Brief Communication

Smad7 attenuates TGF-β-mediated aging-related hypofunction of submandibular glands

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Impact statement

Submandibular gland (SMG) hypofunction is common in the aged population and impairs the quality of life. However, the mechanism of age-related SMG hypofunction has not been well explored. Although TGF-β/Smad is important in the fibrosis of many organs, to the best of our knowledge, this preliminary study is the first one to investigate the role of TGF-β/Smad signaling in the aging-related SMG hypofunction. This study showed that Smad7 attenuates TGF-β-mediated aging-related SMG hypofunction and NFκB-driven inflammatory infiltration. The results suggested a promising treatment target for aging-related dysfunction and sialadenitis of SMG.

Abstract

Submandibular glands have essential functions in taste, mastication, swallowing, and digestion. Submandibular gland hypofunction is prevalent in the elderly, impairing the patients' quality of life. Current clinical treatment strategies have not decelerated or reversed the pathological process of submandibular gland hypofunction. Therefore, novel restoration strategies should be explored. However, studies on the mechanism of aging-related submandibular gland hypofunction remain very limited. The role of the TGF-β/Smad pathway in fibrosis has been studied in other organs. Therefore, this study aimed to elucidate the role of TGF-β/Smad signaling in the aging-related submandibular gland hypofunction. The results showed that Smad7 knockout in mice decreased the salivary flow rate. H&E, Masson trichrome, and immunohistochemistry staining of MCP-1 and α-SMA showed that Smad7 knockout in mice resulted in lymphocytic infiltration, acinar cell atrophy, and interstitial fibrosis. The Western blotting of collagen I and III also confirmed extensive fibrosis. We then found that Smad7 depletion resulted in the TGF-β-mediated fibrosis via mir-21, mir-29, and np_5318, and NFκB-driven inflammation activation. This study confirmed the inhibitory role of Smad7 in the aging-related submandibular gland hypofunction. Therefore, it provided a promising treatment target for aging-related dysfunction and sialadenitis of submandibular gland.

Keywords: Saliva, fibrosis, transforming growth factor, aging, sialadenitis

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Introduction

Submandibular glands (SMG) have essential functions in taste, mastication, swallowing, and digestion. SMG hypofunction is a common problem in patients having received radiation as part of cancer therapy and in the elderly. Hyposalivation might exacerbate dental caries and periodontal disease and cause masticatory, swallowing, and speech difficulties, impairing the patients' quality of life.[1] The clinical treatment strategies for SMG hypofunction are symptomatic, which could only alleviate xerostomia rather than decelerate or reverse the pathological process.[2]

Current research is increasingly focusing on regenerating the SMG tissue and restoring normal salivary functions.[3] Unfortunately, the mechanism of age-related SMG hypofunction is not yet elucidated. Age-related SMG hypofunction might be due to oxidative stress accumulation, resulting in DNA damage and apoptosis.[4] Oxidative stress could promote the endoplasmic reticulum stress, which inhibits the Ca^{2+} entry to the salivary gland acinar cells, impacting saliva secretion.[5] Despite the effect of oxidative free radicals, the transforming growth factor (TGF) pathway might have a role in the SMG hypofunction. It was demonstrated that TGF-α concentrations in unstimulated human saliva decrease with aging, but the increase in oral pathologies is manifested as xerostomia.[6]

Aging-related SMG changes include atrophy of parenchyma cells, fatty degeneration, stromal fibrosis, inflammatory infiltration, and decreased SMG weight ratio.[7,8] TGF-β/Smad activation plays a crucial role in tissue fibrosis, including liver, kidney, and heart tissue.[9–11] TGF-β signaling also functions in the chronic autoimmune disease, Sjögren syndrome.[12] Smad7, an inhibitory molecule in the TGF-β/Smads pathway, inhibits Smad2/3 phosphorylation and blocks TGF-β signaling from being transmitted to the nucleus.[13] Smad7 has an inhibitory effect on TGF-β-induced fibrosis in other organs.[14,15] However, the role of the TGF-β/Smad pathway and Smad7 in the aging-related SMG hypofunction remains unclear. Therefore, this study aims to reveal the position of TGF-β/Smad in the aging-related SMG hypofunction.

Materials and methods

Saliva and SMG tissue collection

Saliva and tissue were collected from Smad7 wild-type (S7WT) and knockout (S7KO) CD-1 mice. Saliva collection was performed following anesthesia by intraperitoneal injection with Avertin (T48402, Sigma-Aldrich, Missouri, USA) (0.75 mg/g mouse weight). Subcutaneous injection of pilocarpine (P6503, Sigma-Aldrich, Missouri, USA) at 0.25 mg/kg body weight was carried out to stimulate saliva secretion. Whole saliva was collected with a dry cotton roll for 30 min and transferred into 1.5-mL preweighed Eppendorf tubes. The saliva volume was estimated by the cotton weight difference before and after the collection. The mice were sacrificed at 12 months with SMG collected for other experiments.

Histopathology and immunohistochemistry

Mouse SMG samples were fixed in formalin and paraffin-embedded, followed by staining the sections with Masson's trichrome, as previously described.[16] Immunohistochemistry was performed on formalin-fixed tissue sections using a microwave-based antigen retrieval method. The primary antibodies used in this study included α-smooth muscle actin (α-SMA) (A5691, Sigma-Aldrich, Missouri, USA) and monocyte chemotactic protein-1 (MCP-1) (sc-1785, Santa Cruz, Dallas, USA). The results were imaged with the Aperio Scan Scope FL+GL system (Leica, Buffalo Grove, USA). The percentage of positive staining was measured using Image-Pro Plus 6.5 (Media Cybernetics, Maryland, USA).[17,18]

Real-time PCR

Total RNA from mouse SMG samples was isolated using TRIzol reagent (15596026, Thermo Fisher Scientific, Waltham, USA). Total RNA (1 µg) was used to synthesize the first strand of cDNA using the TaqMan MicroRNA Reverse Transcription kit (RR047A, Takara, Shiga, Japan). Real-time PCR was performed with SYBR Green (RR820A, Takara, Shiga, Japan) on the 7900HT Fast Real-Time PCR system (4329001, Applied Biosystems, Waltham, USA). The used primers were described as previously.[19] The relative expression levels of target genes were normalized with GAPDH and calculated using the $2^{-\Delta Ct}$ method.

Western blot analysis

Total proteins of SMG tissues were extracted by RIPA lysis buffer (89900, Thermo Fisher Scientific, Waltham, USA) and analyzed by Western blotting as previously described,[17,18,20] and then subjected to the Western blotting analysis with primary antibodies against phospho-p65 (ser276) (p-p65) (ab47395, Abcam, Cambridge, UK), p65 (ab76311, Abcam, Cambridge, UK), phospho-Smad3 (sc-517575, Santa Cruz, Dallas, USA), collagen I (1310–01, Southern Biotech, Birmingham, UK), collagen III (1330–01, Southern Biotech, Birmingham, UK) at a 1:1000 dilution. Secondary antibodies were purchased from ZSGB-BIO (ZF-0512; ZF-0317; ZF-0316, Beijing, China) and used at a 1:5000 dilution. Band densities were quantified with Image J (National Institutes of Health, Bethesda, MD) and normalized with GAPDH.

Statistical analysis

Statistical analyses were performed using one-way ANOVA, followed by Newman–Keuls posttest from GraphPad Software (version 5.0, GraphPad Software, San Diego, USA).

Results and discussion

To understand the role of the TGF-β pathway in aging-related SMG hypofunction, we employed the S7KO mice to observe the aging-related changes in SMG. Because of the known inhibitory effect of Smad7 on TGF-β pathway activation, the salivary flow rate was measured to see if aging-related xerostomia was exacerbated in S7KO mice. The results showed a decrease in the salivary flow rate with aging. In contrast to WT mice, the salivary flow rate of S7KO mice decreased significantly when the mice were 12 or 18 months old (Figure 1). The results suggest that

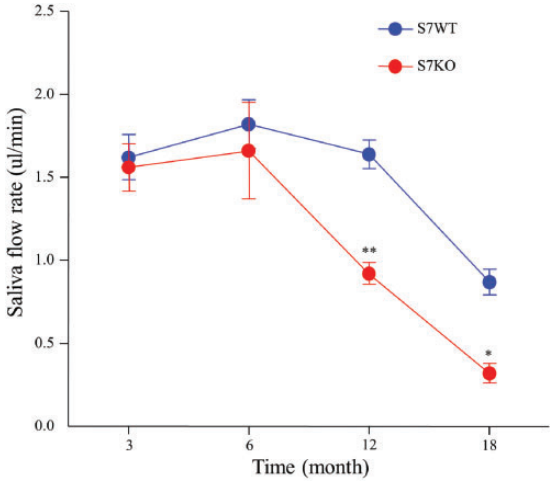

Figure 1. Smad7 depletion promoted aging-related SMG hypofunction. The salivary flow rate was measured. Data are presented as mean ± SD ($n = 5$) (*$P < 0.05$, **$P < 0.01$). (A color version of this figure is available in the online journal.)

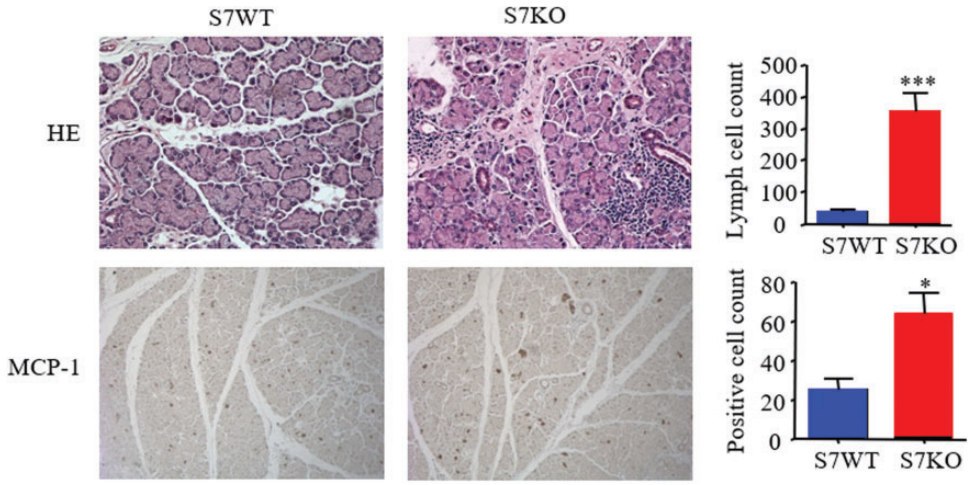

Figure 2. Smad7 depletion increased SMG inflammatory infiltration in aged mice. H&E staining and immunohistochemistry staining of MCP-1 were shown. Data are presented as mean ± SD ($n = 5$) (*$P < 0.05$). (A color version of this figure is available in the online journal.)

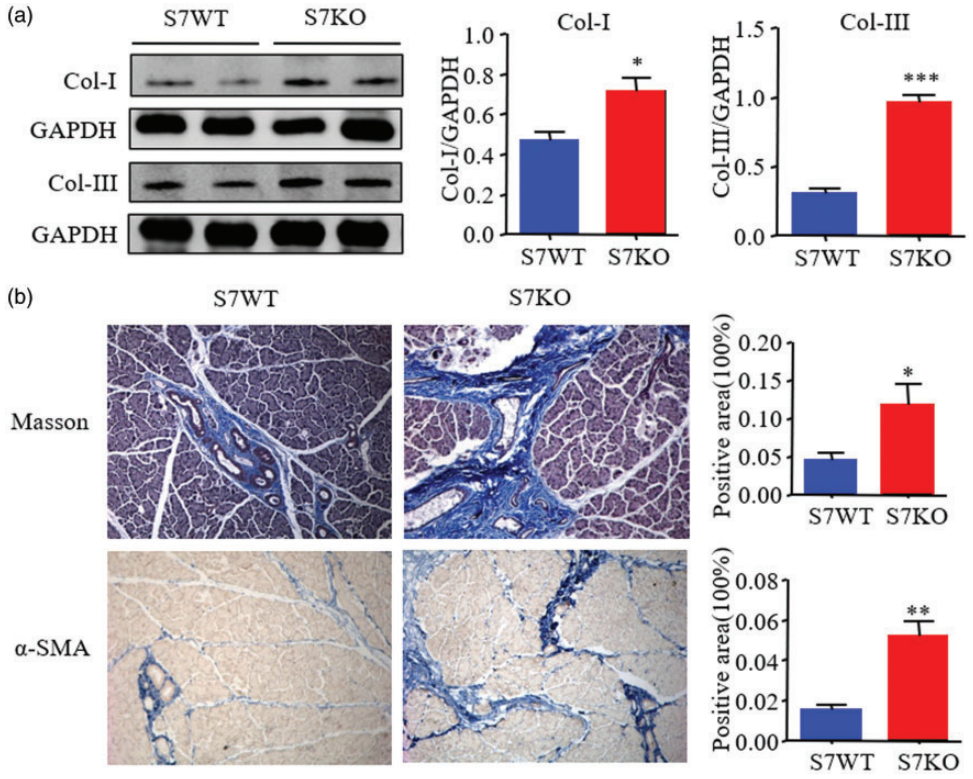

Figure 3. Smad7 depletion increased SMG fibrosis and collagen deposition in aged mice. (a) Western blot of Col I and Col III; (b) Masson trichrome staining and immunohistochemistry staining of α-SMA. Data are presented as mean ± SD ($n = 5$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (A color version of this figure is available in the online journal.)

Smad7 might inhibit the SMG degradation process, especially after six months of age in mice.

Following the confirmation that Smad7 depletion exacerbated the aging-related SMG hypofunction, the histopathological changes were investigated. By 12 months of age, lymphocytic infiltration and acinar cell atrophy were apparent in the SMG of S7KO mice (Figure 2). MCP-1 is a critical mediator of immune cell chemotaxis and is expressed by different cell types, including macrophages. The increased positive staining numbers of MCP-1 further confirmed the gland inflammation with macrophage recruitment (Figure 2). As inflammation could lead to glandular fibrosis, we first examined the Col-I and Col-III expression in the total tissue of SMG, which revealed significantly higher collagen expression in S7KO mice (Figure 3(a)). We next stained the mouse SMG with Masson's trichome and observed increased fibrotic collagen deposition in S7KO mice, suggesting inflammation-mediated interstitial fibrosis (Figure 3(b)). α-SMA reaction showed the degree of myoepithelial cell proliferation

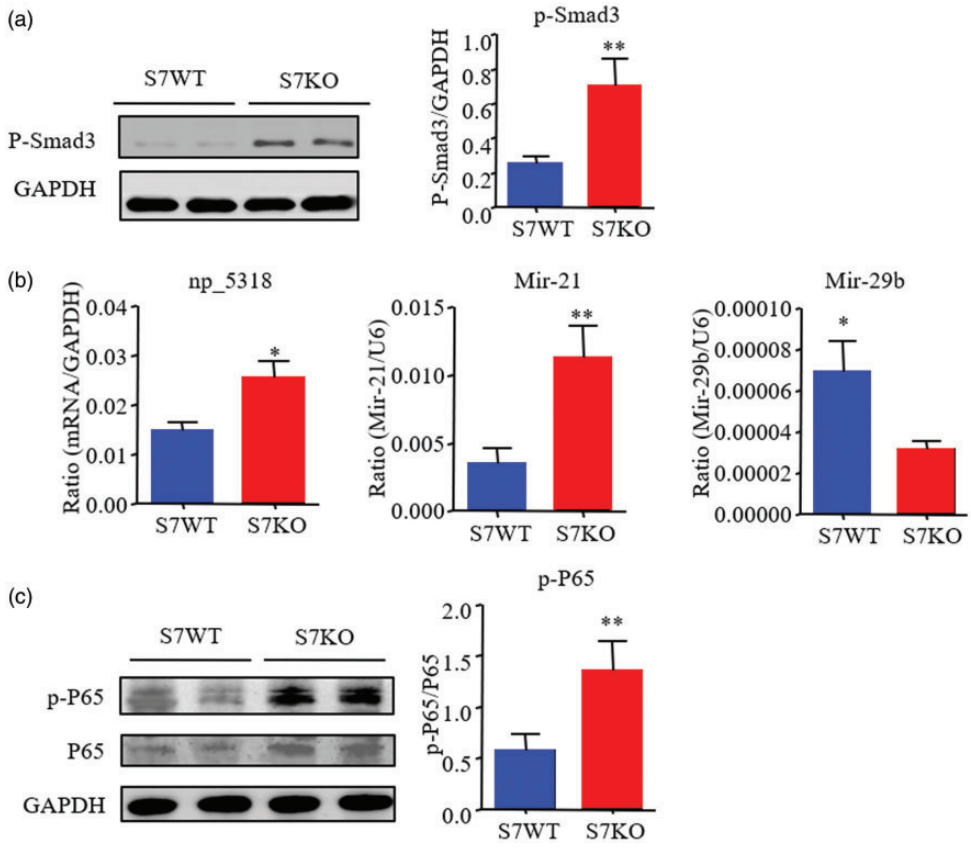

Figure 4. Smad7 depletion caused TGF-β/Smad-mediated fibrosis and NFκB-driven inflammation. (a) Western blot of p-smad3; (b) Real-time PCR of mir-21, mir-29, and np_5318; (c) Western blot of p-p65. Data are presented as mean $\pm$ SD ($n = 5$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (A color version of this figure is available in the online journal.)

surrounding the glandular acini. S7KO mice exhibited more blue deposits of α-SMA, especially in the area of atrophic glandular tissue (Figure 3(b)).

With these histological findings of S7KO mice, we next studied the role of the TGF-β pathway in aging-related inflammation and fibrosis. As Smad7 functioned as an inhibitory factor of Smad3 phosphorylation, we tested the phosphorylation level of Smad3 and confirmed the upregulation of p-Smad3 in the S7KO mice (Figure 4(a)). MiR-21 plays a dynamic role in inflammatory responses and accelerating injury responses to promote organ failure and fibrosis, while mir-29 is negatively correlated with fibrotic diseases.[21,22] Moreover, a functional link has been found between Smad3-dependent lncRNA np_5318 and tissue inflammation and fibrosis.[19] The upregulation of mir-21 and np_5318 and the downregulation of miR-29 were observed in the SMG tissue of S7KO mice (Figure 4(b)), which suggest that the aging-related SMG fibrosis is induced by mir-21, mir-29, and np_5318 via TGF-β pathway activation. To the best of our knowledge, this is the first study that shows the role of TGF-β pathway activation in SMG fibrosis and hypofunction. We also examined the activation of the inflammation-related NFκB pathway and found the upregulation of p65 phosphorylation induced by the Smad7 depletion (Figure 4(c)). Previous studies have demonstrated the correlation between Smad7 depletion and NFκB-driven inflammation,[23] which is highly consistent with the findings of aging-related SMG inflammatory infiltration.

In summary, Smad7 depletion exacerbated the aging-related SMG hypofunction. Inflammatory infiltration, acinar cell atrophy, and interstitial fibrosis were induced in the aging S7KO mice. Loss of Smad7 resulted in TGF-β-mediated fibrosis via mir-21, mir-29, np_5318, and NFκB-driven inflammation. This study confirmed the inhibitory role of Smad7 in the aging-related SMG hypofunction, providing a promising treatment target for aging-related dysfunction and sialadenitis.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, analysis of the data, and manuscript review. MH and WL conducted the experiments. YC wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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