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Dec1 DEFICIENCY RESTORES THE AGE-RELATED DYSFUNCTIONS OF SUBMANDIBULAR GLANDS

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SUMMARY

Age-related organ and tissue-specific cell kinetic and morphological alterations are associated with the incidence of numerous diseases in old age. Salivary dysfunction frequently appears in a wide range of older people and thus is a physiological and biological aspect of aging. The transcription factor Dec1 (differentiated embryo chondrocyte expressed gene 1) is essential for the regulation of cellular senescence. Here, we explored the morphological and physiological abnormalities and the microRNA (miRNA) expression profiles in the submandibular glands (SMGs) of young (3-month-old) and of aged (24-month-old) wild-type (WT) and *Dec1*KO mice. Hematoxylin-eosin (H-E) staining, Masson's Trichrome staining, immunohistochemistry, immunofluorescence, and quantitative real time PCR were employed. MicroRNA (miRNA) expression profiles were examined using an Agilent system with a Mouse 8x60K array. Immunohistochemical analysis revealed an increased oxidative stress response (8-OHdG), increased expression levels of Type I collagen in the fibrotic tissues with substantial amounts of fibroblasts and collagen fibers, the presence of CCl-22-positive lymphocytes infiltrating the SMGs of aged WT mice and a subsequently enhanced expression of fibrosis-associated gene (MMP-2) in the aged SMGs. The water channel protein aquaporin-5 (AQP5) was expressed in the basal cytoplasmic regions of acini in young SMGs but showed a decreased expression in aged SMGs. Myoepithelial cell markers (p63 immunoreactivity and α-SMA immunofluorescence staining) were also decreased in aged SMGs. Quantitative real-time PCR revealed decreased mRNA expression levels of AQP5 and increased mRNA expression levels of Dec1 in aged WT mice. All those characteristics were attenuated

in aged *Dec1*KO mice. There were no apparent differences between young WT and *Dec1*KO mice. Of the miRNAs analyzed, miR-181c-5p, and miR-141-3p are proposed regulatory targets of Dec1 genes that are involved in SMG dysfunction in aged mice. We suggest that a Dec1 deficiency might alleviate the aging-induced hypofunction of SMGs and relevant alterations of Dec1 would be useful to keep SMGs healthy. This study provides clues for determining unique microRNAs concerned with SMG dysfunction. Subsequent activation of such diversely expressed miRNAs be of great value in clarifying the nature of age-related alterations in SMGs.

Key words: *Dec1, AQP5, aging, submandibular gland, miRNA array*

INTRODUCTION

Saliva is fundamental for the preservation of oral homeostasis (1) and salivary glands participate in the barrier properties of the oral mucosa (2). Hypofunction of salivary glands is characterized as salivary gland atrophy associated with decreased saliva flow rates (3). Aging alters the secretion of saliva and its constituents. Aging-related salivary dysfunction is frequently combined with a decrease in secretory or total protein synthesis (4). Identifying factors that influence aging could provide more insight to develop prevention strategies.

The deterioration of salivary gland function followed by numerous physiological and pathological changes including aging (5), results in salivary gland hypofunction and damages acinar cells (6). Parotid glands produce mainly serous secretions, while submandibular glands (SMGs) are responsible for mixed serous and mucous secretions. Aged individuals show a decline in SMG secretion (7). Aging in SMGs causes increases in comparable number of connective and adipose tissues with decreased acini and minor alterations in ducts (8). The production of oxidative stress is a hidden risk factor of aging (9), and the mitigation of reduced prooxidants and heightened antioxidants can increase longevity (10). Hence, elderly individuals can show oxidative damage that leads to salivary secretory dysfunction. Increased inflammation of C-C Motif Chemokine Ligand (CCL)-17 and CCL-22 and the resultant increment in reactive oxygen species (ROS) like 8-hydroxy-2'-deoxyguanosine (8-OHdG) can lead to many chronic diseases and can stimulate the aging process (11). Indeed, 8-OHdG level in saliva was found significantly higher in potentially malignant oral disorders patients exposed to air pollution (12). A peroxide-producing strain of *Lactobacilli spp.* was reported to associate with acidic proline-rich proteins in human saliva which indicates the correlation between ROS and salivary function (13).

Aging associated salivary gland fibrosis and acinar atrophy (14) is affiliated with reduced salivary flow as a result of salivary dysfunction (1, 15). Age-related histological changes in human salivary glands begin with acinar atrophy and result in fibrous and adipose glandular parenchymal replacement (14). Focal to diffuse changes in the infiltration of mononuclear inflammatory cells into salivary glands occurs during

aging (6). Type I collagen, the most abundant protein of the extracellular matrix, is extensively dispersed in fibril structures (16). Extracellular matrix metalloproteinase inducer regulates Matrix metalloproteinases (MMPs) to coordinated extracellular matrix remodeling during aging-related cardiac remodeling in animal model (17). MMP-2 is involved in normal morphogenesis, wound healing and tissue engineering as well as in pathological angiogenesis (18). MMP-2 is normally expressed in ductal cells and is only weakly expressed in acinar cells.

Repairing salivary gland dysfunction due to aging is pivotal for improving the quality of life of older adults. Expression of the core clock gene, *CLOCK*, was detected in the nuclei of acinar cells and in the epithelial cells of salivary ducts (19). Another clock gene is the transcription factor Dec1 (differentiated embryo chondrocyte expressed gene 1, also called Stra13/BHLHE40/Sharp-2/Bhlhb2), which is essential for the regulation of cellular senescence (20). A series of systemic diseases, such as metabolic syndrome, diabetes, etc., are associated with the induction of circadian rhythm disorders (21, 22). Thus, Dec1 plays a fundamental role in the maintenance of physiological rhythms and healthy aging. Dec1 is extensively employed as a marker of cellular aging *in vivo*, and Dec1-induced cellular senescence has also been reported *in vitro* (23). Aging-related molecular circadian clock dysfunction has been poorly characterized in various organs; however, it has great significance for the concept of the aging process. The physiological clock also modulates Aquaporin (AQP) -5, a water channel protein, and the expression of cytokines/chemokines (19). Animal studies have revealed a significant contribution of AQP5 in salivary function (24). In addition, patients with Sjogren‘s syndrome exhibit hyposalivation due to the impaired physiological function of AQP5 (25). Elucidation of the dynamics of Dec1 and AQP5 expressed in SMGs is anticipated to connect alterations in the biology of SMGs in the oral physiological environment and to provide an explanation for the etiology of aging.

Physiological changes in aging and cellular senescence modulate microRNAs (miRNAs) as potential prognostic and diagnostic biomarkers in those processes (26). We hypothesized that disruption of the Dec1 gene impairs functional AQP5 and could be an important factor involved in SMG aging. Our research was aimed at characterizing how physiological aging influences the molecular mechanism of SMG function using WT and *Dec1*KO mice. Our present findings using a miRNA microarray platform in combination with histological analysis identified a novel regulatory miRNA network involved in SMG aging. The putative role of these differentially expressed miRNAs could be instrumental in shedding considerable light on the crucial role of Dec1 in SMG aging and an awareness of homeostasis in oral and systemic health.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Animal Care and Use Committee of the Nihon University School of Dentistry at Matsudo. A total of 24 wildtype (WT)

C57BL/6J and *Dec1* knockout (*Dec1*KO) male mice were used. *Dec1*KO mice were generated as previously described (27). WT and *Dec1*KO mice did not differ significantly in body weight. All mice were housed in 12:12 light/dark cycles (lights on at 8 AM, off at 8 PM). The mice were sacrificed by anesthesia followed by cervical dislocation.

Real-Time PCR

RNAs were extracted as previously described (28). In brief, 1 μg of each total RNA isolated from WT (3 m and 24 m) and from *Dec1*KO (3 m and 24 m) mice using a RNeasy Mini Kit (Qiagen KK, Tokyo, Japan) was reverse-transcribed using a High-Capacity cDNA Archive Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNAs were subjected to real-time PCR using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) to detect target genes (AQP5: Mm00437578_m1; α-smooth muscle actin (α-SMA): Mm00725412_s1; Dec1: Mm00478593_ml; ACTB: Mm02619580_g1;). miRNA Expression Advanced Assays (Thermo Fisher Scientific, Waltham, MA, USA) were used to detect miRNA expression levels: miR-181c-5p (mmu482604_mir), and miR-141-3p: (mmu483016_mir).

Histological Analysis

SMG tissues from the different groups of mice were fixed in 4% paraformaldehyde at 4°C for 48 h and were then embedded in paraffin. Sections of SMG tissues were stained with Hematoxylin-Eosin (H-E) and with Masson's Trichrome (Abcam, Tokyo, Japan). Histopathological observations were performed using a light microscope.

Immunohistochemistry and Immunofluorescence

Sections of SMGs from WT and from *Dec1*KO mice were initially immersed in citrate buffer pH 6.0 (Abcam, Tokyo, Japan), followed by incubation with 3% hydrogen peroxide to block endogenous peroxidase activity and subsequent steps were performed according to the manufacturer's instructions. Anti-Type I Collagen (1:100, ab34710, Abcam, Tokyo, Japan), anti-MMP-2 (1:100, bs-0412R, Bioss, Woburn, USA), anti-CCL-22 (1:75, bs-1761R, Bioss, Woburn, USA), anti-8-OHdG (1:20, MOG-020P, JalCA, Japan), anti-p63 (1:100, ab124762, Abcam, Tokyo, Japan), anti-AQP5 (1:200, ab78486, Abcam, Tokyo, Japan), anti-α-SMA (1:100, ab5694, Abcam, Tokyo, Japan) and anti-Dec1 (1:100, NB100-1800, Novus, CO, USA) antibodies were used as primary antibodies to detect immunoreactivity in SMG tissues. After overnight incubation, the specimens were rinsed with PBS and were incubated with appropriate secondary antibodies (Nichirei Biosciences Inc., Tokyo, Japan) at room temperature for 30 min. For immunohistochemistry, all specimens were color-developed with diaminobenzidine (DAB) solution (DAKO, Carpinteria, CA, USA) and were counterstained with hematoxylin. For immunofluorescence, all specimens were incubated with Zenon labeling complex and mounted with ProLong™ Gold Antifade

Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). The stained cells were photographed, and four randomly selected microscopic fields per specimen were quantified using x 40 objective lens (0.2 mm^2). The ratio of positive stained area to total area was calculated.

miRNA Arrays

miRNA arrays were conducted as previously reported (29). A NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and a Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) were used to determine RNA integrity. About 1.5 µg of each sample was subjected to hybridization to a miRNA 8x60K array (Rel. 21.0, Agilent). Agilent GeneSpring GX software was used to analyze the acquired data.

miRNA target prediction

Targetscan (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/mirdb/index.html), TarBase v7.0 (http://www.microrna.gr/tarbase) and microT-CDS (http://www.microrna.gr/microT-CDS) databases were used for filtering potential miRNAs that could target Dec1 or AQP-5.

Statistical Analysis

SPSS 19.0 was used for statistical analysis. Statistical significance was assessed by an independent two-tailed Student's t-test or analysis of variance (ANOVA).

RESULTS

Aging promotes inflammatory infiltration and oxidative stress, and downregulates p63 in SMG tissues of aged mice

Histological analysis showed acinar cell atrophy, cytoplasmic vacuolization with pleomorphism and the infiltration of inflammatory cells in SMG tissues of 24 m old WT mice leading to the destruction of acini (Fig. 1). The level of 8-OHdG was higher in 24 m old WT mice compared to 3 m old WT mice, which suggested increased oxidative stress with aging (Fig. 1). Expression of the myoepithelial and basal epithelial marker p63 was declined in 24 m old WT mice (Fig. 1). The distribution of 8-OHdG was abundantly observed in the duct cells and scarcely present in acinar cells while p63 protein displayed a diffuse distribution in the basal cells and myoepithelial cells around the ducts and acini of the SMGs.

*Dec1*KO attenuates fibrosis and inflammation in SMG tissues

24 m old WT mice demonstrated fibrosis in SMG tissues that were not observed in young 3 m old WT mice. Masson's Trichrome staining revealed a higher percentage of collagen accumulation in 24 m old WT mice compared to 24 m old *Dec1*KO mice (Fig. 2). Immunohistochemical analysis showed the accumulation of Type I collagen in SMG tissues of aged mice compared to young mice (Fig. 2). The fibrosis-associated gene

MMP2 was similarly expressed in SMG tissues of 24 m old WT mice (Fig. 2). CCL-22 was markedly stained in SMG tissues of 24 m old WT mice (Fig. 2). The fibrosis level was reduced, and the above-mentioned genes were minimally expressed in 24 m old *Dec1*KO mice compared to 3 m old *Dec1*KO mice suggesting that the aging phenotype was attenuated.

Deficiency of Dec1 rescues age-related changes of AQP5 in SMG tissues

Immunofluorescence staining revealed the downregulation of AQP5 in acinar cells and of α-SMA in myoepithelial cells in 24 m old WT mice compared to 3 m old WT mice (Fig. 3A). The expression levels of AQP5 and α-SMA represent the abnormal homeostasis of SMGs in aged WT mice, while there was a marked difference in 24 m old *Dec1*KO mice. Quantitative real-time PCR analysis confirmed those expression levels. AQP5 mRNA expression was significantly decreased in 24 m old WT mice compared to 3 m old WT mice. 24 m old *Dec1*KO mice maintained similar level of AQP5 mRNA expression compared to 3 m old *Dec1*KO mice (Fig. 3B). The level of α-SMA mRNA was also significantly down-regulated in 24 m old WT mice and 24 m old *Dec1*KO mice compared to their young counterparts (Fig. 3B). Interestingly, the AQP5 and α-SMA expression levels were higher in 24 m old *Dec1*KO mice when compared to 24 m old WT mice (Fig. 3B). A significant elevation of Dec1 expression was observed in 24 m old WT mice than 3 m old mice (Fig. 3B).

Subcellular localization of AQP5 and Dec1 during SMGs aging

Dec1 was expressed in both acini and ducts of SMGs in 3 m old WT mice. Interestingly, the degree of staining of Dec1 protein was markedly increased in 24 m old WT mice. Increased Dec1 expression was evident in myoepithelial cells, especially in atrophic glandular tissues of 24 old WT m mice. AQP5 was mainly expressed in the apical membranes of acini of 3 m old WT mice SMGs and the expression was decreased in 24 m old WT mice. (Fig. 4).

miRNA expression levels in WT and in *Dec1*KO mice during aging

miRNA expression patterns displayed distinct differences in 24 m old WT and 24 m old *Dec1*KO mice compared to their young counterparts, respectively (Tables 1, 2) and differentially expressed miRNAs in the SMGs of 24 m old WT and 24 m old *Dec1*KO mice (Table 3). Of the miRNAs screened, 132 miRNAs showed >= 2-fold difference in 24 m old WT mice vs. 3 m old WT mice, 79 miRNAs exhibited >= 2-fold changes in 24 m old *Dec1*KO mice vs. 3 m old *Dec1*KO mice, and 36 miRNAs showed >= 2-fold changes in 24 m old WT and in *Dec1*KO mice compared to their young counterparts.

Binding site prediction

Bioinformatic analysis identified miR-181c-5p and miR-141-3p, which were down-regulated in 24 m old WT mice compared to 3 m old WT mice, that could

potentially bind to the 3'UTR of Dec1 (Fig. 5A). Quantitative RT-PCR confirmed the expression patterns of the miRNAs (Fig. 5B).

DISCUSSION

The strength of this study is that we used young and aged mice to identify aging specific microRNAs and the detailed histological structures that regulate gene function. Further, the combination of this approach with studies using *Dec1*KO mice that lack expression of the Dec1 protein, an aging marker, revealed the mechanism involved in SMG function and aging.

Little data has been published so far on aging salivary glands, with a focus on circadian genes with physiological influences. This study unequivocally demonstrated that inhibition of the clock gene Dec1 and the resulting microRNA pathway activation attenuates the progression of SMG aging. Multiple parameters involved in the aging of SMGs were examined, including fibrosis, inflammation, and oxidative stress. Age-related changes in those factors were all attenuated by the deficiency of Dec1. This is the first report revealing the antiaging effects of the Dec1 pathway in the physiological aging of SMGs. We propose that deleting the function of Dec1 would maintain oral health by invigorating salivary glands. This result suggests that the progression of age-related phenotypes detected in this study is primarily caused by changes through the Dec1 pathway.

Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death (30). Age-associated changes in the proportional volume of fat and connective tissues might be related to changes in acini type and the SMG cell microenvironment (31). Older mice have acinar cell atrophy, cytoplasmic vacuolization with pleomorphism, lymphocyte infiltration, decrease of mucin and increase of periductal and perivascular fibrosis (32). Likewise, our present study proves that an age-dependent decrease of acinar components also occurred in SMGs of WT mice. We observed a comparatively stable structure of SMGs in aging *Dec1*KO mice.

Dec1 was expressed in both acini and ducts of SMGs in young and old mice, and Dec1 expression was significantly increased with aging. Conversely, AQP5 was mainly expressed in the basal cytoplasmic membranes of acini and its expression was altered in the process of aging. This difference could be attributed to the different anatomical structures and functions of Dec1 and AQP5 in SMGs of mice. AQP5, a water channel protein required for functional salivary glands (33), is expressed in the apical membrane of salivary acinar cells. Targeting the AQP5 gene has been shown to dramatically decrease salivary secretion (24), and the amount of saliva produced is reduced in AQP5 knockout mice (24, 34). Additionally, the incidence of dry mouth resulting from radiation (35) and from Sjogren's syndrome (36) has been associated with decreased levels of AQP5 mRNA expression. In this study, we also observed decreased mRNA expression levels of AQP5 with aging. Therefore, the decrease in AQP5 mRNA

expression is a likely cause of SMG hypofunction in aged mice, although the mechanism for this decrease is still unknown. One hypothesis is that Dec1 might lead to decreased AQP5 mRNA expression since aging induces the expression of Dec1. Surprisingly, a Dec1 deficiency restored the expression of AQP5 in aging SMGs (Fig. 5), suggesting that the water channels are open. The expression of Dec1 was upregulated at the mRNA level during aging of SMGs. Immunohistochemical analyses also revealed that the expression of Dec1 was upregulated primarily at interstitial spaces between and within the lobules, where fibroblastic cells, fibroblasts and collagen fibers accumulated, in aging SMGs, indicating that these fibroblasts express Dec1.

Salivary glands are ideal for demonstrating organ reserve capacities, since these exocrine glands are readily accessible, well-characterized and reflect many systemic conditions (e.g., poorly controlled diabetes, HIV immunosuppression, etc.) (3). Aging leads to oxidative stresses, contributes to the reduction of AQP5 expression, which in turn impairs submandibular gland function. Aging is associated with the accumulation of ROS and the impairment of antioxidant systems (37). ROS-induced oxidative stress in salivary gland tissues induces an alteration in their secretory functions and reduces salivary proteins (33). In this study, the number of 8-OHdG-positive cells in SMGs increased with age in WT mice. Thus, the present study shows that oxidative stress with increasing age as seen by disruption of the redox regulatory mechanism occurs in SMGs. In addition, age-related alterations in antioxidant gene expression in the liver have previously been reported in response to a Dec1 deficiency (38). Dec1 negatively regulates FGF21 and the expression of FGF21 was decreased in the WT 24 m mice liver (38). Since FGF21 was noted to abrogate the activity of the endogenous antioxidant, superoxide dismutase (SOD), scavenging of ROS and inhibiting their production, which aids our hypothesis that FGF21 can inhibit the aging of Dec1KO mice via the repression of oxidative stress. The unexpected findings signal the need for additional studies to understand more about FGF21 and Dec1 in SMG aging. Thus, age-related changes in the oxidative stress pathway may be involved in decreased response of Dec1 to stress in aged mice.

Decreases of cellular components both in organs and in tissues with aging have been described as physiological senile atrophy. Tissue fibrosis is a common cause of the dysfunctions and diseases in various organs, including SMGs (39). We found that p63 is highly expressed in young SMGs but is decreased in aged SMGs, suggesting that the absence of p63 contributes to the maintenance of salivary gland function during aging. Given the significance of myoepithelial cells in SMG physiology, we examined the expression of αSMA and p63, classical markers of myoepithelial cells, during SMG aging, to comprehend the senescence process of these cells and explore the feasible use of these markers in the diagnosis of SMG aging. The differences in staining patterns possibly contemplate peculiarities in myoepithelial tissue as well as subtle distinct regulations of myoepithelial cells in various diseases. MMP-2 and Type I collagen are important factors involved in proinflammation, profibrosis and aging (40). We observed high expression levels of Type I collagen, MMP-2, and CCL-22 in the SMGs

of aged WT mice and a Dec1 deficiency had the ability to restore salivary homeostasis (Fig. 2). This suggests that an increase in fibrosis and inflammation in aging negatively influences salivary function, which could be improved in a Dec1 deficient environment. Our results clearly demonstrate that CCL-22 expression increased significantly in aged SMGs, suggesting that accelerated cellular aging induced inflammaging in SMGs. Conversely, minimal increases in CCL-22 expression and inflammaging were observed in aged *Dec1*KO mice. CCL-22 is a validated biomarker of the development of tissue dysfunction, which suggests an underlying pathophysiological relationship between the levels of CCL-22 and the development of aged salivary dysfunction. The production of CCL-22 could govern the degree, intensity, and period of the inflammatory response by recruiting Treg cells (41) and increased Treg activity during aging can facilitate higher risk of various diseases (42). CCL-17 and CCL-22 were detected in/around the ductal epithelial cells that is strongly linked to lymphocytic accumulation in Sjögren's syndrome patients (43). CCL-17 expedites effector T cell chemotaxis and sustains interaction between T cells and dendritic cells, and CCL-22 instigates chemotaxis of Treg cells. This study is limited to examine the plausible accumulation of CCL-22-positive cells in SMGs during aging, however, future studies are warranted to explore the role of CCL-17 for therapeutic approaches targeting immune senescence in SMGs. Thus, the inflammatory response generated by aging may alter the expression of Type I collagen and MMP-2 in SMGs. Our data suggest that the disruption in the salivary environment resulting from aging could lead to the hypofunction of SMGs, and that a deletion of Dec1 could improve their function.

The biological aging process is dependent on differential miRNA expression profiles and multiple signaling pathways. Using miRNA microarray analysis in WT mice together with *Dec1*KO mice, we exposed a unique role of Dec1 in the structure and function of SMGs. In addition, our data demonstrate that aging dysregulates the Dec1-mediated pathway *in vivo*, resulting in the regulation of certain types of miRNA expression and subsequent effects on SMG function. Bioinformatics analysis identified the possible binding of the up-regulated miR-181c-5p and miR-141-3p to the Dec1 3'UTR. miR-181c-5p is differentially expressed in the process of thymus aging and plays a role in aging-related diseases such as inflammation and osteoporosis (44, 45). miR-141-3p has been identified as a human liver aging marker that is involved in inflammation (46). A sharp increase in Dec1 transcription activates TGF-β signaling to reset the molecular clock (47) and endogenous Dec1 silencing revoked TGF-β-mediated cell survival (48) In addition, Dec1 can collaborate with various signaling molecules to regulate specific biological actions of TGF-β (49). The repressive function of Smad7, a downstream target of TGF-β signaling, caused fibrosis and inflammation leading to age-dependent submandibular gland dysfunction (50). Recently, Kwon et al. reported the PARP-γ transcriptional network in the submandibular salivary gland aging (51) and HIF-1α responsive gene Dec1 inhibits PPARγ2 transcription, thus illustrating a possible link between adipogenesis and obesity (52). Based on these considerations, inclusive of the dynamic transcriptional regulation of Dec1, we anticipated that the

Dec1 deficiency contributes to considerable defense to the molecular actions of TGF-β and PPARγ2. Thus, to shed light on the mechanisms behind aging, it is of fundamental importance to analyze Dec1 signaling and to target microRNA function in salivary biology.

A complete assessment of SMG aging is appropriate since Dec1 deficiency comparatively reclaimed a few anomalies. Thereby, distinct elements, including variations in miRNA function, are involved in the age-related deterioration of SMG function. However, this study broadens contemporary knowledge of molecular changes accompanying the aging of SMGs and will open new possibilities for its control.

We previously demonstrated that Dec1 is involved in innate immune responses in periodontal tissues, since *Dec1*KO mice challenged with lipopolysaccharides produced restricted inflammation in periodontal ligament cells (27). Subsequently, the present study indicates that Dec1 also governs differential adaptive immune responses to the aging of SMGs, suggesting that a connection between transcription signals and miRNA function is essential to avoid aging.

In the present study, there are substantial differences in the structure and function of SMGs in aged WT or *Dec1*KO mice. SMGs play a substantial role in the production of saliva. Hence, optimizing antiaging therapeutic approaches for SMGs is a high priority for oral homeostasis in the elderly. However, the sublingual and parotid salivary glands counterbalance the salivary gland function in this process. A recent study has demonstrated that AQP5 level was increased in sublingual and parotid salivary glands with aging, which might be a compensatory response to maintain the water and electrolyte balance and acinar atrophy (53). In addition, understanding the molecular mechanism of aging in *in vitro* cell culture experiments is indispensable.

In summary, aging leads to inflammation and reduces AQP5 expression in SMGs. Furthermore, aging induces Dec1 expression in SMGs undergoing fibrosis along with the profiles of certain miRNAs. Additional research is underway to ascertain putative miRNAs and their target genes that are involved in SMG aging.

Authors' contributions statement: KY and UKB contributed to the concept of study and the design of experiments, animal studies, data analysis and manuscript writing; XW contributed to the data analysis, the manuscript writing, and critical review of data. The final manuscript was approved by all authors.

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Figure Legends

Fig. 1. Aging promotes inflammatory infiltration, oxidative stress and downregulates p63 in SMGs of 24 m old mice. The inflammatory cell infiltration and oxidative stress level of 8-OHdG was increased in 24 m old WT mice compared with 3 m old WT mice, while p63 expression was decreased in aged WT mice. H&E staining exhibited acinar cell atrophy (black arrow), cytoplasmic vacuolization with pleomorphism (yellow arrows) and lymphocyte infiltration (green arrow), Scale bars = 50 μm. The number of cells in four independently chosen fields (430 μm x 330 μm) were counted and averaged. Data are the mean ± s.d. (n = 4). *$p < 0.05$, as determined using the t-test.

Fig 2. *Dec1*KO attenuates fibrosis and inflammation in SMG tissues. The expression of Type I collagen, MMP-2 and CCL-22 were induced with aging in WT mice, however, a deficiency of Dec1 attenuated the changes of those age-related genes. Scale bars = 50 μm. The number of cells in four independently chosen fields (430 μm x 330 μm) were counted and averaged. Data are the mean ± s.d. (n = 4). *$p < 0.05$, as determined using the one-way ANOVA.

Fig 3. A deficiency of Dec1 rescues age-related changes of AQP5 and α-SMA.
A) Immunohistochemical analysis of AQP5 and α-SMA**.** The expression of AQP5 and α-SMA was decreased in 24 m old WT mice, compared with 3 m old WT mice. A deficiency of Dec1 partially rescued those changes. Scale bars = 50 μm. B) Quantitative RT-PCR analysis of AQP5 and α-SMA revealed that the expression levels of AQP5 and α-SMA were reduced in aging mice, whereas *Dec1*KO rescued this effect. Dec1

mRNA expression was increased during aging in WT mice. Data are the mean ± s.d. (n = 4). *p < 0.05, as determined using the one-way ANOVA and the t-test.

Fig 4. Subcellular localization of AQP5 and Dec1 during aging. Dec1 was induced in ductal cells of 24 m old WT mice compared with 3 m old WT mice, while the expression level of AQP5 was reduced in acinar cells in 24 m old WT mice. All specimens were incubated with Zenon labeling complex and mounted with ProLong™ Gold Antifade Mountant with DAPI. Scale bars = 50 μm. Arrow indicates myoepithelial cell.

Fig 5. miRNAs potentially bind to Dec1. A) Schematic illustration of miRNA targeting sequences. B) The expression levels of miR-181c-5p and miR-141-3p were examined using quantitative RT-PCR.

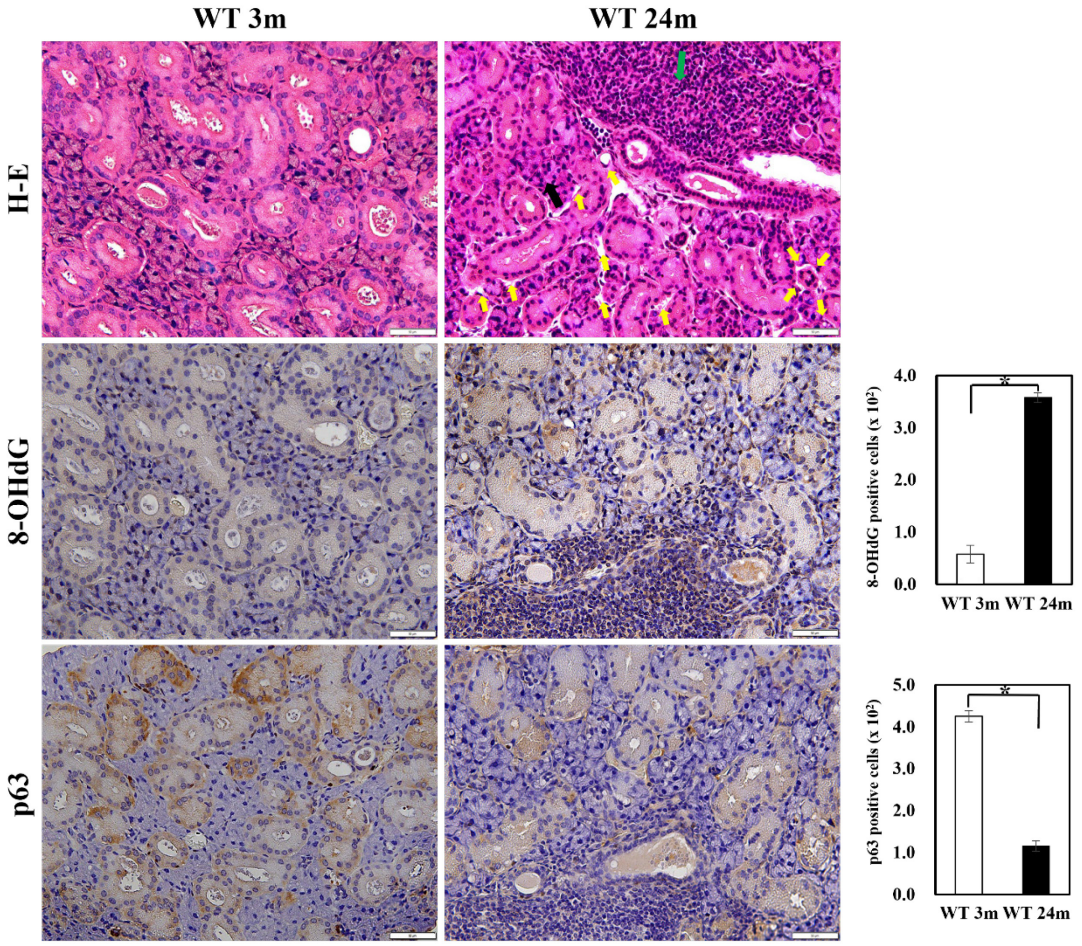

Figure 1

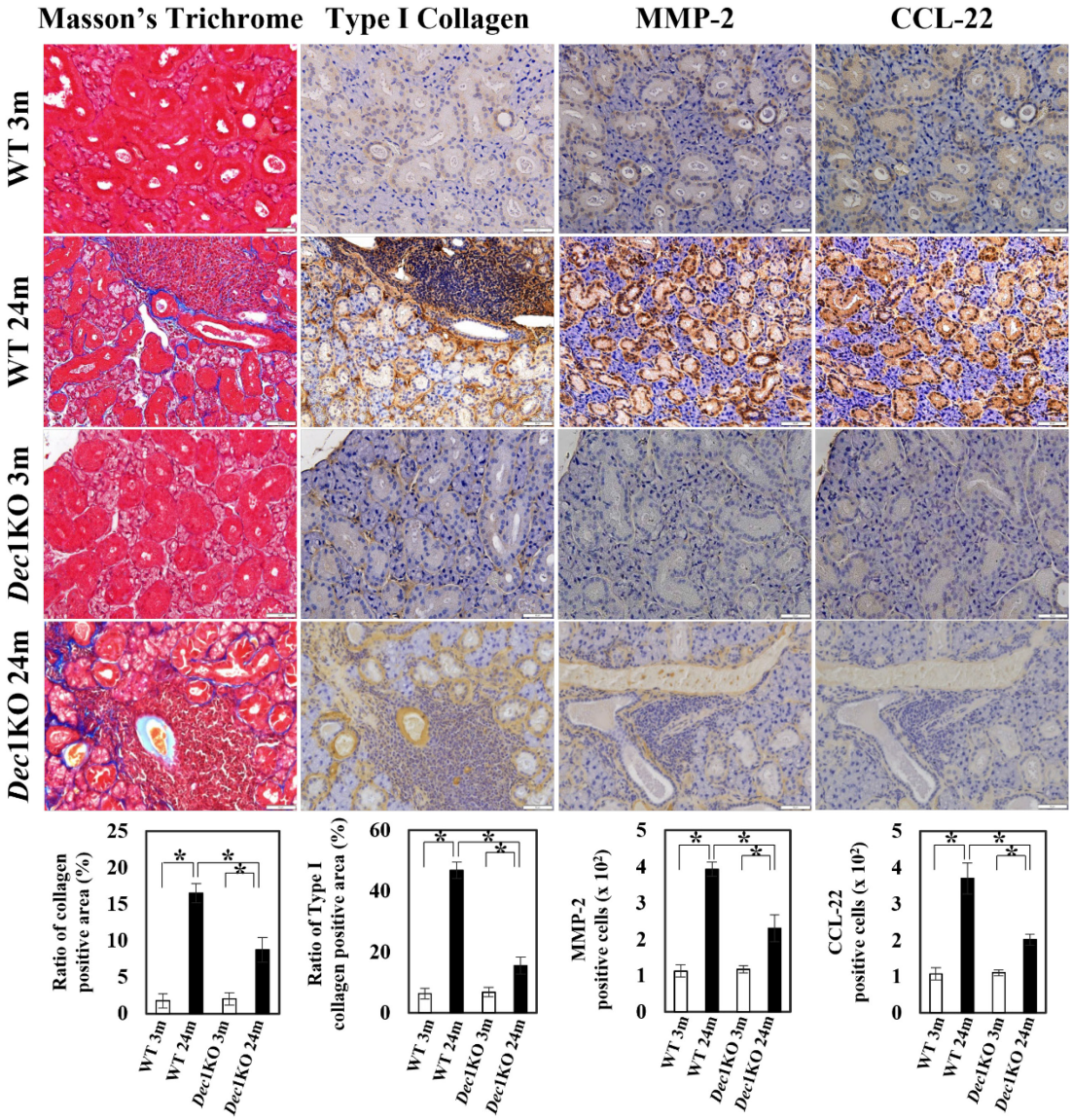
Masson's Trichrome
Type I Collagen
MMP-2
CCL-22
WT 3m
WT 24m
Dec1KO 3m
Dec1KO 24m
Ratio of collagen positive area (%)
Ratio of Type I collagen positive area (%)
MMP-2 positive cells (x 10²)
CCL-22 positive cells (x 10²)
WT 3m
WT 24m
Dec1KO 3m
Dec1KO 24m

Figure 2

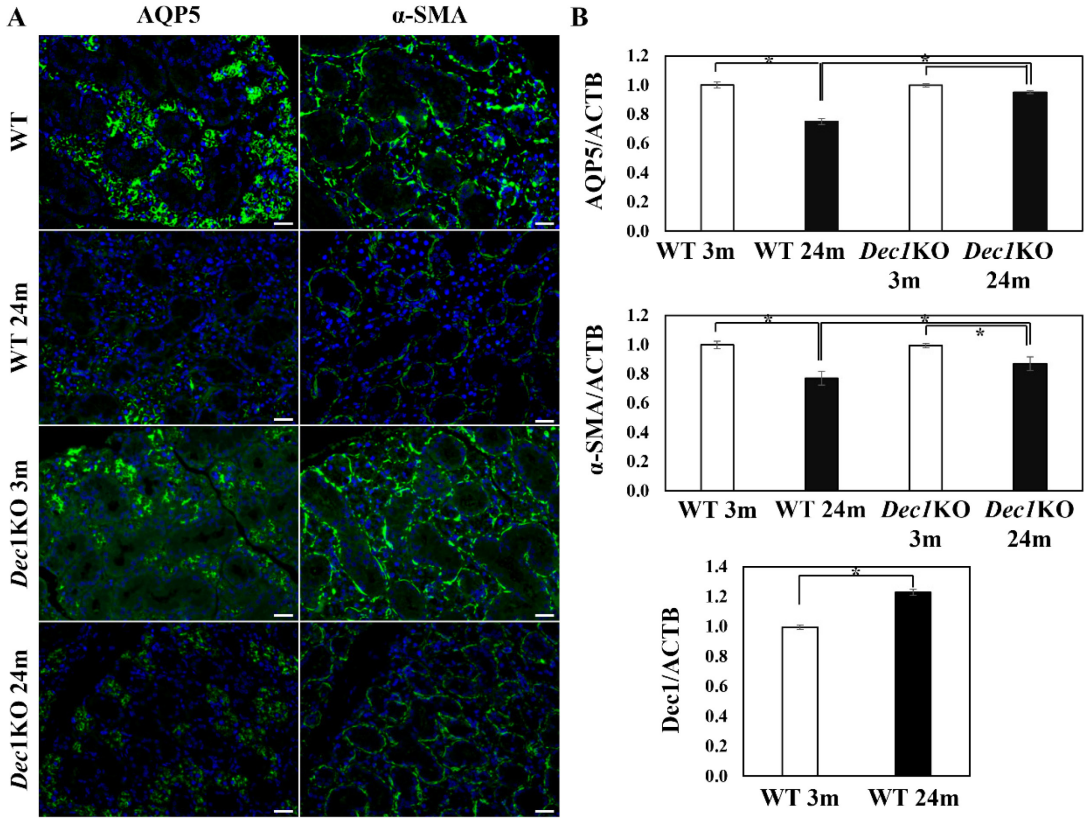

Figure 3

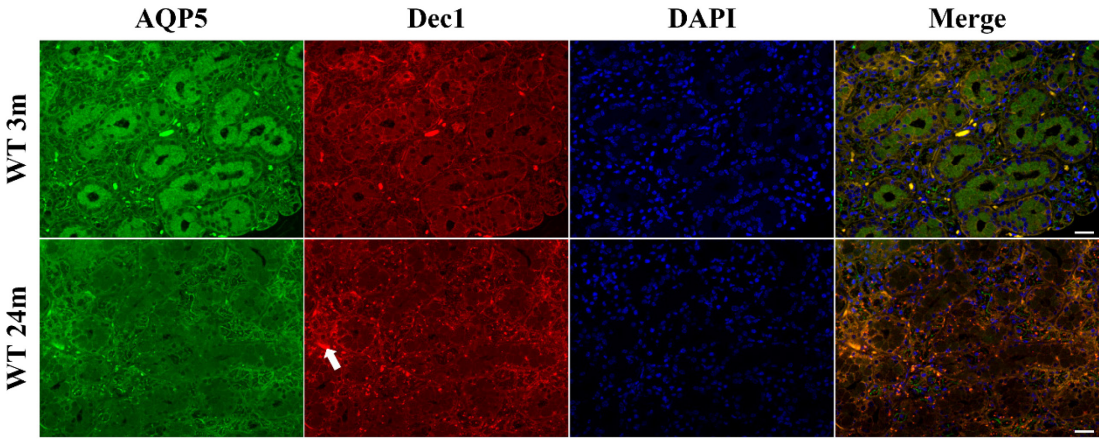

Figure 4

A

Putative binding sites

WT-24m mice

Dec1 3'UTR 5' ...CAGUGAGGAAAAAAAUGAAUGUA…

miR-181c-5p 3' UGAGUGGCUGUCCAACUUACAA

Dec1 3'UTR 5' ...GGAGAGGCGAGGUUACAGUGUUU...

miR-141-3p 3' GGUAGAAAUGGUCUGUCACAAU

B

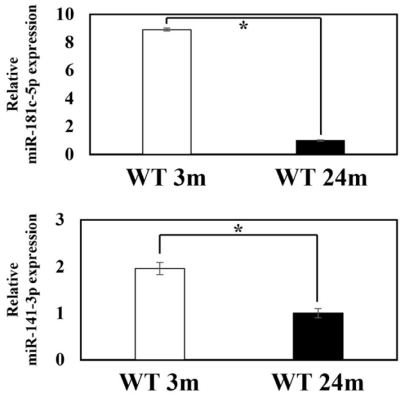

Figure 5

Table 1. Top 50 up-regulated and down-regulated miRNAs in the SMGs of 24 m old vs. 3 m old WT mice

Up-regulated		Down-regulated	
miRNA	**Fold change**	**miRNA**	**Fold change**
miR-379-5p	199.81696	miR-181c-5p	-94.558716
miR-376b-3p	122.81426	miR-181d-5p	-66.458786
miR-154-5p	122.33054	miR-6981-5p	-60.75694
miR-376a-3p	100.4966	miR-6385	-51.8502
miR-382-5p	73.364075	miR-483-5p	-36.57288
miR-411-5p	65.66278	miR-8107	-31.779459
miR-7075-5p	56.188683	miR-18a-5p	-29.74537
miR-300-3p	55.844925	miR-130b-3p	-28.539318
miR-290b-3p	50.437103	miR-30d-3p	-28.25226
miR-154-3p	49.83145	miR-135a-5p	-27.562778
miR-410-3p	46.196533	miR-34b-5p	-25.175987
miR-7055-3p	41.58707	miR-200c-5p	-23.93462
miR-290a-5p	39.275246	miR-5103	-23.56524
miR-377-3p	38.1631	miR-7687-3p	-22.85625
miR-434-5p	34.349976	miR-7009-5p	-16.815466
miR-299b-5p	30.813198	miR-32-5p	-12.72515
miR-329-3p	30.34377	miR-30c-1-3p	-11.848365
miR-376b-5p	29.712257	miR-6351	-3.983692
miR-337-3p	27.328604	miR-218-5p	-3.6718698
miR-1843a-5p	23.343672	miR-551b-3p	-3.516133
miR-376c-3p	23.311296	miR-193a-5p	-3.2998319
miR-7040-5p	22.48002	miR-24-2-5p	-3.2015045
miR-7242-5p	21.83485	miR-15a-5p	-3.1389964
miR-1896	21.619215	miR-322-5p	-3.0818486
miR-7046-3p	21.307453	miR-511-3p	-3.0817351
miR-7045-3p	21.269669	miR-192-5p	-3.0392346
miR-1957b	20.870308	miR-194-5p	-3.0028672
miR-877-5p	20.73392	miR-497a-5p	-2.927727
miR-495-3p	20.411978	miR-195a-5p	-2.8648775
miR-8095	20.357862	miR-191-3p	-2.7460196
miR-7069-5p	20.339333	miR-31-3p	-2.7156298
miR-139-5p	15.105884	miR-22-3p	-2.715377
miR-705	12.833992	miR-674-3p	-2.6797814
miR-127-3p	10.918511	miR-193b-3p	-2.652176
miR-134-5p	10.528893	miR-210-3p	-2.6518328
miR-7031-5p	10.397103	miR-16-5p	-2.6399817
miR-136-5p	8.110494	miR-31-5p	-2.5763404
miR-1892	8.099368	miR-378a-3p	-2.530043
miR-375-3p	4.425905	miR-378d	-2.5050595
miR-1894-3p	4.34991	miR-19a-3p	-2.4938507
miR-1895	3.8705657	miR-144-3p	-2.488031
miR-7226-5p	3.8662179	miR-204-5p	-2.477432
miR-3072-5p	3.6615777	miR-378b	-2.4706855
miR-2861	3.6453784	miR-183-3p	-2.4612074
miR-7018-5p	3.626475	miR-378a-5p	-2.4316125
miR-3102-5p	3.5573506	miR-295-5p	-2.3772154
miR-1934-3p	3.519809	miR-340-5p	-2.3559802
miR-8110	3.5090365	miR-374c-5p	-2.3537552
miR-7045-5p	3.3385758	miR-141-3p	-2.348805
miR-149-3p	3.067138	miR-450a-2-3p	-2.3442028

Table 2. Top 50 up-regulated and down-regulated miRNAs in the SMGs of 24 m old vs. 3 m old *Dec1*KO mice

Up-regulated		Down-regulated	
miRNA	**Fold change**	**miRNA**	**Fold change**
miR-7235-5p	82.61279	miR-6981-5p	-68.87777
miR-3470a	79.87196	miR-7211-5p	-55.463055
miR-877-5p	77.501976	miR-7115-3p	-54.70367
miR-328-5p	73.65352	miR-193a-5p	-38.91574
miR-7040-5p	62.823017	miR-295-5p	-33.00701
miR-290a-5p	43.82995	miR-136-5p	-32.11722
miR-1931	29.980944	miR-135a-5p	-31.246845
miR-3547-5p	29.844212	miR-3544-3p	-28.04442
miR-5131	28.478014	miR-434-3p	-27.870644
miR-21a-3p	28.133991	miR-6897-3p	-27.538784
miR-150-5p	24.25329	miR-200c-5p	-27.133745
miR-8109	23.403822	miR-7117-3p	-26.281788
miR-466i-3p	21.979048	miR-292b-3p	-25.914322
miR-7075-5p	21.812136	miR-7687-3p	-25.91124
miR-7088-5p	21.79595	miR-3473c	-25.304619
miR-7216-5p	21.128874	miR-664-3p	-17.447115
miR-6934-5p	20.845695	miR-30c-1-3p	-13.432029
miR-5120	20.748692	miR-450a-2-3p	-2.592218
miR-7046-3p	20.49051	miR-30d-3p	-2.572094
miR-3472	20.107819	miR-193b-3p	-2.4723194
miR-224-5p	19.78144	miR-6934-3p	-2.2625623
miR-7684-5p	19.182404	miR-8107	-2.0522668
miR-6407	18.292986	miR-183-3p	-2.0342863
miR-155-5p	14.43364		
miR-706	13.309501		
miR-142a-5p	9.992599		
miR-466f-3p	9.957185		
miR-714	9.125459		
miR-142a-3p	8.082744		
miR-721	6.9932785		
miR-344i	5.4550395		
miR-5121	4.3644133		
miR-705	4.1173215		
miR-7031-5p	4.036817		
miR-130b-3p	3.7079687		
miR-669p-3p	3.5424016		
miR-3102-5p.2-5p	3.5338595		
miR-135a-1-3p	3.417257		
miR-139-5p	3.4089675		
miR-5128	3.3973656		
miR-342-3p	2.9022486		
miR-10a-5p	2.6809459		
miR-3082-5p	2.5264573		
miR-1187	2.4589677		
miR-18a-5p	2.3874025		
miR-2861	2.3009174		
miR-7044-5p	2.2369106		
miR-6516-3p	2.2091596		
miR-1897-5p	2.095239		
miR-3102-5p	2.0685105		

Table 3. Differentially expressed miRNAs in the SMGs of 24 m old WT and 24 m old *Dec1*KO mice

Up-regulated		Down-regulated	
miRNA	**Fold change**	**miRNA**	**Fold change**
miR-877-5p	77.501976	miR-6981-5p	-68.87777
miR-7040-5p	62.823017	miR-7211-5p	-55.463055
miR-290a-5p	43.82995	miR-193a-5p	-38.91574
miR-7075-5p	21.812136	miR-295-5p	-33.00701
miR-7046-3p	20.49051	miR-136-5p	-32.11722
miR-721	6.9932785	miR-135a-5p	-31.246845
miR-705	4.1173215	miR-3544-3p	-28.04442
miR-7031-5p	4.036817	miR-200c-5p	-27.133745
miR-669p-3p	3.5424016	miR-7687-3p	-25.91124
miR-139-5p	3.4089675	miR-3473c	-25.304619
miR-18a-5p	2.3874025	miR-30c-1-3p	-13.432029
miR-2861	2.3009174	miR-450a-2-3p	-2.592218
miR-7044-5p	2.2369106	miR-30d-3p	-2.572094
miR-6516-3p	2.2091596	miR-193b-3p	-2.4723194
miR-3102-5p	2.0685105	miR-8107	-2.0522668
miR-7045-5p	2.0636866	miR-183-3p	-2.0342863
miR-149-3p	2.0621123		
miR-1934-3p	2.01501		
miR-7018-5p	2.0030885		