathway. The gene expression profile was clearly different in SMGs compared with fibroblasts. Specifically, expression levels of ROCK signaling pathway-related genes such as *Rhoa*, *Rhob*, *Rhoc*, *Rock1*, and *Rock2* were downregulated in SMGs compared with their counterparts in mouse skin fibroblasts (Fig. 1B). Extended PPIN derived from 18 genes analyzed in expression profiling (Fig. 1B) was visualized using STRING (Fig. 1C). *Rhoa*, *Rhob*, *Rhoc*, *Rock1*, and *Rock2* were placed in the center of the protein network.

Discussion

ROCK signaling pathway regulates several cellular functions including contractility, proliferation, and apoptosis [19,20]. Rho GTPases (RhoA, RhoB, and RhoC) and Rho kinases (ROCK1 and ROCK2) are the main regulators of the ROCK signaling pathway. Previously, as a potential therapeutic target of a variety of pathological conditions, studies on ROCK signaling pathway and ROCK inhibitors were reported [21]. Increased activity of RhoA was observed in human pulmonary artery smooth muscle cells from patients with pulmonary hypertension (PH) and this indicates that RhoA is a promising therapeutic target for PH [22]. In kidney, ROCK2 mediates the development of diabetic kidney disease by regulating TGF-β-induced expression of profibrotic genes [23]. In this report, the expression pattern of ROCK signaling pathway-related genes of mouse SMGs and skin fibroblasts was confirmed using RNA-seq. *Rhoa* was included in profiling analyses as a main regulator of ROCK signaling pathway although its *p*-value (0.107) was statistically out of range. Downregulated expression of *Rhoa*, *Rhob*, *Rhoc*, *Rock1*, and *Rock2* was observed in mouse SMGs and these proteins were placed in the center of the PPIN. These results indicate that downregulated expression of ROCK signaling pathway-related genes might be required for SMG maintenance and used as a therapeutic target of salivary gland diseases accompanied by the activation of Rho GTPases or Rho kinases.

Acknowledgements

This work was supported by a grant from the National Research Foundation of Korea (NRF-2020R1F1A1070433).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

References

1. Napeñas JJ, Brennan MT, Fox PC. Diagnosis and treatment of xerostomia (dry mouth). Odontology 2009;97:76–83. doi: 10.1007/s10266–008–0099–7.
2. Sui Y, Zhang S, Li Y, Zhang X, Hu W, Feng Y, Xiong J, Zhang Y, Wei S. Generation of functional salivary gland tissue from human submandibular gland stem/progenitor cells. Stem Cell Res Ther 2020;11:127. doi: 10.1186/s13287–020–01628–4.
3. Jensen SB, Pedersen AM, Reibel J, Nauntofte B. Xerostomia and hypofunction of the salivary glands in cancer therapy. Support Care Cancer 2003;11:207–25. doi: 10.1007/s00520–002–0407–7.
4. Amerongen AV, Veerman EC. Saliva––the defender of the oral cavity. Oral Dis 2002;8:12–22. doi: 10.1034/j.1601–0825.2002.1o816.x.
5. Martinez–Madrigal F, Micheau C. Histology of the major salivary glands. Am J Surg Pathol 1989;13:879–99. doi: 10.1097/00000478–198910000–00008.
6. Mattingly A, Finley JK, Knox SM. Salivary gland development and disease. Wiley Interdiscip Rev Dev Biol 2015;4:573–90. doi: 10.1002/wdev.194.
7. Vissink A, Mitchell JB, Baum BJ, Limesand KH, Jensen SB, Fox PC, Elting LS, Langendijk JA, Coppes RP, Reyland ME. Clinical management of salivary gland hypofunction and xerostomia in head–and–neck cancer patients: successes and barriers. Int J Radiat Oncol Biol Phys 2010;78:983–91. doi: 10.1016/j.ijrobp.2010.06.052.
8. Hall EJ, Giaccia AJ. Radiobiology for the radiologist. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2006.
9. Aure MH, Konieczny SF, Ovitt CE. Salivary gland homeostasis is maintained through acinar cell self–duplication. Dev Cell 2015;33:231–7. doi: 10.1016/j.devcel.2015.02.013.
10. Albersen M, Shindel AW, Mwamukonda KB, Lue TF. The future is today: emerging drugs for the treatment of erectile dysfunction. Expert Opin Emerg Drugs 2010;15:467–80. doi: 10.1517/14728214.2010.480973.
11. Komers R, Oyama TT, Beard DR, Tikellis C, Xu B, Lotspeich DF, Anderson S. Rho kinase inhibition protects kidneys from diabetic nephropathy without reducing blood pressure. Kidney Int 2011;79:432–42. doi: 10.1038/ki.2010.428.
12. Olson MF. Applications for ROCK kinase inhibition. Curr Opin Cell Biol 2008;20:242–8. doi: 10.1016/j.ceb.2008.01.002.
13. Rath N, Olson MF. Rho–associated kinases in tumorigenesis: re–considering ROCK inhibition for cancer therapy. EMBO Rep 2012;13:900–8. doi: 10.1038/embor.2012.127.
14. Schaafsma D, Gosens R, Zaagsma J, Halayko AJ, Meurs H. Rho kinase inhibitors: a novel therapeutical intervention in asthma? Eur J Pharmacol 2008;585:398–406. doi: 10.1016/j.ejphar.2008.01.056.
15. Shiratsuchi H, Shimizu O, Saito T, Mashimo T, Yonehara Y. Immunohistological study of small Rho GTPases and β–catenin during regeneration of the rat submandibular gland. J Mol Histol 2012;43:751–9. doi: 10.1007/s10735–012–9437–8.
16. Koslow M, O'Keefe KJ, Hosseini ZF, Nelson DA, Larsen M. ROCK inhibitor increases proacinar cells in adult salivary gland organoids. Stem Cell Res 2019;41:101608. doi: 10.1016/j.scr.2019.101608.
17. Lee J, Park S, Roh S. Y–27632, a ROCK inhibitor, delays senescence of putative murine salivary gland stem cells in culture. Arch Oral Biol 2015;60:875–82. doi: 10.1016/j.archoralbio.2015.03.003.
18. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta–Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome–wide experimental datasets. Nucleic Acids Res 2019;47(D1):D607–13. doi: 10.1093/nar/gky1131.
19. Etienne–Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002;420:629–35. doi: 10.1038/nature01148.
20. Pertz O. Spatio–temporal Rho GTPase signaling – where are we now? J Cell Sci 2010;123(Pt 11):1841–50. doi: 10.1242/jcs.064345.
21. Feng Y, LoGrasso PV, Defert O, Li R. Rho kinase (ROCK) inhibitors and their therapeutic potential. J Med Chem 2016;59:2269–300. doi: 10.1021/acs.jmedchem.5b00683.
22. Guilluy C, Eddahibi S, Agard C, Guignabert C, Izikki M, Tu L, Savale L, Humbert M, Fadel E, Adnot S, Loirand G, Pacaud P. RhoA and Rho kinase activation in human pulmonary hypertension: role of 5–HT signaling. Am J Respir Crit Care Med 2009;179:1151–8. doi: 10.1164/rccm.200805–691OC.
23. Nagai Y, Matoba K, Kawanami D, Takeda Y, Akamine T, Ishizawa S, Kanazawa Y, Yokota T, Utsunomiya K, Nishimura R. ROCK2 regulates TGF–β–induced expression of CTGF and profibrotic genes via NF–κB and cytoskeleton dynamics in mesangial cells. Am J Physiol Renal Physiol 2019;317:F839–51. doi: 10.1152/ajprenal.00596.2018.