

Original Article

Rapamycin Attenuates Age-Related Changes in Marmoset Submandibular Gland: A Non-Human Primate Model of Human Oral Aging

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ABSTRACT: The aging of salivary glands (SGs) leads to xerostomia, impaired oral defenses, and a compromised quality of life, yet the biology of human submandibular gland (SMG) aging remains poorly understood due to limited availability of human tissues and differences in anatomy and physiology between rodent and human SGs. The common marmoset (*Callithrix jacchus*) is a short-lived non-human primate (NHP) with SMG histology that closely resembles humans and has the potential to provide a translational model for investigating SG aging. In this study, we examined the age-associated structural and molecular alterations that occur in marmoset SMGs and evaluated whether chronic oral rapamycin treatment, a gerotherapeutic mTOR inhibitor, mitigates these age-related changes. Histological and immunofluorescence analyses revealed that there was a substantial decline in mucous and serous acinar units, expansion of ductal and collagen-rich stromal compartments, increased extracellular matrix remodeling, elevated dysregulation of lipid metabolism, and increased numbers of apoptotic and senescent cells in the SMG with aging. Rapamycin treatment was associated with partial preservation of acinar structure and a reduction in fibrosis, lipid accumulation, and number of apoptotic and senescent cells that resulted in an overall tissue phenotype that more closely resembled that of younger animals. These findings suggest that the aging marmoset SMG recapitulates key features of human SG aging and that rapamycin treatment was associated with an attenuation in the hallmarks of SG degeneration. The results suggest that the marmoset represents a valuable NHP model for studying SG aging biology and testing therapeutic strategies aimed at attenuating age-related structural degeneration associated with SG dysfunction.

Keywords: Aging, Submandibular gland, Marmoset, Rapamycin, mTOR, Acinar atrophy, Fibrosis, Senescence, Extracellular matrix, Oral health.

INTRODUCTION

Oral health in older populations has not only been associated with decreased nutritional status and quality of life but a variety of chronic and neurological diseases [1].

Since age-related dental diseases in humans do not generally occur in rodents (or other common lab animals), the establishment of an animal model that naturally develops dental diseases is needed so that we can increase our understanding of changes in human oral health with

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aging. The common marmoset (*Callithrix jacchus*) is a small NHP that has been used in a wide range of research studies. With respect to studies of aging biology, marmosets are relatively short-lived (maximum lifespan ~21 years; average lifespan of 8-10 years), as compared to other commonly studied NHP species, and recapitulate a number of aging phenotypes found in humans but on an accelerated time scale [2]. We have previously reported that the marmoset naturally develops dental diseases, similar to those found in humans, and experiences a deterioration in oral health with advancing age [3].

Salivary gland (SG) aging has broad systemic implications in the elderly as SG dysfunction contributes to malnutrition, an unbalanced oral microbiome, and chronic inflammation [4,5]. As a result, an increased understanding of the cellular and molecular mechanisms underlying SG aging is essential for achieving improved oral and systemic health outcomes in aging populations. Saliva, which is produced by SGs, is one of the main physiologic defense mechanisms used to maintain both oral and general health and consists of a complex fluid secreted into the oral cavity by three major (i.e., parotid, submandibular and sublingual) and numerous minor SGs. Our prior studies and a meta-analysis by others have shown that salivary flow rates, especially for the submandibular and sublingual SGs, decrease with age and that this loss is independent of xerostomia-inducing drug use or underlying systemic diseases [6,7]. Moreover, histological studies have shown that secretory acinar units are replaced by non-functional fibrotic and fatty tissues during human aging [8–12]. Additional recent work has demonstrated an ~30% decline in acinar volume and increased lipid infiltration and fibrosis in aging submandibular glands (SMGs) [13].

Among all the major SGs, the SMG plays a dominant role in maintaining oral homeostasis. In humans, this gland contributes approximately 60-70% of unstimulated (resting) saliva, making it the primary determinant of basal oral lubrication, antimicrobial defense, and mucosal integrity [14, 15]. As a result, an age-related decline in SMG structure and function has a disproportionately large impact on xerostomia, oral inflammation, and quality of life in older individuals [16]. In contrast, the parotid gland contributes predominantly to stimulated saliva and retains greater functional reserve with aging, while the sublingual gland is smaller, predominantly mucous, and less amenable to quantitative morphometric analysis [17]. For these reasons, the SMG represents the most clinically relevant and anatomically informative gland for studying age-associated SG degeneration and therapeutic intervention. Therefore, the present study will focus on changes in the SMG.

Changes in SG tissue biology during aging remain largely unevaluated due to their superficial position and

anatomical proximity to facial nerves, which make obtaining clinical samples difficult. Further, differences in histophysiology (e.g., sexual dimorphism, presence of granular convoluted ducts and different secretory proteins) between rodent and human SMGs create obstacles to using small animals (e.g., rats & mice) in studies of SG aging biology [18–20]. However, we have observed that SMG histology in the marmoset is remarkably similar to that in humans, including mucin- and serous-producing acinar cells with a similar ductal system. Therefore, the marmoset has significant potential as a small animal model for studying SG aging biology. Early histopathological studies in the common marmoset described the basic organization of major SGs, including mixed serous–mucous acini, ductal architecture, and age-associated structural variation [21, 22]. Subsequent work further documented glandular remodeling and degenerative changes across the lifespan [23, 24]. These foundational studies established the anatomic framework upon which modern investigations of primate SG aging are built.

The mechanistic target of rapamycin (mTOR) signaling pathway has been identified as a key regulator of the aging process in yeast, *C. elegans*, *Drosophila*, and mice [25]. The pharmaceutical inhibitor of mTOR, rapamycin, has been reported to extend mouse lifespan and also delays or prevents the incidence of several age-related diseases, including atherosclerosis, Alzheimer's disease, and cancer; moreover, it significantly delays the age-related decline of a number of physiological functions in pre-clinical mouse models [26–29].

mTOR inhibition has demonstrated some translational benefits with the rapalog, RAD001, promoting enhanced immune function by increasing the response to influenza vaccination and reducing markers of T-cell exhaustion in older subjects [30]. Rapamycin also attenuates the normal age-related loss of periodontal alveolar bone in mice [31]. However, the effect of rapamycin on SG tissues has not been previously studied. Therefore, as a step toward understanding the potential translational relevance of mTOR inhibition, in the current study we examine the long-term effect of rapamycin on marmoset aging to identify whether this treatment prevents or slows the loss of SG functional units [2] [32,33].

In the current studies, we examined the histological changes that occur in marmoset SMGs during aging to determine whether they experience an age-related loss of acinar cells as has been documented in humans [6–9]. In addition, we also evaluated the potential protective effects of rapamycin on the structural and functional changes that occur with aging in the marmoset.

MATERIALS AND METHODS

Marmoset SMGs

All tissues used in this study were obtained from a repository of tissues collected from studies performed using marmosets at the Barshop Institute for Longevity and Aging Studies at the University of Texas Health Science Center at San Antonio (UTHSCSA). The research protocols for the use of these animals were reviewed and approved by the UTHSCSA Institutional Animal Care and Use Committee (IACUC), which complies with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals, and adheres to the American Society of Primatologists (ASP) principles for the ethical treatment of NHPs. All marmosets were either born at UTHSCSA, raised in a modified specific pathogen free (SPF) barrier facility, or transferred from the Southwest National Primate Research Center (SNPRC, San Antonio, TX) to the UTHSCSA facility. While at the UTHSCSA Lab Animal Facility, marmosets were maintained in a modified SPF barrier facility and pair- or family-housed (male–female breeding pair with offspring).

Tissues used in the current studies were collected and preserved after euthanasia and then archived in a tissue biobank. Euthanasia was performed, typically within 24–48 hours, after it was determined by the veterinarian in charge that the animal was unlikely to survive, based on a

standardized set of criteria (e.g., loss of >20% body weight, lack of activity including feeding and drinking, etc.). A subset of these animals was enrolled in a study to test the effect of chronic rapamycin administration on lifelong health in marmosets; details of this study are provided in a separate publication [32]. Rapamycin was administered orally at an approximate dose of 1 mg/kg body weight once daily (Monday to Friday) using an encapsulated formulation mixed with food (yogurt). Treatment was initiated in adult animals (middle-aged, approximately 7–9 years) with chronic treatment thereafter. Blood rapamycin concentrations (~ 5–8ng/ml) were monitored as previously described [32, 33]

Since tissues were collected after euthanasia and archived for future use, standardized gross morphologic measurements, including absolute SMG weights, were not consistently recorded at the time of tissue banking. Marmoset SMGs were obtained from the repository of formalin-fixed tissues from animals maintained in the UTHSCSA colony [3]. A total of 12 pairs of SMGs from 10 male and 2 female marmosets were randomly selected and assigned to 4 different groups: Young (<6 years old), Middle-aged (>6 and <10 years old), Old (>10 years old), and RAPA-Old (old animals treated with rapamycin (RAPA) during adult life) (Table 1). Due to the nature of the tissue repository, the cause of death and/or type of disease responsible for death, beyond gross necropsy, are not available.

Table 1. Demographic and experimental characteristics of the NHPs included in the study.

Animal ID	Age (Mos) ¹	Date of Birth	Date of Death	Age group	Gender	Treatment category	Euthanized/Found dead
2584	4 yrs, 7 mos, 11 days	5-21-18	1-20-23	Young	Male	General population	Euthanized
1583	5 yrs, 3 mos, 22 days	05-19-13	10-09-18	Young	Male	General population	Found dead
2283	4 yrs, 3 mos, 21 days	07-28-16	11-18-20	Young	Female	General population	Euthanized
1722	8 yrs, 10 mos, 5 days	04-12-14	02-17-23	Middle-age	Male	General population	Euthanized
1805	7 yrs, 4 mos, 12 days	10-30-14	11-10-22	Middle-age	Male	General population	Euthanized
940	9 yrs, 2 mos, 8 days	12-18-07	03-26-17	Middle-age	Male	General population	Euthanized
31055	13 yrs, 5 mos, 1 day	12-07-10	19-07-22	Old	Male	General population	Euthanized
34080	12 yrs, 0 mos, 2 days	03-12-07	05-12-19	Old	Male	General population	Euthanized
1358	12 yrs, 1 mos, 30 days	2-28-11	01-09-21	Old	Male	General population	Euthanized
33703	13 yrs, 3 mos, 5days	6-29-09	04-10-22	RAPA-Old	Male	Rapamycin	Euthanized
33700	11 yrs, 10 mos, 7 days	01-18-09	25-11-20	RAPA-Old	Male	Rapamycin	Euthanized
1241	9 yrs, 2 mos, 24 days	09-01-10	02-04-19	RAPA-Old	Female	Rapamycin	Euthanized

Demographic details of common marmosets (*Callithrix jacchus*) used in the study, including animal ID, age, sex, date of birth and death, experimental group, and mode of death (euthanized or found dead).

¹Yrs = years, Mos = Months

Histochemical and immunofluorescence staining

SMGs were fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned (5 µm) using routine histological methods. Sections for brightfield microscopy were stained using Haematoxylin & Eosin (H&E) (ab245880, Abcam), Alcian Blue for mucins (ab150662,

Abcam) [24], and Masson's Trichrome for collagenous proteins (ab150686, Abcam) [25], following the manufacturers' protocols. For immunofluorescence microscopy, sections were incubated at 65 °C for 1 h, deparaffinized, and then rehydrated using a graded alcohol (ethanol) series. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) using microwave

heating for 3 min at 20% power. Sections were cooled for 30 min and then blocked for 1 h using 10% donkey serum (Santa Cruz Biotechnology) in PBS containing 0.3% Triton X-100. Sections were subsequently incubated overnight with primary antibodies (diluted 1:50) at 4 °C in 1% BSA/PBS containing 0.3% Triton X-100. The following primary antibodies were used: amylase (66133-AP, Proteintech), cytokeratin-19 (CK19) (MA5-15884, Invitrogen), type 1 collagen (PA5-95137, Invitrogen), ceramide synthase 2 (CerS2) (BS-5077R, Invitrogen), matrix metalloproteinase-7 (MMP-7) (PA5-13197, Invitrogen), matrix metalloproteinase-9 (MMP-9) (MA5-15886, Invitrogen), tissue inhibitor of metalloproteinases-1 (TIMP-1) (MA1-773, Invitrogen), and senescence-associated β -galactosidase (GLB1) (15518-1-AP, Proteintech). After incubation with primary antibody, the slides were washed with PBS and then incubated for 1 h at 4 °C with secondary antibodies (1:500): goat anti-rabbit Alexa Fluor 647 (ab150079, Abcam) or goat anti-mouse Alexa Fluor 647 (A-21236, ThermoFisher). Primary antibody specificity was ensured using appropriate species matched rabbit and mouse isotype controls as described above (Invitrogen RI238244; BD 550878). Sections, after immunostaining, were mounted using Fluoroshield containing the nuclear stain DAPI (Sigma-Aldrich).

Apoptosis detection by TUNEL Assay

Apoptosis in SMG tissues was evaluated using the TUNEL (TdT-mediated dUTP nick end labeling) assay. Formalin-fixed, paraffin-embedded SMG sections (5 μ m) from Young, Middle-aged, Old, and RAPA-Old animals were processed using the *In-Situ* Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. Following deparaffinization, rehydration, and antigen retrieval, sections were incubated with the TUNEL reaction mixture and counterstained with DAPI to visualize nuclei.

Image Processing and Quantification

Histological slides were examined using a bright-field microscope (Olympus BX53, Tokyo, Japan), and immunofluorescence slides were visualized using an inverted fluorescence microscope (Olympus IX73, Waltham, MA, USA). Image processing and quantification were performed in FIJI/ImageJ (version 1.54), following standard procedures described by Schneider et al. [34]. Images were obtained from archived specimens and analyzed using representative regions across all groups. Histological interpretation and quantitative analyses were performed by investigators trained in histopathology using standardized

morphometric criteria. For H&E morphometry, the acinar and ductal areas were calculated as a percentage of the total gland area. For Alcian blue and Masson's trichrome staining, the positive stained area was measured in pixels. For all immunofluorescence analyses, signal was quantified as fluorescence-positive area normalized to total tissue area and expressed in arbitrary units (a.u.). This approach was applied consistently across MMP-7, MMP-9, TIMP-1, CerS2, GLB1, and amylase datasets. It should be noted that fluorescence intensity (mean gray value) was not used as a quantitative metric in this study.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, Boston, MA, USA). Given the small sample size ($n = 3$ animals per group), assumptions of normality and equal variance required for parametric testing could not be reliably verified. Therefore, all quantitative comparisons among groups were performed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple-comparisons test. Each analysis included four groups (Young, Middle-aged, Old, and RAPA-Old) with six pairwise comparisons. Adjusted p-values were reported using Dunn's correction. Data are presented as the median with Interquartile range (IQR). Statistical significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Given the number of endpoints evaluated, the analyses are considered exploratory and hypothesis-supporting, reflecting coordinated biological changes rather than independent confirmatory tests.

RESULTS

Age-related changes in marmoset SMG secretory units are ameliorated by treatment with Rapamycin

H&E staining of marmoset SMG histological sections revealed the presence of mixed serous, mucous, and demilune (i.e., crescent-shaped clusters of serous cells that cap the mucous acini) acinar organization and ductal architecture systems (i.e., intercalated, striated, and excretory ducts) that were comparable to those found in human SMGs. Moreover, the H&E sections also showed the presence of a progressive, age-dependent loss of secretory parenchyma (Fig. 1A). SMGs from young marmosets contained densely packed acini and intact lobular architecture, whereas glands from middle-aged animals had larger, granule-rich acini. Glands from old animals exhibited an extensive loss of acinar units with a concomitant expansion of the ductal and stromal compartments. Morphometric changes were confirmed by measuring the percentage of acinar or ductal area as a

fraction of the total gland area (Fig. 1B&C); these data indicated that acinar area was significantly reduced in old versus young ($p = 0.0045$) and old versus middle-aged ($p = 0.0109$) animals, while ductal area increased by ~30% in old glands ($p < 0.005$). Chronic rapamycin treatment

(i.e., RAPA-Old animals) preserved SMG secretory architecture and retained significantly more acinar area than SMGs from untreated old animals ($p = 0.0014$) and maintained ductal proportions comparable to younger marmosets.

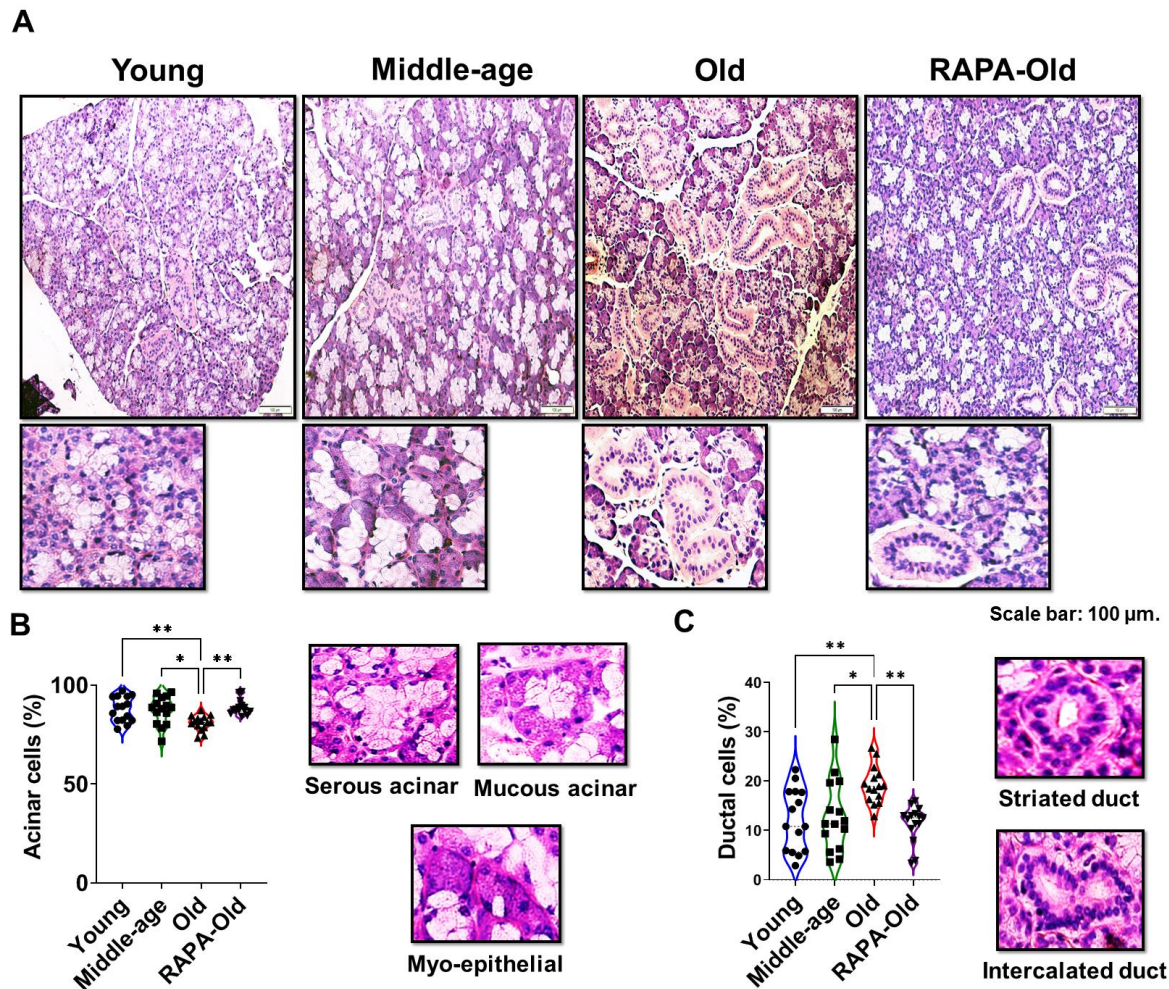


Figure 1. Rapamycin treatment preserves marmoset SMG architecture during aging. (A) Representative hematoxylin and eosin (H&E)-stained sections of SMGs from Young, Middle-age, Old, and Rapamycin-treated Old (RAPA-Old) marmosets. Low-magnification images show a progressive depletion of acinar cells and an enrichment of stromal/ductal cells with aging; this is in contrast to the preservation of acinar organization in RAPA-Old animals. (B) Quantification of acinar cells (as a percentage of total gland area) demonstrates a significant age-associated decrease in acinar cells and partial restoration with rapamycin treatment. Adjacent high-power insets illustrate typical serous and mucous acini used for quantitation. (C) Quantification of ductal cells (as a percentage of total gland area) demonstrates a significant age-associated increase in ductal cells and preservation of acinar cells with rapamycin treatment. Adjacent high-power insets illustrate typical striated and intercalated duct areas used for quantitation. Data shown in the violin plots with overlaid individual points (Panels B & C) represented as median with interquartile range (IQR) ($n=3$ animals/group; five fields were analyzed/gland for a total of 15 measurements for each group). Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's multiple-comparisons test, as described in the Methods. Significance levels were defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Rapamycin treatment preserves mucous and secretory features in marmoset SMGs during aging

Age-related remodeling of the marmoset SMG was further assessed by Alcian blue staining, which highlights

mucous-producing acinar elements (Fig 2A,B). Sections from Young and Middle-aged marmosets exhibited abundant Alcian blue staining acini, distributed throughout the parenchyma, which was consistent with a glandular architecture rich in functional secretory units.

In contrast, old glands displayed a marked reduction in Alcian blue positive areas, reflecting substantial loss or shrinkage of mucous acini with aging. In contrast, sections of SMGs from RAPA-Old animals displayed a qualitative increase in Alcian blue positive regions compared with untreated old animals, suggesting a trend toward preservation of mucous acinar structures with

rapamycin treatment. This pattern was confirmed by quantitating the area of positive staining, which showed a marked reduction in staining of old glands and a partial restoration of staining in RAPA-Old animals. However, this difference did not reach statistical significance ($p = 0.0793$) (Fig. 2B).

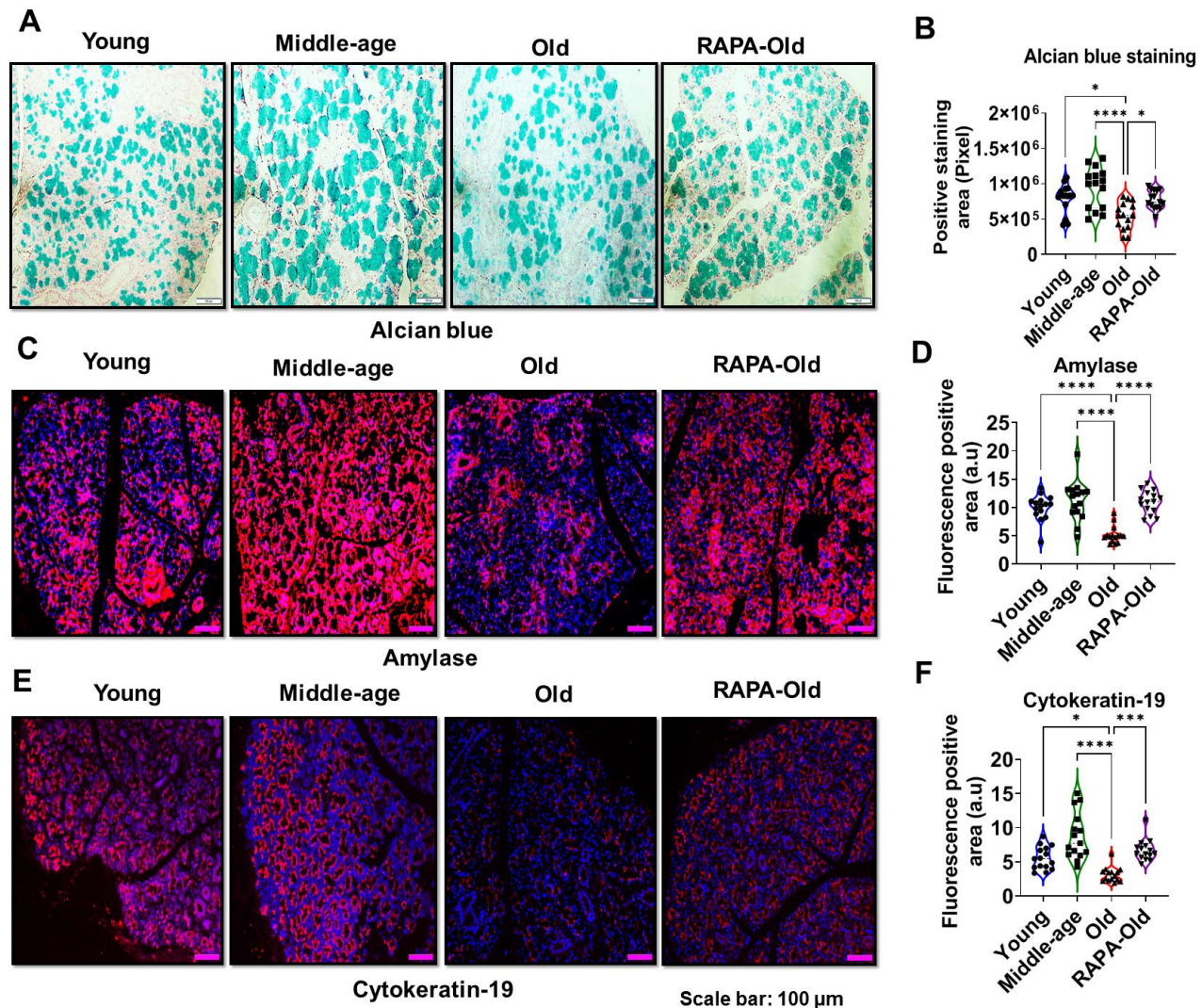


Figure 2. Rapamycin treatment preserves the number of mucous and serous secretory units in marmoset SMGs during aging. (A) Representative Alcian blue stained sections of SMGs from Young, Middle-aged, Old, and RAPA-Old marmosets. (B) Quantification of the total area stained for the presence of mucous acini (expressed as positive staining area normalized to total tissue area, a.u.) in histological sections in panel A. (C) Immunofluorescence staining for the presence of amylase (in serous acini) in sections of SMGs from Young, Middle-aged, Old, and RAPA-Old marmosets. (D) Quantification of fluorescence-positive area normalized to total tissue area for amylase expression (in serous acini), expressed in arbitrary units (a.u.), in histological sections in panel C. (E) Immunofluorescence staining for the presence of cytokeratin-19 (CK19) (in acini and ductal systems) in sections of SMGs from Young, Middle-aged, Old, and RAPA-Old marmosets. (F) Quantification of fluorescence-positive area normalized to total tissue area for CK19 expression, expressed in arbitrary units (a.u.), in histological sections in panel E. The results showed that mucous, serous, and overall acini in marmoset SMGs decreased in Old marmosets, while this aging-related loss was ameliorated in the RAPA-Old group. The data shown in panels B, D, & F represent the median with interquartile range (IQR) ($n=3$ animals/group; five fields were analyzed/gland for a total of 15 measurements for each group). Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's multiple-comparisons test, as described in the Methods. Significance levels were defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To evaluate serous acinar function in the marmoset SMG, amylase immunofluorescence was examined across the four study groups (Fig. 2C&D). Young and Middle-aged glands showed strong amylase expression in almost all acinar cells, consistent with preservation of serous acinar phenotype. Tissue sections from old glands exhibited a dramatic reduction in amylase positive area, consistent with loss of serous acinar marker expression during aging and loss of serous acinar activity. In

contrast, sections of SMGs from RAPA-Old animals had an increased amylase signal relative to untreated old animals, reflecting a rapamycin-mediated preservation of the acinar secretory phenotype. Quantification of fluorescence positive area supported these findings, with old glands showing a significant reduction in fluorescence positive area (i.e., decreased amylase expression) and RAPA-Old glands displaying maintenance of fluorescence positive area ($p < 0.0001$).

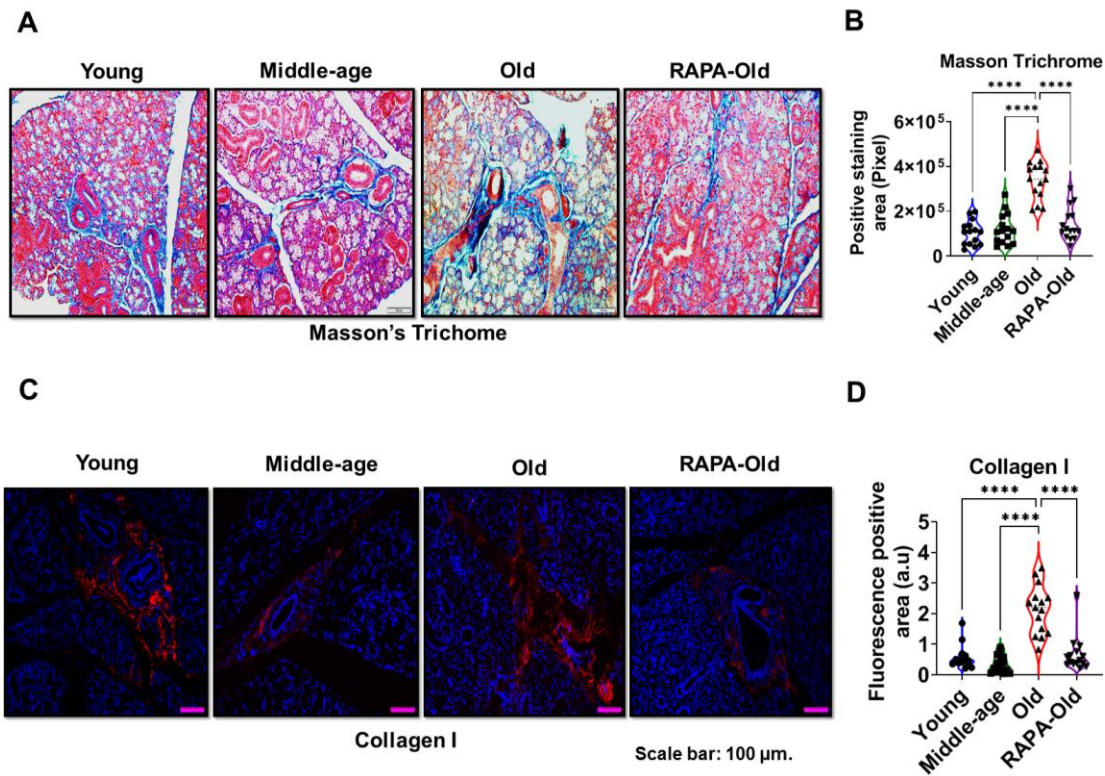


Figure 3. Rapamycin attenuates age-associated fibrosis in marmoset SMGs. (A) Representative histological sections of marmoset SMGs from Young, Middle-aged, Old, and RAPA-Old animals stained with Masson's trichrome to demonstrate interstitial connective tissue (i.e., collagen) accumulation. (B) Quantification of the total area stained for the presence of interstitial connective tissue based on positive staining area normalized to total tissue area of SMG sections shown in panel A. The results show an extensive amount of connective tissue staining in old SMGs which was substantially reduced in rapamycin treated animals. (C) Representative histological sections of marmoset SMGs from Young, Middle-aged, Old, and RAPA-Old animals processed for fluorescence immunostaining for the presence of type 1 collagen (collagen I). The results show the presence of dense periductal and periacinar collagen in tissue from old animals and a significant reduction in collagen production in tissue from RAPA-Old animals. (D) Quantification of fluorescence-positive area normalized to total tissue area for collagen I expression, expressed in arbitrary units (a.u.), in histological sections shown in panel C. The presence of collagen staining corroborates the results of staining with Masson's trichrome. The data shown in panels B & D represent the median with interquartile range (IQR) (n=3 animals/group; five fields were analyzed/gland for a total of 15 measurements for each group). Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's multiple-comparisons test, as described in the Methods. Significance levels were defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CK-19, a marker expressed in both acinar and ductal epithelial cells in the marmoset SMG, revealed striking age-associated architectural changes (Fig. 2E&F). Histological sections of SMGs from Young and Middle-aged marmosets displayed strong immunofluorescence for CK-19 in both acinar units and ductal structures,

reflecting a predominantly acinar-rich, functional gland. In old animals, however, the ducts were more abundant histologically, but total CK-19 positive staining was markedly reduced. This reduction reflects the loss of both CK19-positive acinar epithelium and epithelial marker intensity within the remaining glandular structures, rather

than ductal expansion alone. In contrast, RAPA-Old animals retained more positive CK-19-staining acinar structures and exhibited a stronger overall CK-19 staining than the untreated old animals, producing a pattern more closely resembling that of middle-aged glands. Quantification of CK-19 fluorescence positive area confirmed these observations, showing a significant decline in old glands and a maintenance of CK-19 staining in RAPA-Old animals ($p < 0.05$ to $p < 0.0001$ across comparisons) (Fig. 2F).

Taken together these results demonstrate that in the marmoset SMG aging leads to a decline in acinar mucous and serous content, reduced epithelial marker expression, and a shift toward ductal-predominant architecture. Long-term rapamycin treatment partially preserves acinar structure, secretory protein expression, and epithelial marker integrity, supporting an association between mTOR inhibition and preservation of SG structural integrity during aging.

Rapamycin attenuates age-associated fibrosis in marmoset SMGs.

Masson's trichrome (blue) and type 1 collagen immunofluorescence staining indicated the presence of progressive interstitial fibrosis in the SMGs of aged marmosets (Fig. 3A-D). Masson's trichrome staining revealed abundant collagen deposition surrounding acini and ducts in the Old group, whereas SMGs from Young and Middle-aged marmosets exhibited minimal staining and interstitial fibrosis. Quantitative image analysis showed an ~3-fold increase in collagen-positive areas in Old animals compared to the Young and Middle-aged groups ($p < 0.0001$). In contrast, collagen deposition in the RAPA-Old animals was markedly reduced and had glandular parenchymal architecture that was similar to that of Middle-aged animals ($p < 0.007$ vs. Old) (Fig. 3A&B). Immunofluorescence analysis of type 1 collagen confirmed these findings and showed intense periductal and interstitial collagen accumulation in the Old group, which was substantially diminished in the RAPA-Old group (Fig. 3C&D). Taken together, these results indicate that long term rapamycin treatment attenuates age-related extracellular matrix (ECM) accumulation and fibrosis in the marmoset SMG.

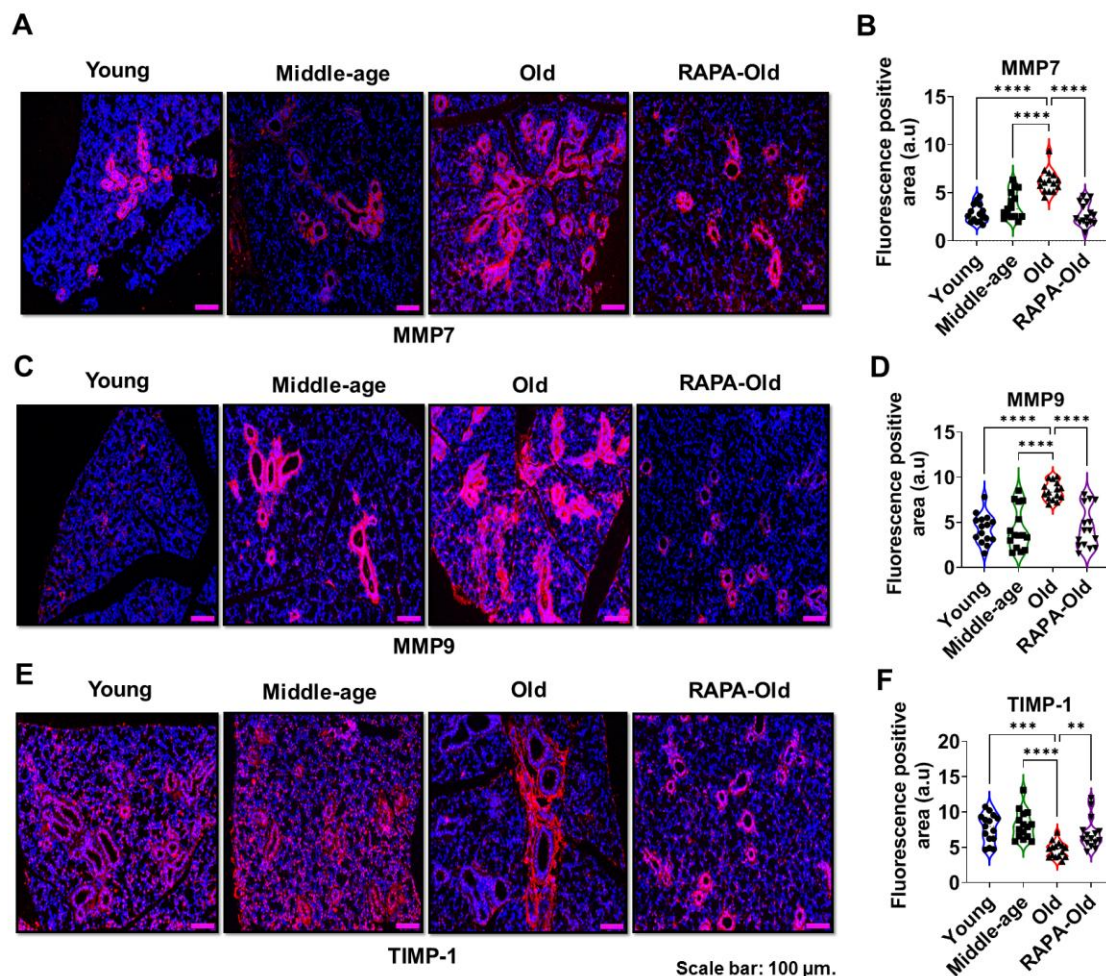


Figure 4. Rapamycin treatment reverses the pro-fibrotic phenotype favored during aging in marmoset SMGs by restoring the MMP/TIMP balance. Representative histological sections of marmoset SMGs from Young, Middle-aged, Old, and RAPA-Old animals were processed for fluorescence immunostaining for the presence of MMP-7 (A), MMP-9 (C), and TIMP-1 (E). The results show that the amount of staining for MMPs is increased with aging, while the amount of TIMP staining is decreased. Long-term treatment with rapamycin reversed this pro-fibrotic trend and restored the MMP/TIMP balance found in young marmoset SMGs. Quantification of fluorescence-positive area normalized to total tissue area for MMP-7 (B), MMP-9 (D), and TIMP-1 (F) expression, expressed in arbitrary units (a.u.), in histological sections shown in panels A, C, and E, respectively. The quantitative analyses corroborate the qualitative results. The data shown in panels B, D, & F represent the median with interquartile range (IQR) (n=3 animals/group; five fields were analyzed/gland for a total of 15 measurements for each group). Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's multiple-comparisons test, as described in the Methods. Significance levels were defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Rapamycin attenuates age-associated pro-fibrotic remodeling in marmoset SMGs

Aging markedly altered the expression of matrix metalloproteinases (MMPs) and their endogenous inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1), in marmoset SMGs (Fig. 4A-F). In SMG tissue from Old marmosets, strong MMP-7 and MMP-9 immunoreactivity was detected in periductal and interstitial regions, indicating active ECM degradation and tissue remodeling. Quantitative analysis of fluorescence positive area revealed a significant upregulation of both MMP-7 ($p < 0.001$ and $p < 0.005$) and MMP-9 ($p < 0.0002$ and $p < 0.0001$) in Old vs. Young and Middle-aged animals (Fig. 4B&D). In RAPA-Old animals, long term rapamycin treatment partially corrected this imbalance (or blocked its development), leading to MMP-7 and MMP-9 expression levels comparable to those found in the Middle-aged group ($p < 0.0001$ vs. Old). Conversely, TIMP-1 expression was markedly decreased in aged SMGs; however, inhibitor expression was significantly restored (or maintained) by long term rapamycin treatment ($p < 0.014$ vs. Old), suggesting re-establishment or maintenance of the MMP/TIMP balance. Together, these data demonstrate that long term rapamycin treatment mitigates age-related ECM remodeling by suppressing the expression of MMPs and promoting the expression of TIMP-1, thereby maintaining tissue integrity in the RAPA-Old animals.

Rapamycin ameliorates dysregulated lipid metabolism (CerS2) and increased levels of apoptosis (TUNEL) and senescence (GLB1) found in SMGs during aging

Lipid metabolism in marmoset SMGs becomes dysregulated during aging as characterized by an accumulation of ceramide synthase 2 (CerS2) (Fig. 5A-D). CerS2 immunofluorescence staining in the acinar region of SMGs from Young and Middle-aged animals was weak, while SMGs from Old animals exhibited strong staining for CerS2 in both acinar and ductal cells, suggesting enhanced ceramide biosynthesis and lipid deposition with aging. When fluorescence positive area

was analyzed quantitatively, CerS2 was found to be significantly upregulated in old versus young ($p < 0.0001$) and middle-aged ($p < 0.0001$) animals. Interestingly, long term rapamycin treatment markedly suppressed the accumulation of CerS2, resulting in levels that were comparable to those of younger animals ($p < 0.024$ vs. Old).

In parallel, TUNEL staining revealed increased numbers of apoptotic nuclei in aging marmoset SMGs, primarily within acinar and periductal regions. Quantitation of fluorescence positive area showed that apoptosis in old animals was significantly elevated ($p < 0.0005$ vs. Young), while rapamycin-treated marmosets had substantially fewer numbers of apoptotic cells ($p < 0.0003$ vs. Old).

The number of potentially senescent cells in SMGs also increased during aging as reflected in the elevated expression of GLB1, a senescence-associated β -galactosidase (Fig. 5E-F). Young and Middle-aged SMGs showed minimal immunofluorescence staining for GLB1, while SMG tissue from Old animals had widespread and intense staining ($p < 0.0001$ vs. Young). SMG tissue from Rapamycin-treated animals contained significantly reduced GLB1 levels compared to old animals ($p < 0.004$ vs. Old). Taken together, these findings showed that during aging SMG lipid metabolism was altered and levels of apoptotic and senescent cells were increased; these changes in SMGs were attenuated by long term rapamycin treatment.

DISCUSSION

The results of the current study have confirmed that the marmoset SMG recapitulates the histological appearance of SMGs in humans, including the presence of mixed serous and mucous acini with demilune. These findings build on classic anatomical and histological descriptions of marmoset SGs [21-24] by providing quantitative assessment of acinar composition and molecular characterization of salivary epithelial markers; these observations provide objective evidence that the marmoset SMG closely recapitulates key structural features of human SMG. The findings, across multiple

endpoints, are best understood as reflecting a coordinated biological phenotype rather than independent confirmatory comparisons. Interpretation is further limited by the small sample size and absence of a study-wide multiplicity correction and therefore should be considered exploratory. Further, the number of mucous and serous acini were decreased in old marmosets which is consistent with age related changes reported in human

SMGs [35]. Along with acinar degeneration, the volume of connective tissue and immunofluorescence staining for CerS2, a lipid metabolism biomarker, were increased in SMGs of old marmosets. These changes may be related to the increased levels of MMP-7 and MMP-9, and decreased levels of TIMP-1. Increased numbers of apoptotic cells were also seen in SMGs from old marmosets.

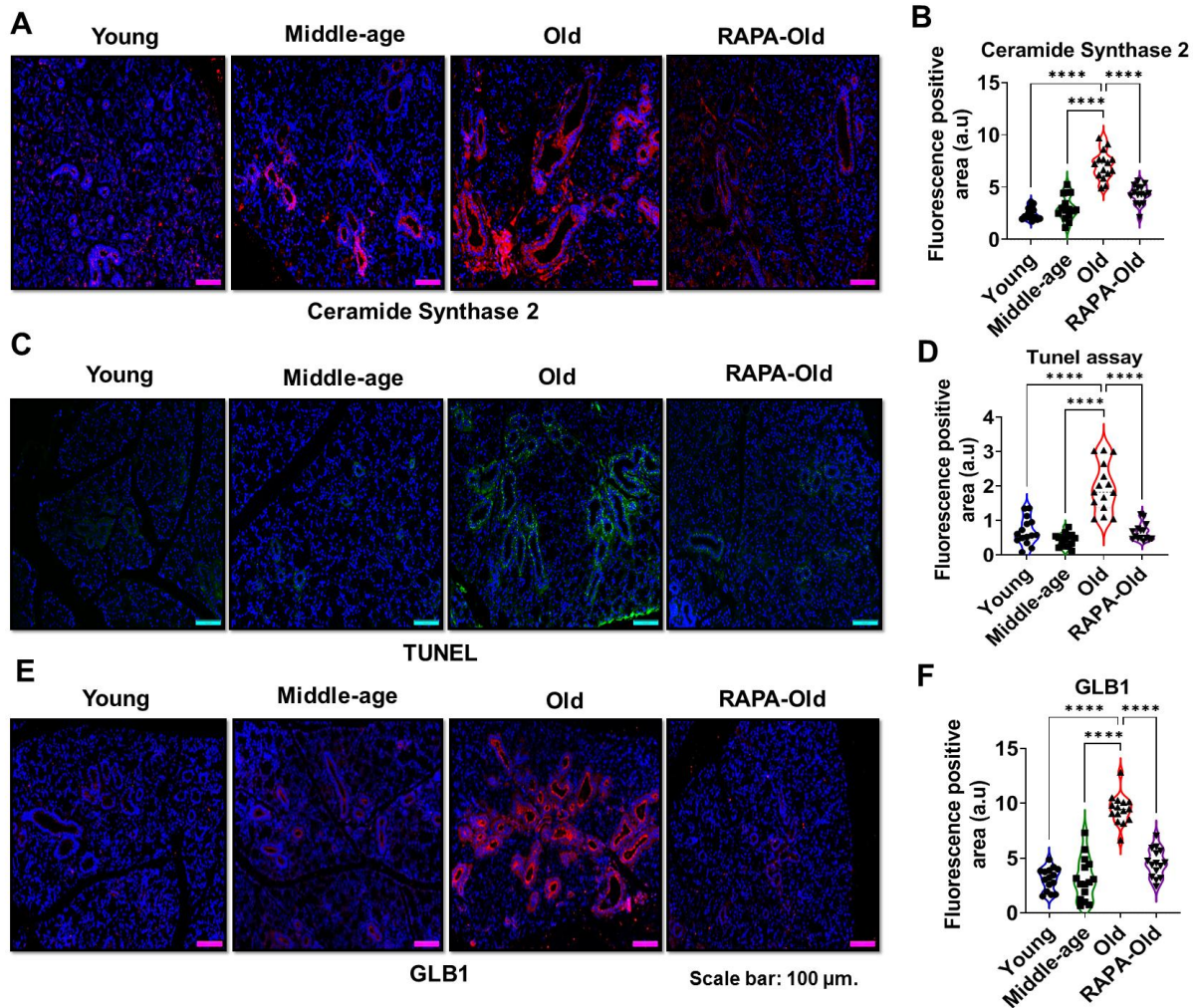


Figure 5. Rapamycin treatment ameliorates dysregulated lipid metabolism and the increased levels of apoptosis and senescence found in marmoset SMGs during aging. Representative histological sections of marmoset SMGs from Young, Middle-aged, Old, and RAPA-Old animals were processed for fluorescence immunostaining for Ceramide Synthase 2 (CerS2) (A), TUNEL (C), and senescence-associated β -galactosidase (GLB1) (E). CerS2 immunofluorescence (pink color) was substantially increased in SMGs from Old marmosets, indicating lipid metabolic stress in these animals; long term treatment with rapamycin significantly reduced dysregulated lipid metabolism in the RAPA-Old group. TUNEL staining (green color) revealed an elevated level of apoptosis in marmoset SMGs during aging (Old), which was markedly attenuated following rapamycin treatment. Immunostaining for GLB1 (pink color) revealed the presence of widespread accumulation of senescent cells in marmoset SMGs from Old animals and a near complete absence of this senescence marker in RAPA-Old animals. Quantification of fluorescence-positive area normalized to total tissue area for CerS2 (B), TUNEL (D), and GLB1 (F) staining, expressed in arbitrary units (a.u.), in histological sections shown in panels A, C, and E, respectively. The quantitative analyses corroborate the histological (i.e., qualitative) results. The data shown in panels B, D, & F represent the median with interquartile range (IQR) ($n=3$ animals/group; five fields were analyzed/gland for a total of 15 measurements for each group). Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's multiple-comparisons test, as described in the Methods. Significance levels were defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Interestingly, long term rapamycin treatment was associated with attenuation of these age-related changes in RAPA-Old animals; specifically, the presence of abundant acini, along with minimal changes in fibrous tissue volumes, were found in the RAPA-Old marmosets just as found in the younger animals. Moreover, the levels of MMP-7, MMP-9, and CerS2, and number of apoptotic cells were significantly lower, while TIMP-1 levels were significantly higher, in the RAPA-Old versus Old marmosets. The mechanistic interpretations presented in this study are based on established literature and should be considered as plausible pathways rather than directly measured processes. Moreover, because a detailed cause-of-death and comorbidity data were not consistently recorded for each animal in the archived records, the potential contribution of terminal health status to the findings reported here is unknown.

Histological sections of human SGs obtained at autopsy showed that the secretory apparatus (i.e., acini) of both major (parotid, submandibular and sublingual) and minor (i.e., labial) SG tissues gradually decreased with advancing age [12,13]. In sections from older human subjects, serious and mucus acinar volume was generally decreased by ~30% as compared to younger subjects. In parallel, the volume of fibrotic and fatty tissue was increased in the aging SG. These observations are similar to the reported reduction in acinar cell compartment during aging in mice [36]. In the current study, we demonstrated that loss of secretory components in the SMG of older marmosets also occurred with a parallel increase in the presence of ECM proteins and collagen I and an accumulation of fatty tissue based on CerS2 staining.

CK19 expression in marmoset SMGs showed a broader epithelial distribution than typically described in rodent SGs. In mice, CK19 is widely expressed in embryonic salivary epithelium but becomes progressively more restricted to ductal cells as the acinar cells mature, resulting in CK19 negative acini in the adult gland [37]. In contrast, multiple human studies demonstrate that CK19 is not exclusively a ductal marker as it is expressed in luminal duct cells as well as in subsets of serous and mucous acinar cells during development and in certain adult glandular compartments [38,39]. More recently, other human studies have further confirmed that CK19 exhibits a broad distribution across ductal epithelium and variable expression in epithelial subpopulations of the SMG [40]. Evidence from other exocrine tissues further support this interpretation as CK expression in human pancreas is detectable in subsets of normal acinar cells and upregulated during epithelial stress or the acinar-to-ductal transition, suggesting that epithelial plasticity rather than strict lineage identity exists in this tissue [41–43]. Given that pancreas and salivary glands originate from the same

developmental program and share conserved exocrine programs, these findings imply that CK19 may be a useful marker for detecting acinar populations with high plasticity or regenerative potential. Our finding that CK19 was robustly expressed in both acinar and ductal cells in young and middle-aged marmosets was therefore more consistent with a primate-like epithelial pattern rather than a more restricted ductal profile found in rodents. It should be noted that although the proportion of ductal cells in aged marmoset glands was increased, the expression of CK19 was decreased; this suggests that aging primarily reduced the number of CK19-positive acinar epithelial cells rather than the population of ductal cells. In the current study, we found that treatment with rapamycin preserved CK19 expression in RAPA-Old glands, suggesting that mTOR inhibition helps maintain epithelial integrity and acinar-ductal lineage stability during aging. Taken together, our findings underscore species specific differences compared to the rodent model for interpreting CK19 biology and emphasize that marmosets are a better model for studying human SG epithelial cell biology during aging.

Xerostomia, the subjective experience of mouth dryness, is a common oral health problem in older adults and is estimated to affect up to 50% of older individuals. Current consensus is that salivary flow rates, especially for SMG/Sublingual (SLG) saliva, decrease with advancing age independent of polypharmacy and age-related chronic diseases such as diabetes and hypertension. In contrast, parotid glands retain more reserve capacity even after aging-associated loss of acinar cells [4,5]. Human SMG produces ~65% of unstimulated (resting) saliva and thus plays an essential role in maintaining moisture in the mouth as well as defense of oral health. Thus, age-related changes in the SMG make older adults more susceptible to oral diseases and compromised quality of life since they are also more likely to suffer from chronic diseases which often require drug treatments, potentially leading to further loss in saliva secretion and other clinical challenges.

Degeneration of SG tissues during aging is generally attributed to cellular senescence (i.e., cells at cell cycle arrest but remaining metabolically active) [13,44], which occurs in response to accumulated DNA damage, mitochondrial dysfunction, and other cellular insults. It's possible for senescent cells to be rejuvenated, undergo apoptosis which leads to cell death, or remain senescent. Senescent cells in aging tissues typically acquire a senescence-associated secretory phenotype (SASP) and release paracrine factors which include many pro-inflammatory cytokines and chemokines that promote inflammaging (i.e., a low-level of inflammation associated with aging). Cellular senescence and apoptosis have been previously reported in the SMG of aging mice

[37,45,46]. In the current study, we confirmed that markers of senescence (e.g., GLB1) and apoptosis (e.g., TUNEL) were increased in the SMG of Old marmosets, as compared to young and middle-aged counterparts. These changes are consistent with the possibility that they play a role in the age-related loss of acinar function in marmoset SMGs. GLB1 reflects increased lysosomal β -galactosidase activity associated with cellular senescence, however it should not be interpreted as a direct measure of irreversible replicative arrest.

In the aged marmoset SMG, increased MMP-7 and MMP-9 expression accompanied by a marked reduction in TIMP-1 creates a pro-fibrotic environment characterized by dysregulated extracellular matrix (ECM) turnover [47,48]. Because TIMP-1 is the principal endogenous inhibitor of MMP-9, its age-related decline would be expected to disproportionately enhance MMP-9 activity, accelerating basement membrane degradation and promoting secondary fibrotic remodeling [49–51]. Restoration of TIMP-1 expression by rapamycin likely rebalanced MMP-9 activity toward more physiological levels which may contribute to preservation of tissue architecture observed in RAPA-Old animals.

An increase in fatty tissue is another common manifestation of human SG aging [8–12]. CerS2 is one of six ceramide synthases in mammals and is a key enzyme in producing ceramides containing long fatty acid chains. CerS2 is implicated in lipid metabolism and may contribute to lipid accumulation [52–54]. In the studies reported here, we showed that the expression of CerS2 (based on fluorescence immunostaining) was increased in the SMG of aged marmosets and that fat accumulation also occurred in the SG of marmosets with aging. In exocrine tissues, ceramide accumulation has been linked to altered membrane integrity and stress-responsive signaling pathways, which may compromise secretory cell homeostasis during aging. Interestingly, CerS2, which showed strong fluorescence in aged SMGs, has been implicated in lipid metabolism and context-dependent pro-survival signaling in other tissues. In oligodendrocytes, age-dependent CerS2 upregulation and extracellular vesicle mediated lipid transfer to neurons activates compensatory survival pathways under aging-related stress conditions [55]. Thus, while increased CerS2 production may reflect metabolic dysregulation, it might also contribute adaptively to cell survival under stress, suggesting a nuanced role in aging and rapamycin effects.

Autophagy plays a key role in cell survival under nutrient-limiting conditions as well as providing a vital means for removing damaged cellular components. Currently, one report suggests that autophagy regulates salivary gland homeostasis and stress response [56]. A salivary gland-specific reduction of autophagy in mice

causes enlarged salivary acinar cells, isoproterenol-induced accumulation of secretory granules, and elevated apoptosis and cellular senescence in response to radiation or ductal ligation; however, these outcomes are prevented by rapamycin administration [57,58]. Rapamycin has been shown to attenuate/block cellular senescence under many conditions including aging models [59]. In the current study, chronic rapamycin treatment was associated with a reduction in markers of cellular senescence and decreased MMP-7 & MMP-9 expression, which is consistent with potential modulation of the SASP related process. Moreover, there was greater preservation of acinar structures, less cellular apoptosis and fibrotic connective tissue formation, and reduced fatty deposition in SMGs of Old marmosets receiving treatment. Our data are the first to show that rapamycin treatment attenuates age associated structural and molecular changes in salivary glands. The potential benefit of rapamycin therapy during aging in humans has recently been reviewed, with promising outcomes for healthy aging [60]. The anti-aging effect of rapamycin warrants further investigation and may lead to novel strategies for preventing or treating age-related salivary gland degeneration and other salivary gland diseases/disorders. While traditional small animal models have provided a number of breakthrough discoveries regarding the biology of aging and the efficacy of various anti-aging interventions, the translational implications of these findings have been limited due to histologic, physiological and pathological differences between rodent and human SMGs. NHPs are closely aligned evolutionarily with humans and develop age-related diseases that are similar to humans. The common marmoset (*Callithrix jacchus*) has several advantages over other NHPs for aging research because of their relatively short life span (e.g., 12-15 years vs. 40-60 years for other bigger NHPs), smaller size, rapid reproduction rate, and ability to be maintained under SPF barriers [2]. Further, marmosets develop a number of common age-related changes similar to humans, such as bone loss, diffuse amyloid plaques, tau protein conformational changes in brain, weight loss pattern, blood aging biomarkers, cardiac function, and decrease of testosterone in males [2,61]. Our results indicate that the histological and histochemical changes during marmoset aging are consistent with the age-related changes found in human SMGs. Moreover, we have shown that rapamycin treatment is consistent with the reduction of age-related changes in marmoset SMG. These findings suggest that rapamycin mediated mTOR pathway suppression may play a role in preserving the cellular and structural integrity of the primate SG, highlighting mTOR as a promising target for oral-aging interventions. The cellular mechanisms by which inhibition of mTOR by rapamycin

prolongs lifespan are still not clear, although cell and rodent studies point to the stimulation of autophagy, a cellular process by which proteins and organelles are recycled through lysosomal degradation [19,62]. Our current study suggests that marmosets are an appropriate model for further exploration of the biology/pathology of aging in human SG tissues and for evaluating new/novel pharmaceuticals for reversing age-related changes or other treatments of SG diseases.

Limitations of the Study

This work was performed using archived, formalin-fixed, marmoset tissues, which is a common practice in NHP aging research. The lack of standardized gross morphologic data limits contextual interpretation; however, histological and quantitative imaging analyses were internally consistent across age and treatment groups. Analyses were performed on representative regions of archived specimens, which may not fully reflect whole gland heterogeneity. Given the very small group size ($n = 3$), these findings should be interpreted cautiously, and effect estimates may be sensitive to individual variability. The study is therefore best considered hypothesis-generating.

Additionally, because tissues were collected from animals euthanized due to declining health, potential confounders due to terminal illness, systemic inflammation, nutritional decline, and age-associated comorbidities cannot be excluded. A limitation of this study is the absence of direct functional measurement such as salivary flow rate or saliva composition. Therefore, the present findings support preservation of structural and molecular features associated with salivary gland integrity rather than direct demonstration of functional rescue. Future studies integrating physiological saliva measurement together with molecular and transcriptomic profiling will be important for establishing the functional significance of rapamycin mediated tissue preservation.

Conclusions

This study supports adoption of the common marmoset as a translationally relevant model for studies of oral biology as it replicates many key features of human SMG aging and supports rapamycin as a candidate intervention associated with attenuation of age-related changes in the SMG. We showed that aging drives coordinated acinar loss, fibrosis, lipid dysregulation, matrix remodeling, apoptosis, and senescence in the marmoset SMG. Long-term rapamycin treatment was associated with a reduction in a number of degenerative features, partially preserving glandular structure and attenuating multiple molecular

hallmarks associated with aging. These findings highlight mTOR inhibition as a promising strategy for combating age-related SG decline and position the marmoset as an essential platform for future therapeutic studies.

Author contributions

P.A.A.A. and H.W. performed the experiments, processed the samples, acquired and analyzed the data, designed the figures, and drafted the manuscript. J.C. and M.M. assisted with data processing, verified analytical methods, curated the datasets, and contributed to data interpretation and manuscript revision. D.D.D. and X.D.C. contributed to data interpretation, validated data curation, provided administrative and technical oversight, and critically reviewed and edited the manuscript. A.B.S. and C.K.Y. conceived and designed the study, supervised the project, secured funding, and contributed to data analysis, interpretation, manuscript writing, and final approval of the work. All authors provided critical feedback, contributed to shaping the research and analysis, and reviewed and approved the final version of the manuscript.

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Declarations

All procedures in this study were conducted in accordance with the guidelines and approval of the University of Texas Health Science Center at San Antonio (UTHSCSA) Institutional Animal Care and Use Committee (IACUC), and were compliant with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the American Society of Primatologists (ASP) principles for the ethical treatment of non-human primates.

Conflict of interest

The authors declare that they have no competing or conflicts of interest.

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