Retinoic acids regulate the differentiation of salivary gland epithelial cells through interactions between the RAR and TGF-β2

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It is a new feeling to write this letter of thanks after finishing my master's course and dissertation. Before I go to the new world again, I would like to express my gratitude through this page, even a little.

First of all, I would like to express my sincere thanks to Professor Jae-Yol Lim, who gave me great lessons during my master's course. I was able to learn the attitude toward science by seeing the open mind and passion for science shown by the professor. I was able to finish my thesis well because of his generous encouragement and guidance until the very end. I would also like to express my genuine gratitude to Professor Sang Ho Oh and Professor Hyunki Kim for pointing out the systematic refinement of the thesis. I will not miss learning continuously so that the teachings of the professors I have learned here are not shameful.

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Dear my family, I would like to thank my mother, Eun-sil Ko, and my father, Yong-jun Kim, who constantly supported education for me. I am where I am today because they have always back on me so that I can do what I want. And my younger brother Kim Seok-hyun, his mischievous attitude was a great comfort in my academic life. It may not be enough on this paper, but thanks to my precious friends, their warm hugs who have always been by my side and listened to my long and long complaints, and their cool appearances in society, I was able to finish my degree. Someday I will be grateful and be the person who can give back everything I received. Thank you.

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ABSTRACT

Retinoic acids regulate the differentiation of salivary gland epithelial cells through interactions between the RAR and TGF-β2

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Retinoic acid (RA) is essential in the cell growth, differentiation, and apoptosis of cells in various organs. Recent studies have shown that RA signaling is required for submandibular gland morphogenesis and is involved in lumen formation in salivary glands. In our previous study, we reported that salivary gland organoid culture is a useful model for investigating the niche factors associated with salivary morphogenesis and RA can promote lumen formation via RA signaling-mediated regulation of Keratin7 (KRT7). However, the underlying mechanism for the differentiation of KRT7+ luminal ductal cells remains unclear. In this paper, we found that luminal marker, KRT7, and epithelial-to-mesenchymal transition (EMT)-related genes increased with RA concentration while the pro-acinar marker, submandibular gland protein C (SMGC), decreased. We hypothesize that RA's ductal cell differentiation is correlated with transforming growth factor-beta (TGF-β) signaling because TGF-β was upregulated by RA treatment. We also found that RA promoted the enrichment of KRT7+ luminal ductal cell genes along with TGF-β2 genes in a RAR-dependent manner. It was also confirmed that the RA directly increased TGF-β2 through the pathways of P38-MAPK-mediated activation of the transcription

factor, ATF2, which binds to the TGF-β2 promoter. When P38-MAPK was inhibited in salivary gland organoids, recovery of mucous acinar cells was confirmed. Finally, we demonstrate that RAs can enrich the gene expression of *Krt7*, *Muc1*, and *Muc4*, which were representative markers of mucoepidermoid carcinoma in salivary glands.

This study suggests that RAs can control the differentiation of luminal ductal cells and mucous acinar cells in salivary glands through the interactions between RAR and TGF-β2 pathways. The salivary gland organoid culture system is a useful platform for studying salivary gland organogenesis and tumorigenesis.

Key words : salivary gland, organoid, differentiation, retinoic acid, TGF-β

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I. INTRODUCTION

The salivary glands are divided into three major glands. These three major salivary glands produce more than 90% of 1-1.5 L of saliva daily[1]. Salivary glands are composed of acinar cells, which mainly produce saliva; ductal cells, which modify the secretion; and myoepithelial cells, which help the secretion of saliva throughout acinar and ductal cells. Acinar cells are divided into serous acinar, which secrete watery saliva, and mucus acinar, which secretes viscous saliva. The parotid gland secretes watery saliva in humans, and the sublingual gland secretes mucus saliva. The submandibular gland has both serous and mucous acinar.[2] Like every other tissue and organ, salivary glands maintain their homeostasis, which is regulated by progenitor cells to regenerate and differentiate.[3] Although acinar and ductal cells are known to replenish and replace the damaged cells in salivary glands, it remains unknown about the mechanisms involved in plasticity and differentiation.[4,5,6]

We have published in a previous paper that retinoic acid (RA) increases luminal cells in mouse submandibular gland (mSMG) organoids.[7] RA is known to contribute to

morphogenesis, development, and growth in various organs. RA is essential to the salivary glands' development process.[8,9] RA is metabolized by two successive oxidation reactions. Retinol is mainly converted to retinaldehyde by the enzyme RDH10[10], and retinaldehyde is metabolized to retinoic acid by ALDH1A.[11] RA-mediated signal transmission is carried out through receptor activation of RAR and RXR through metabolic processes of retinol, retinal, and retinoic acid.[12,13] It has been shown in our previous study that RA-RAR signals mainly induce an increase in luminal cells. RAR consists of RARα, RARβ, and RARγ. RA transmits various roles to cells by increasing various signals. WNT, BMPs, SOX9, and FGFs are regulated by RA and are known to be involved in differentiation.[14,15,16] However, it has not been established whether RA directly affects the differentiation of luminal cells. Therefore, the main purpose of this study is to understand how RA regulates luminal and acinar cells in salivary glands using mSMG organoid culture system.

II. MATERIALS AND METHODS

1. Cell isolation and Organoid culture

mSMG organoids were raised from primary cells of 8 to 12 weeks old female B6 mice. SMG tissues were minced with a razor blade, and the homogenate was incubated in digestion buffer consisting of DMEM/F12 F12 (Gibco, ThermoFisher Scientific, Waltham, MA, USA) with penicillin /streptomycin (Gibco, ThermoFisher Scientific), 10 mM HEPES (Biowest, Nuaille, France), Glutamax-L (Gibco, ThermoFisher Scientific). collagenase type II (Gibco, ThermoFisher Scientific) and 10 μM of Y-27632 (Tocris Bioscience, Bristol, UK) were supplemented to the basal medium to make enzyme supplemented medium. Minced mSMG fragments were incubated with enzyme supplemented medium for 1 hour at 37°C in shaking incubator at 200 rpm. After the incubation, debris was filtered through a 70 μm strainer (SPL, Seoul, Korea). Isolated cells were mixed with 40 μL of Matrigel (Corning, Corning, NY, USA) and seeded in each well of 24-well culture plates (Greiner, Kremsmünster, Austria). primocin (InvivoGen, San Diego, CA, USA), B27 minus vitamin A (Gibco, ThermoFisher Scientific), N-acetyl cysteine (Sigma-Aldrich, St. Louis, MO, USA), Noggin (peprotech), NRG1 (peprotech), FGF1 (peprotech), FGF7 (peprotech), A83-01 (Tocris Bioscience), and 10% R-spondin 1-conditioned media (homemade) were supplemented to the basal medium for consist culture medium. 10 μM of Y-27632 were added until first media change for enhancing survival of mSMG cells and 500 μL of the culture medium was added per well. organoids were maintained in a 37°C humidified atmosphere under 5% CO_2 culture conditions. 300 nM of all-trans retinoic acid (atRA), 300 nM of TTNPB (Tocris Bioscience), 300 nM of Bexarotene (Tocris Bioscience), AGN 193109 (Sigma-Aldrich), HX531 (Sigma-Aldrich), AM580 (Tocris Bioscience), CD2314 (Tocris Bioscience), BMS961 (Tocris Bioscience), AM580 (Tocris Bioscience), CD2314 (Tocris Bioscience),

BMS961 (Tocris Bioscience) and 10 μM of SB203580 (Sigma-Aldrich) as P38 inhibitor were added to culture medium.

2. Immunofluorescence analysis

In case of immunofluorescent staining of organoid, organoids were washed using cold PBS (Welgene, Kyungsan, Korea) 2 times each 5 min. To dissolve solidified Matrigel, 1x Cell recovery solution (Corning) for 1 hour with gently pipetting on ice to retain organoid formation. Centrifuge for 5 minutes at 130 G, Eliminate supernatant. Organoids were fixed by 4% Paraformaldehyde (Wako, Osaka, Japan) for 1 hour at room temperature (RT). Fixed organoids were transferred to paraffin blocks. In case of immunofluorescence staining of mSMG tissues, they were harvested from 8 weeks old mouse at least. Harvested mSMG tissues were fixed by 4% Paraformaldehyde for overnight at 4°C, and paraffin-embedded blocks were cut into 5 μm sections. The sections were stretched at 60°C incubator for 2 hours and conduct de-paraffin and rehydration in order. Next, I performed antigen retrieval to the samples for 40 minutes in 98°C heated Tris-EDTA buffer (pH 9.0) containing 0.05% Tween-20 (Sigma-Aldrich). The heated buffer was maintained high temperature in water bath. After cooling down the samples for 10 minutes at tap water, samples were washed using Tris-buffered saline (TBS) (Biosesang, Seongnam, Korea) containing 0.025% Triton X-100 (Sigma-Aldrich) 1 times, and TBS 2 times for 5 minutes each. The samples were blocked by TBS containing 5% normal serum (Jackson ImmunoReaserach, West Grove, PA, USA) at RT for 1 hour, and incubate primary antibodies overnight at 4°C. Following antibodies were used for staining on mouse SG organoids; anti-KRT7 (1:50, Santa Cruz

biotechnology, Dallas, TX, USA), anti-SMGC (1:500, Abnova, Taipei, Taiwan). Subsequently organoids were washed with 1x TBS with 0.05% Tween-20 1 time, and 1x TBS 2 times for 5 minutes each. The samples were stained with Alexa-fluor (-488, -555, -647) conjugated secondary antibodies (1:1,000; Invitrogen, ThermoFisher Scientific) for 1 hour in dark condition at RT. And I performed DNA staining with NucBlue Live cell Stain ReadyProbes reagent (Invitrogen, ThermoFisher Scientific) at RT for 15 minutes and mounting slides using ProLong glass antifade mountant with NucBlue (Invitrogen, ThermoFisher Scientific)

3. Bright-Field Image Acquisition

Bright-field images (40× or 100× magnification) of each culture were acquired using the Nikon Eclipse Ti 2-U microscope with NIS-Elements BR software (Nikon, Tokyo, Japan).

4. Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was obtained from organoids using TRIZOL reagent (Ambion, ThermoFisher Scientific, Waltham, MA, USA), as manufacturer's instruction. The total RNA was used to conduct reverse transcription to complementary DNA (cDNA) using PrimeScript RT Reagent Kit (TaKaRa Bio, Kusatsu, Japan) as manufacturer's instruction. qRT-PCR was performed using SensiFAST SYBR Lo-ROX Kit (BIOLINE, London, UK). Primer sequences are shown in Table 1. All reactions were conducted triplicate. The data were normalized by Ct value of housekeeping gene.

5. Western blot

Equal amounts of proteins were separated by SDS-PAGE gel. And proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Once all the proteins were transferred onto the membranes, the membranes were blocked with 1x TBS containing 0.1% Tween-20 (TBS-T) and 5% Bovine serum albumin (BD Bioscience) for 1 hour at room temperature. The membranes were incubated for overnight at 4℃ with primary antibody in 40 rpm shaking condition. The used antibodies were Phosphorylated-p38(p-p38) (1:1000, Cell Signaling Technology), P38 (1:1,000, Cell Signaling Technology), phosphorylated ATF2(p-ATF2) (1:1,000, Santa Cruz Biotechnology), ATF2(1:1,000, Santa Cruz Biotechnology) and anti-beta-actin (1:3,000, Cell Signaling Technology). The membranes were washed 3 times for 5 minutes with TBS-T and incubated with HRP-conjugated secondary antibody (1:3000, Cell Signaling Technology) for 1 hour at RT. The membranes were washed 3 times for 5 minutes with TBS-T and developed using ECL Western Blotting Substrate (Invitrogen, ThermoFisher Scientific).

6. Statistical analysis

Each experiment was replicated more than 3 times. Relative quantification of gene expression of calculated as $2^{-\Delta\Delta Ct}$. The results of qRT-PCR were statistically analyzed by One-way ANOVA or Two-way ANOVA using Graphpad prism 7 software (GraphPad Software, San Diego, CA, USA). Data were shown as the mean ± standard deviations (SD). Values of $p < 0.05$ were considered statistically significant.

Table 1. A list of primers used for qRT-PCR

Mouse Gene	**Primer sequence (5' → 3')**
Smgc	F : CTG ACA GAG GAT CAT GGA CCA AC
	R : TCC TGA CAC CTT GGA GAG TCC A
Bpifa2	F : TGA ACA CAG CGG ACC TTG GCA A
	R : CCA TTG CCG TTG GAA GAC AGC T
Krt7	F : GTC CTG AAG GCT CAG ACT TGG T
	R : CCA GCC GTA AAT ACT GCAA GT GG
Krt19	F : GCC ACC TAC CTT GCT CGG ATT G
	R : GTC TCT GCC AGC GTG CCT TC
Krt5	F : TTG GTG TTG GCA GTG GCT TT
	R : CCC GCT ACC CAA ACC AAG AC
P63	F : GTA TCG GAC AGC GCA AAG AAC G
	R : CTG GTA GGT ACA GCA GCT CAT C
Vim	F : CGG AAA GTG GAA TCC TTG CAG G
	R : AGC AGT GAG GTC AGG CTT GGA A
Snai1	F : TGT CTG CAC GAC CTG TGG AAA G
	R :CTT CAC ATC CGA GTG GGT TTG G
Snai2	F : TCT GTG GCA AGG CTT TCT CCA G
	R : TGC AGA TGT GCC CTC AGG TTT G
Twist	F : GAT TCA GAC CCT CAA ACT GGC G
	R : AGA CGG AGA AGG CGT AGC TGA G
Sma	F : GCC ATC ATG CGT CTG GAC TT
	R : ATC TCA CGC TCG GCA GTA GT
Tgfb1	F : TGA TAC GCC TGA GTG GCT GTC T
	R : CAC AAG AGC AGT GAG CGC TGA A
Tgfb2	F : CCT GCC CGA CCC TAC AAA AT
	R : TGC TGC TTC TCC TTC ACT GG
Tgfb3	F : TTG GTG TTG GCA GTG GCT TT
	R : CCC GCT ACC CAA ACC AAG AC
Human gene	**Primer sequence (5' → 3')**
KRT7	F : TGT GGA TGC TGC CTA CAT GAG C
	R : AGC ACC ACA GAT GTG TCG GAG A

MUC1	F : CCT ACC ATC CTA TGA GCG AGT AC
	R : GCT GGG TTT GTG TAA GAG AGG C
MUC4	F : AAC ACA GCC TGC TAG TCC AGC A
	R : TGG AGA GGA TGG CTT GGT AGG T

III. RESULTS

1. Retinoic acids induce EMT and reciprocally regulate the luminal and pro-acinar cell differentiation in adult salivary gland organoids.

1-1 AtRA increased luminal cells and decreased pro-mucous acinar cells in mSMG organoids.

In a previous study, we confirmed that *Krt7*, a luminal cell marker of mSMG organoid, increased when treated with RA. It was confirmed whether the luminal cell marker increased and also the pro-acinar marker decreased even when all-trans-Retinoic acid (atRA), the metabolite of retinoid, was treated under the improved media conditions. As a result of performing qPCR with mSMG organoids treated with atRA 0, 30, 100, and 300 nM, the luminal cell gene, *Krt7* increased according to the concentration of atRA, and the intermediate ductal cell gene, *Krt19*, showed a tendency to increase, but decreased at 300 nM of atRA (Figure 1A). This was due to the complete differentiation into *Krt7*+ cells. On the other hand, among the pro-acinar markers, the serous acinar gene, *Bpifa2*, showed no significant difference, but the pro-mucous acinar gene, *Smgc*, decreased according to the concentration. It was confirmed that the expressions of basal cell markers, *Krt5* and *p63*, were not related to the atRA (Figure 1A). Immunofluorescent staining confirmed whether there was a difference in the expression of KRT7 and SMGC at the protein level. As a result of the staining of SMGC in green and KRT7 in red, SMGC was expressed only in the group without atRA, and KRT7 was increased in organoids treated with atRA (Figure 1B). These results indicate that atRA induced the differentiation of luminal cells, whereas

atRA inhibited the pro-mucous cells in mSMG organoids.

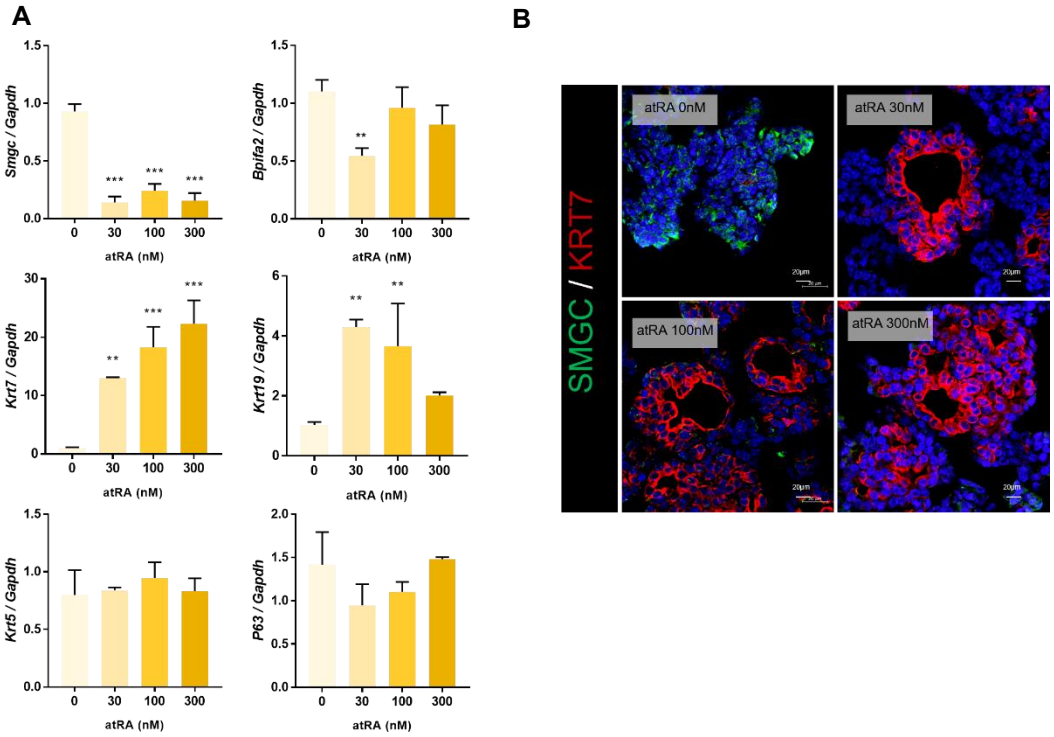

Figure 1. atRA increased luminal cells and decreased pro-mucous acinar cells in mSMG organoids. (A) mSMG organoids were treated with varying doses of RA and subjected to qRT-PCR for the evaluation of gene expression. *Smgc, Bpifa2* as a pro-acinar marker, *Krt7, Krt19* as a luminal cell marker, *Krt5,* and *P63* as basal markers. *P<0.05, **P<0.01, ***P<0.001 (B) Immunofluorescent analysis of the mSMG treated with varying doses of RA. Merged images of SMGC (green) and KRT7 (red) are shown.

1-2 atRA induced epithelial-to-mesenchymal transition in SG organoids.

When atRA was treated with mSMG organoid, we could confirm the change in epithelial cell differentiation and observe the epithelial-to-mesenchymal transition (EMT) phenomenon. EMT is essential for development, wound healing, and stem cell behavior. The cells attached to the bottom were increased in the RA-treated groups compared to those not treated with atRA (Figure 2A). qRT-PCR confirmed that the expressions of EMT-related genes, *Vimentin*, *Twis1*, *Snail*, *Slug*, and *Sma*, were increased (Figure 2B).

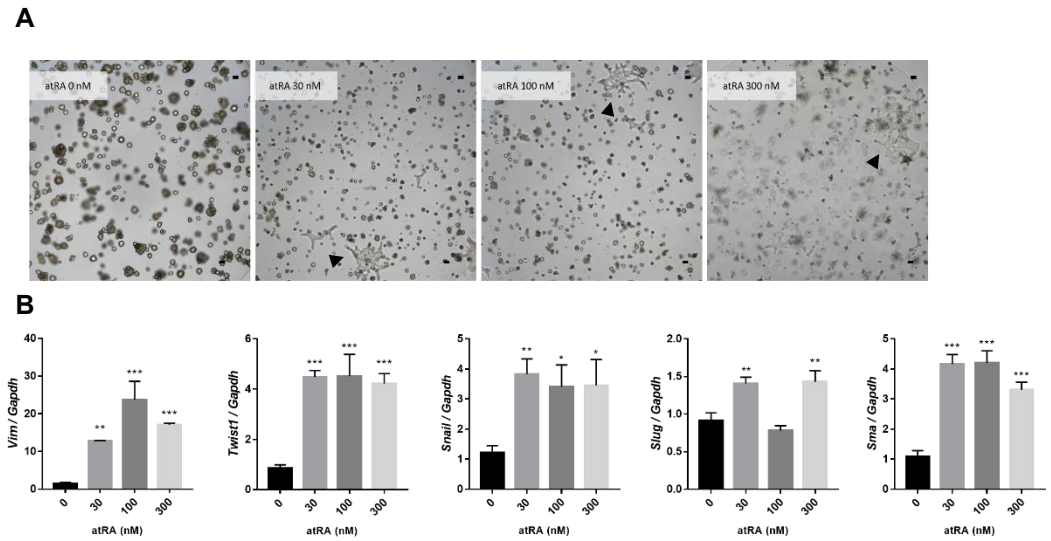

Figure 2. atRA induced EMT in SG organoids. (A) Representative bright-field images of mSMG organoids with atRA. The scale bar indicates 100 μm. Black triangles indicate EMT morphology. (B) Relative expression of mSMG organoids cultured with atRA were determined by performing qRT-PCR. Results are expressed as mean ± SD (n = 3); *P<0.05, **P<0.01, ***P<0.001.

1-3 atRA treatment increased TGF-β2 signaling in mSMG organoids, and TGF-β2 mediated the differentiation of luminal cells and pro-mucous cells in mSMG organoids.

Transforming growth factor-beta (TGF-β) is well-known as an EMT-inducing cytokine, and in particular, Snail and Slug have been well-documented as mediators of TGF-β induced EMT.[17] TGF-β also plays an important role in the development of mSMG and is known to be expressed in terminal buds, luminal cells, and ducts during development. Therefore, before confirming whether atRA regulates the differentiation through TGF-β, we checked whether atRA increases TGF-β signaling. qRT-PCR revealed that all of the TGF-β series were increased when atRA was treated. Among the TGF-β families, TGF-β2 was expressed the highest in adult salivary glands (Figure 3A).

When TGF-β2 was treated with mSMG organoid at 0, 1, 5, and 10 ng, it was confirmed that *Krt7* and *Krt19* genes were enriched and *Smgc* were declined, as when atRA was treated (Figure 3B). To verify whether this finding is due to the effect of TGF-β2, A83-01, TGF-β inhibitor, was treated with atRA 300nM into mSMG organoids. When A83-01 was treated, the expression of *Smgc* was recovered, and *Krt7* and *Krt19* were decreased, as opposed to when treated with atRA and TGF-β2 (Figure 3C). These results indicate that atRA and TGF-β2 regulate the differentiation pattern of mouse salivary gland organoids.

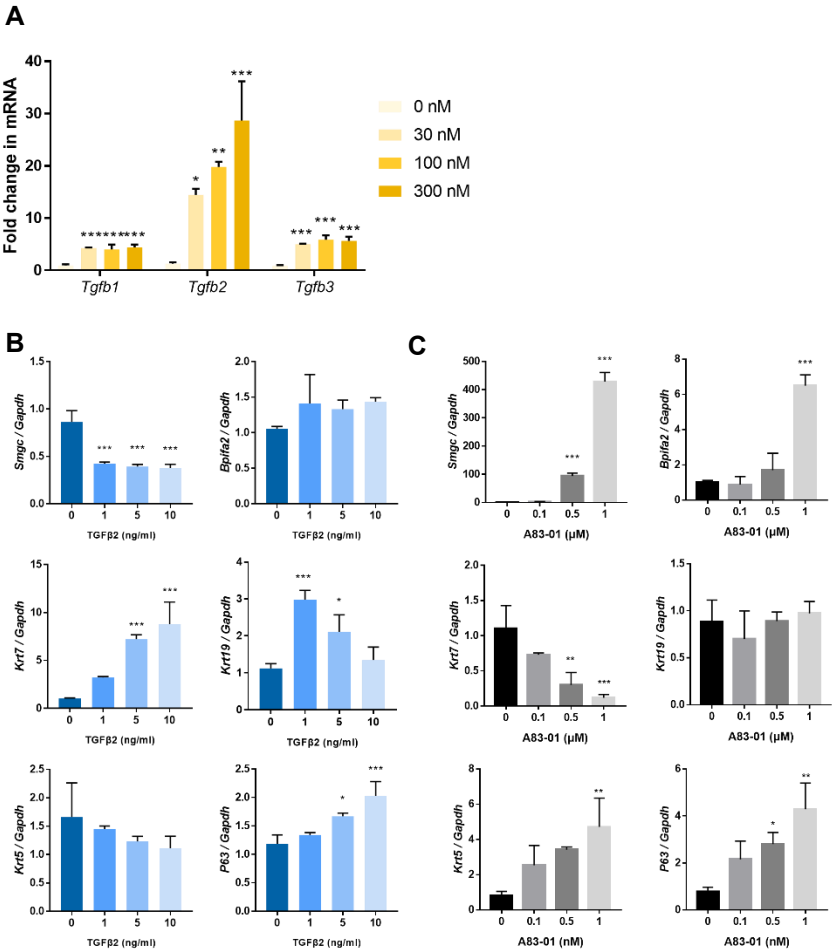

Figure 3. TGFb2 increased luminal cells and decreased pro-mucous acinar cells in mSMG organoids. (A) Relative expression of *Tgfb1, Tgfb2, Tgfb3* was determined by performing qRT-PCR. Results are expressed as mean ± SD (n = 3); p values shown in the graph were determined using One-way ANOVA with the Tukey's post-hoc test. *p < 0.05, **p < 0.01, and ***p < 0.001 (B) m**S**MG organoids were treated with varying doses of TGF-β2 and subjected to qRT-PCR for the evaluation of gene expression. Results are expressed as mean ± SD (n = 3); *P<0.05, **P<0.01, ***P<0.001. (C) m**S**MG organoids were treated with varying doses of A83-01(TGF-β inhibitor) and subjected to qRT-PCR to evaluate gene expression. Results are expressed as mean ± SD (n = 3); *P<0.05, **P<0.01, ***P<0.001.

2. RAR-mediated regulation of TGF-β2 promotes *Krt7*+ cells and inhibits *Smgc*+ cells in mouse SMG organoid.

2-1 RAR signal induced TGF-β2 and differentiation in mSMG organoids.

Our previous studies have also shown that RAR-mediated regulation of RA binding factors, RAR and RXR, promotes the development of luminal cells in mSMG. To confirm whether the expression of TGF-β2 is also RAR-mediated, AGN-1931099 (RAR selective antagonist) or HX-531 (RXR selective antagonist) were treated under the condition that atRA 300nM was contained. When AGN1931099 was treated, branching morphology was relatively lower than that when HX531 was treated or atRA alone (Figure 4A), and the expression of *Tgfb2* and *Krt7* was lower when treated with the RAR antagonist AGN-1931099. The expression of *Smgc* was increased (Figure 4B). To confirm that TGF-β2 expression and differentiation are RAR-dependent regulation, TTNPB (RAR agonist) or Bexarotene (RXR agonist) instead of RA was treated in the absence of atRA condition. Branching morphology could be observed in the organoids of the group treated with TTNPB compared to the condition without RA (Figure 5A). Also, it was confirmed that *Tgfb2* and *KRT7* gene expression was significantly higher in TTNPB. On the other hand, when the agonist was treated, *Smgc* was also regulated by RXR (Figure 5B).

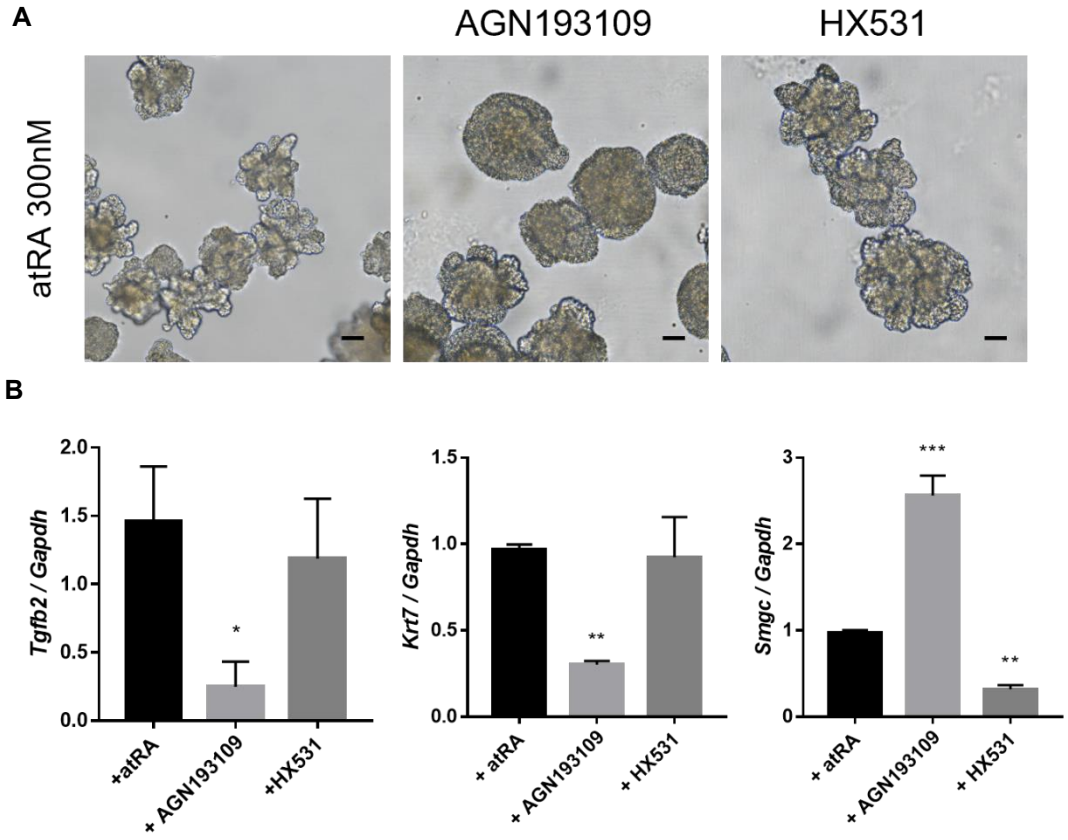

Figure 4. RAR antagonist reduced RA-mediated *TGF-β2*, and *Krt7* expression and increased pro-mucous acinar cells in mSMG organoids. (A) Representative bright-field images of mSMG organoids with atRA 300nM, AGN193109, HX531. The scale bar indicates 50 μm. (B) Relative expression of mSMG organoids cultured with atRA 300nM treated group, atRA treated with AGN193109 group, and atRA treated with HX531 group were determined by performing qRT-PCR. Results are expressed as mean ± SD (n = 3); *P<0.05, **P<0.01, ***P<0.001.

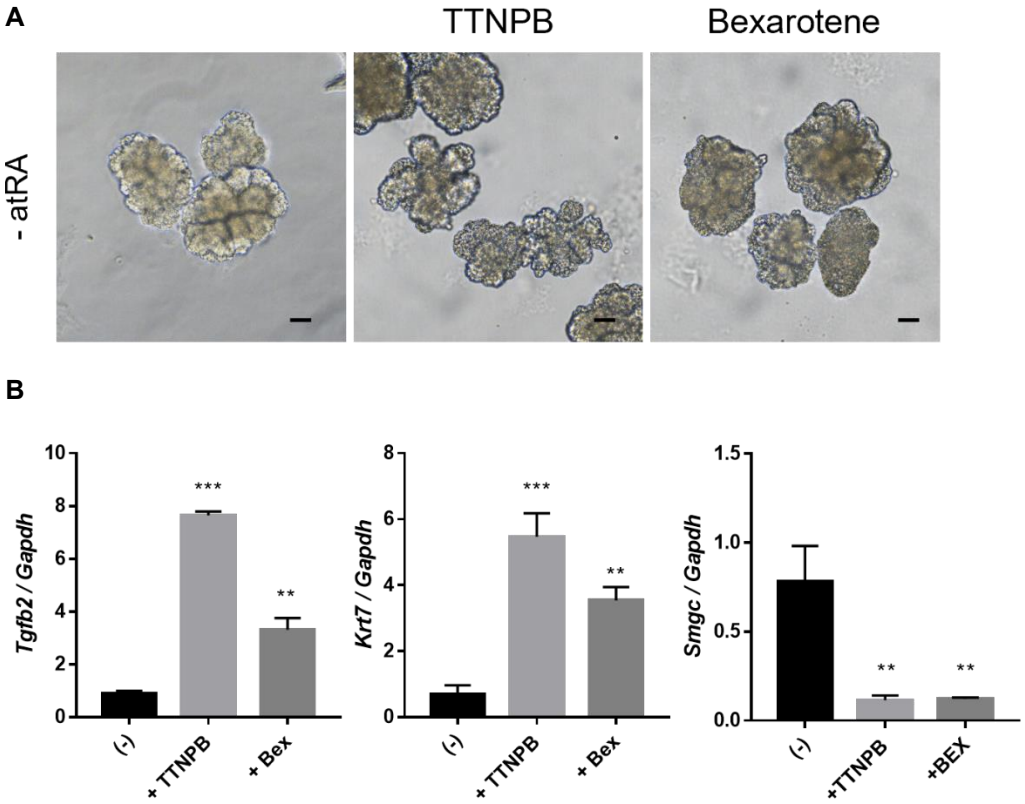

Figure 5. RAR agonist regulated *TGF-β2*, *Krt7*, and *Smgc* RNA expression in mSMG organoids. (A) Representative bright-field images of mSMG organoids with TTNPB, Bexarotene. The scale bar indicates 50 μm. (B) Relative expression of mSMG organoids cultured with atRA untreated group, TTNPB-treated group, and Bexarotene-treated group was determined by performing qPCR. Results are expressed as mean ± SD (n = 3); *P<0.05, **P<0.01, ***P<0.001.

2-2 RAR-β makes significant contributions while all three RAR isotypes complement each other to increase TGF-β2

RAR is divided into RAR-α, β, and γ. Each plays a complementary role as an isotype of RAR, but also shows a unique function that cannot be replaced in development and differentiation.[18] When AM580 (RAR-α selective agonist), CD2314 (RAR-β selective agonist), and BMS961 (RAR-γ selective agonist) were treated instead of atRA, *Tgfb2* and *Krt7* were increased and *Smgc* decreased compared to -RA for each isotype. However, the group treated with CD2314 showed a higher difference than the group treated with AM580 and BMS961(Figure 6). Therefore, it was found that the expression changes of luminal cells and pro-mucous cells by RA were complementary effects of RAR-α, β, and γ. and among them, it was found that RAR-β was mainly induced.

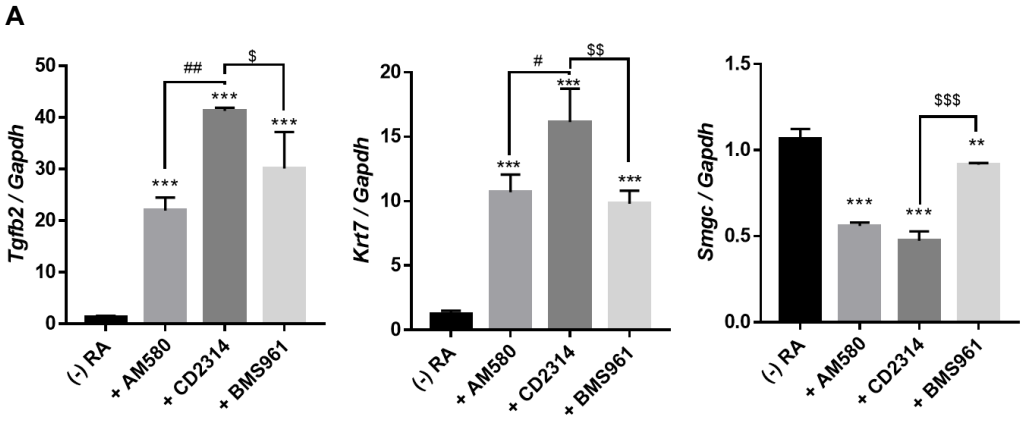

Figure 6. *Tgfb2*, *Krt7*, and *Smgc* RNA expression are regulated by RAR, mainly RAR-β, in mSMG organoids. (A) Relative expression of mSMG organoids cultured with atRA untreated group, AM580-treated group, CD2314-treated group, and BMS961-treated group were determined by performing qPCR Results are expressed as mean ± SD (n = 3); .*P<0.05, **P<0.01, ***P<0.001 *compared to (-)RA; #P<0.05, ##P<0.01 #compared to AM580/CD2314; $P<0.05, $$P<0.01, $$$P<0.001 $compared to CD231/BMS961.

3. atRA-induced TGF-β2 expression in mouse SMG organoid is mediated via p38 MAPK and ATF2.

Studies on the pathway by which atRA directly increases TGF-β2 have been reported to be through the p38 MAPK and ATF2 pathways in intestinal epithelial cells and human keloid fibroblasts.[19,20] Therefore, in order to confirm the RA-induced TGF-β2 expression pathway in mSMG organoids, p38 and ATF2 phospho-forms were identified. First, the temporal changes of phosphor-p38 and phosphor-ATF2 expression in mSMG organoids after atRA treatment were measured. Phospho-p38 increased from 15 min after atRA treatment and decreased after 6 h. phspho-ATF2 showed the greatest increase at 15min when phosphor-p38 was increased, and it was confirmed that it increased at 2 h, 6 h, and 12 h (Figure 7A). In order to verify the increase of phospho-p38 and phospho-ATF2 by RAR, the same Western blot was performed 15 min after treatment TTNPB, RAR agonist, and 300 nM of atRA / AGN193190, the RAR antagonist. Compared with the group treated with AGN193190, it was confirmed that the increase in phospho-p38 and phosphio-ATF2 was higher in the group treated with TTNPB (Figure 7B). We performed immunofluorescence staining using SMAD2/3, TGF-β-induced signaling, and KRT7, a luminal marker, to verify that luminal cells respond to TGF-β2 when atRA is treated. It was confirmed that SMAD2/3 and KRT7 markers were stained at the same location (Figure 8). Also, when SB203580, a p38 inhibitor, was treated by concentration, the luminal cell markers *Krt7*, *Krt19* decreased and *Smgc* increased (Figure 9). It was confirmed that RA increased TGF-β2 through the p38 and Atf2 pathway and that TGF-β2 affects mSMG organoid differentiation.

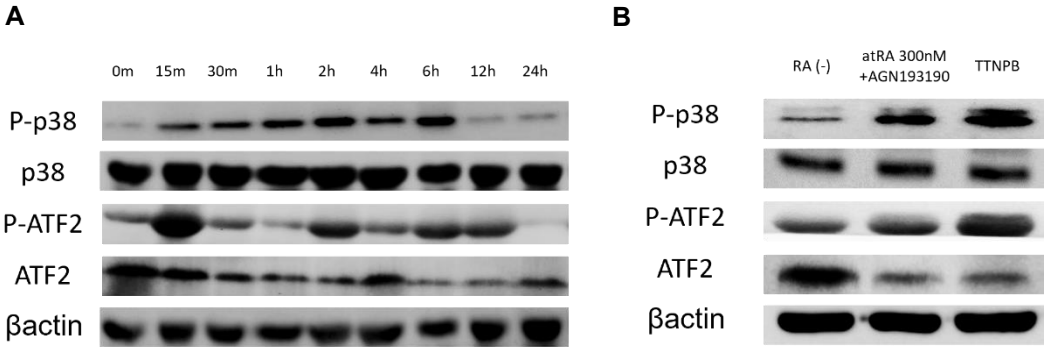

Figure 7. AtRA-induced TGF-β2 expression in mSMG is mediated via p38 and ATF2. (A) Western blot analysis of p38-ATF2 axis protein levels of mSMG organoids treatment of atRA. Samples were acquired in 0, 15, 30 minutes and 1, 2, 4, 6, 12, and 24 hours after treatment of atRA. The expression levels of p-p38, p38, p-ATF2, and ATF2 were detected by Western blots. Beta-actin was used as a loading control. (B) Western blot analysis of p38-ATF2 axis protein levels of mSMG organoids treatment of atRA with AGN193190 and treatment of TTNPB. Samples were acquired in 15 minutes after treatment of atRA with AGN193190 and treatment of TTNPB. The expression levels of p-p38, p38, p-ATF2, ATF2 were detected by Western blots. Beta-actin was used as a loading control.

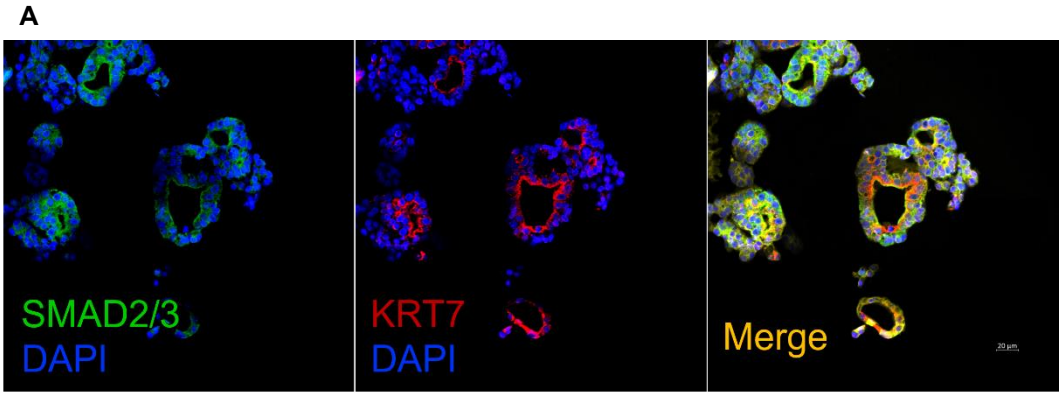

Figure 8. SMAD2/3 is expressed in KRT7+ cells. Immunofluorescent analysis of the mSMG atRA treated conditions. SMAD2/3(green), KRT7(red) and merged images are shown. Several cells co-expressed SMAD2/3 and KRT7. Scale bar, 20 μm.

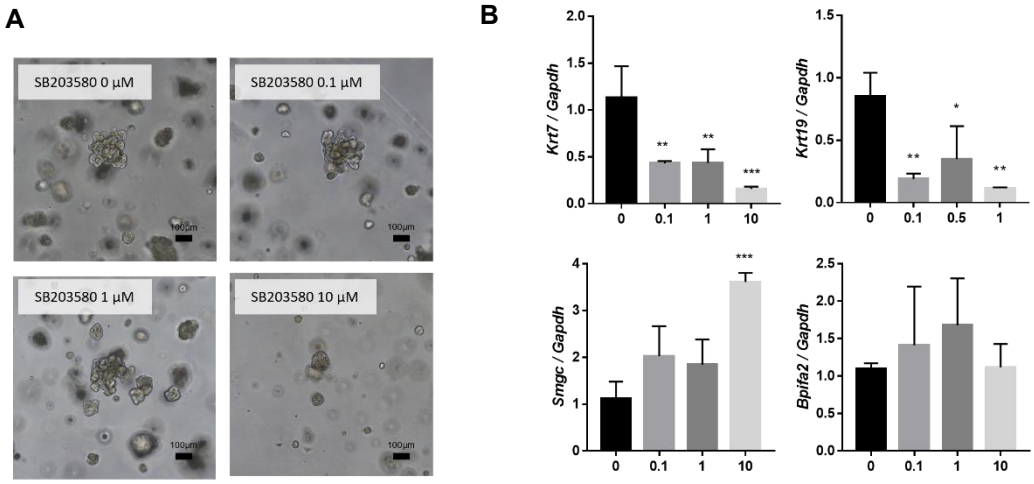

Figure 9. The SB203580, p38 inhibitor, significantly changed TGF-β2 mediated gene expression. (A) Representative bright-field images of mSMG organoids with SB203580 0, 0.1, 1, 10 μM. The scale bar indicates 50 μm. (B) Relative expression of mSMG organoids cultured with SB203580 0, 0.1, 1, 10 μM treated group was determined by performing qPCR. Results are expressed as mean ± SD (n = 3); .*P<0.05, **P<0.01, ***P<0.001

4. RA Signaling induced the differentiation of cells of mucoepidermoid carcinoma tumoroids.

Lastly, to see whether the regulation of differentiation in the salivary gland by atRA also can be applied to disease models, the salivary gland tumoroidwas employed as shown in our recently published data. Among them, mucoepidermoid carcinoma (MEC) is the most common type of salivary gland cancer. MECs are characterized by containing mucocytes, intermediate and epidermoid cells. In particular, mucocytes have a duck-like structure. Low-grade tumors have more mucocytes, and high-grade tumors have more epidermoid and undifferentiated cells.[21] To confirm that atRA induces differentiation in MEC, cells were isolated from low-grade MEC to make a tumoroid. When looking at the tumoroid bright field image, it was confirmed that lumen was formed in the group containing atRA compared to the non-treated group (Figure 10A). In order to confirm more quantitatively, qPCR was performed. KRT7 is also used as a luminal cell marker in normal salivary gland cells or a marker for diagnosing MEC[22]. Similarly, MUC1 and MUC4 are known to be expressed mainly in duct cells in normal cells[23], but are used as characteristic markers in MEC. When RA was treated, *KRT7* and *MUC1* were significantly increased in MEC tumoroid #1. In MEC #2 tumoroid, *KRT7* was not increased, but *MUC1* and *MUC4* gene expression levels were increased. *KRT7*, *MUC1*, and *MUC4* were all increased when atRA was treated in MEC #3 (Figure 10B). These results suggest that atRA can induce similar differentiation, although not completely identical in MEC.

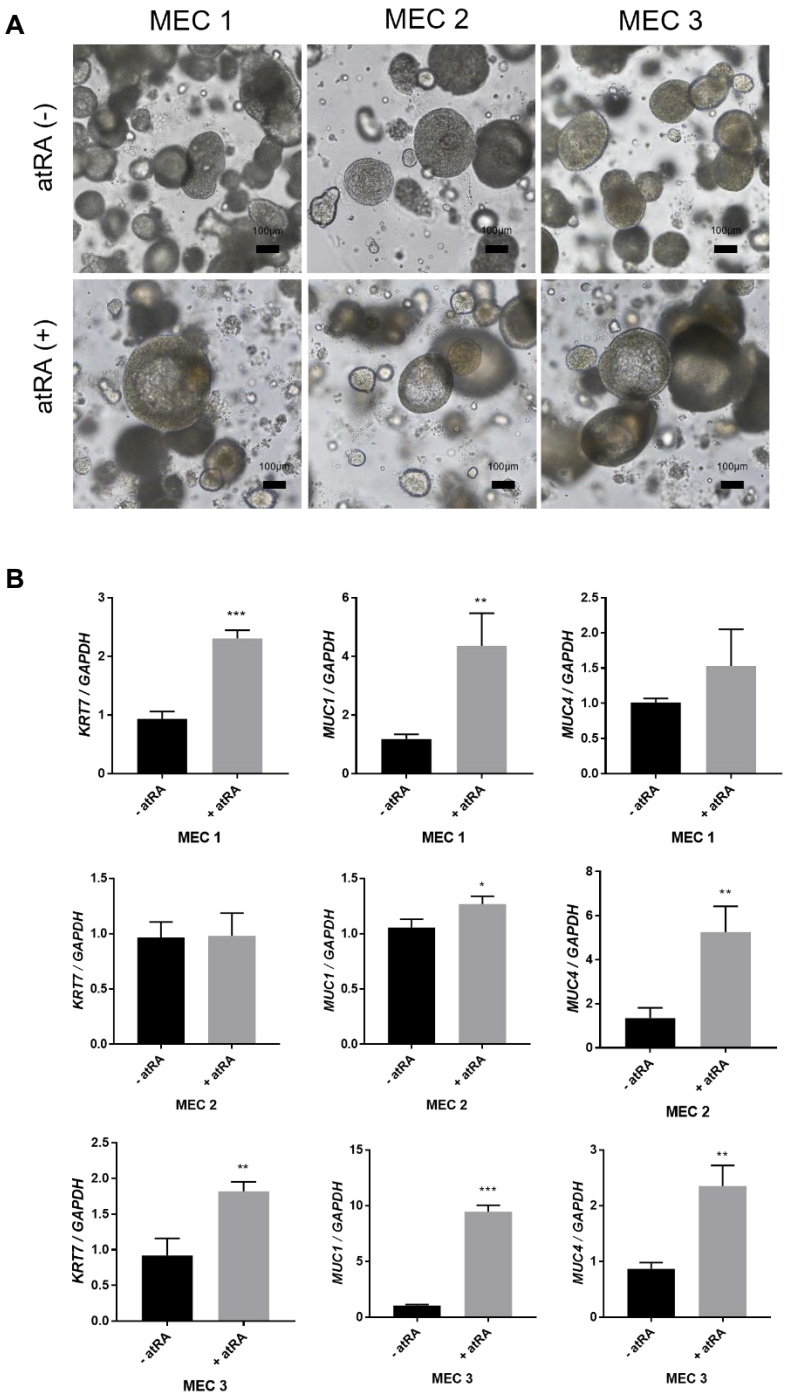

Figure 10. RA may also affect MEC tumoroid differentiation. (A) Representative bright-field images of MEC tumoroids 1 / 2 / 3 without, with atRA 300nM. The scale bar indicates 100 μm. (B) Relative expression of MEC tumoroids cultured with atRA 0, 300 nM treated group was determined by performing qRT-PCR. Results are expressed as mean ± SD (n = 3); .*P<0.05, **P<0.01, ***P<0.001

IV. DISCUSSION

Our previous study showed that RA contributes to lumen formation in salivary gland organoids and RA decreased the pro-acinar marker *Aqp5* while increasing the luminal cell marker, *Krt7*. It was confirmed that the recently announced improved media for organoid maintenance are conditions that can support acinar cells compared to the previous media. An experiment was conducted to check whether there was a differentiation difference as in the previous study when atRA was treated under improved media conditions. Consequently, in this study, we found that RA inhibited the expression of mucous acinar cells but not serous acinar cells while inducing lumen formation in salivary gland organoids. However, since atRA, the final metabolite of vitamin A, was used to induce an immediate cellular response, there may be limitations in applying metabolic regulation *in vivo*. Although atRA has been studied to regulate acinar cells in tissues such as the pancreas and lacrimal glands[24,25], studies of the effects of atRA on acinar cell differentiation in the development of salivary glands have been lacking. Acinar cells, as known in the pancreas, are sensitive to damage and rapidly dedifferentiate, making it difficult to maintain within the organoid. However, acinar cell maintenance is essential for organoids' structural and functional recapitulation. Therefore, our findings may facilitate research into the maintenance and regeneration of diversity within salivary gland organoids.

We found that atRA mediated TGF-β2 induced contrast regulation of luminal and acinar cells. TGF-β family plays an important role in the process of salivary gland formation. While TGF-β3 is mainly expressed in mesenchyme cells, TGF-β1,2 is expressed in terminal buds, luminal cells, and ducts. Although TGF-β1 affects SMG branching morphogenesis, only TGF-β2 is consistently expressed in adult salivary glands. Therefore, our results have important implications for understanding salivary gland cell differentiation in adult tissues. In addition, although cells from an adult mouse were used, it is difficult to fully explain the mature state due to some similarities between embryonic

developmental stages and adult tissue regeneration.[26,27] Even if TGF-β2 secreted from adults showed the highest expression difference in organoids, the relatively high increase in TGF-β1 and TGF-β3 can be applied to this. Though the roles of TGF-β1 and TGF-β3 may overlap in regulating the expression of luminal and pro-acinar cells in organoids, we found that TGF-β2 can regulate the expression of luminal and pro-acinar cells in adults.

We verified the result that atRA increases TGF-β2 through RAR. Furthermore, we investigated whether one of the RAR isoforms, RAR-α, RAR-β, or RAR-γ, independently induces differentiation. According to the experimental results, it was found that each isotype played a complementary role, but RARβ mainly played a role. In case of mucoepidermoid carcinoma, which is the most common carcinoma among salivary gland cancers, it has been reported that the RARβ gene is methylated at high grade.[28] Based on the results that RA regulates differentiation in normal salivary glands, we hypothesized that the duct-type structure of lower-grade MECs and the contrasting form of undifferentiated cells of higher-grade MECs would be related to RA.

It has been shown in previous studies that MEC tumoroids derived from MEC patients mimic tumor type-specific characteristics. Although the reactivity to atRA was different for each patient, the luminal cell marker showed an increasing direction, and it was confirmed that the expression levels of *MUC1* and *MUC4* increased. It was confirmed that RA affects differentiation in MEC tumoroids and in normal salivary glands. However, they were all low-grade MECs, and it can be speculated that the differentiation by RA can inhibit cancer stem cell proliferation. However, a more in-depth study is needed to determine what it means.

V. CONCLUSION

In summary, this study provides new insight into differentiation in adult salivary glands by demonstrating that differentiation regulation of atRA is TGF-β2-mediated. This differentiation regulation was validated with TGF-β2 induced by RAR and was validated using an *in vitro* mSMG organoid model. We also verified that atRA could induce differentiation in cancer organoids as well. Collectively, atRA regulates RAR-TGF-β2-mediated mouse salivary gland organoid differentiation, which may support *in vivo*-like cellular diversity in studying salivary gland morphogenesis.

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ABSTRACT (IN KOREAN)

RAR - TGF-β2 사이의 상호작용을 통한 레티노산의 타액선 상피 세포 분화 조절

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김 지 선

레티노산(RA)은 다양한 장기에서 세포의 성장, 분화, 세포사멸에 필수적이다. 최근 연구에서 RA 신호가 타액선 턱밑샘 형태 형성에 필요하며 침샘에서 내강 형성에 관여한다는 것을 보여주었다. 이전 연구에서, 우리는 침샘 오가노이드가 RA 신호에 의한 Keratin7 (KRT7) 분화 조절을 통해 내강 형성과 관련된 요인을 조사하는 유용한 모델이라고 보고했다. 그러나 여전히 KRT7+ 내강관세포의 분화를 위한 기작은 불분명했다. 본 연구에서 우리는 내강 관세포 마커인 *Krt7*, 상피-중간엽 전이(EMT) 관련 유전자가 RA 농도에 따라 증가하는 반면, 점액질 선포세포 지표인 *Smgc* 는 감소한다는 것을 발견하였다. 우리는 *Tgfb2* 가 RA 에 의해 상향 조절되었기 때문에 RA 에 의한 내강 관세포 분화가 TGF-β 신호와 상관관계가 있다고 가정했다. 우리는 또한 RA 가 RAR 의존적인 방식으로 *Tgfb2* 유전자와 함께 *Krt7*+ 내강관세포 유전자의 증가를 촉진한다는 것을 발견했다. 또한 RA는 *Tgfb*2 프로모터에 결합하는 전사인자 ATF2가 p38-MAPK 매개 활성화 경로를 통해 TGF-β2 를 직접 증가시키는 것을 확인하였다. 침샘 오가노이드에서 p38-MAPK 경로를 억제하였을 때, 점액 선포 세포의 회복이 확인되었다. 마지막으로 우리는 RA가 침샘 점막표피모양암종의 대표적인 지표였던 *KRT7, MUC1, MUC4* 의

유전자 발현을 풍부하게 할 수 있음을 보여준다. 본 연구는 RA 가 RAR 과 TGF-β2 경로의 상호작용을 통해 침샘에서 내강 관 세포와 점액성 선포 세포의 분화를 제어할 수 있음을 시사한다. 타액선 오가노이드 배양 시스템은 타액선 오기노이드 형성과 종양 형성을 연구하는 데 유용한 플랫폼임을 증명하였다.

핵심되는 말 : 레티노이드, 타액선, 오가노이드, 타액선 분화, 전환성장인자 베타