1 Long-term administration of whey alters atrophy, gene expression profiles
2 and dysfunction of salivary glands in elderly rats

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Abstract

Salivary glands in elderly individuals commonly exhibit morphological changes and dysfunction resulting in xerostomia. Long-term (4-week) drinking of whey prevented and/or restored age-dependent decline of salivary volume and protein concentration, and atrophy of sublingual glands (SLGs) significantly in 88-week-old rats. The transcripts of 42 genes were up-regulated and 7 genes were down-regulated by more than 1.5-fold change with $FDR \leq 0.1$ after whey-drinking. The expression levels of genes associated with salivary proteins and tissue repair were significantly increased, while those associated with lipid metabolism were decreased. Venn diagram analysis revealed that expressions of 13 genes, including *Tcfap2b* and *Abpa*, were induced significantly by whey-drinking. Furthermore, secretory protein levels in SLGs and saliva were revealed by immunoblot analysis. This is the first study to report that whey-administration can prevent and/or restore age-dependent atrophy and functional decline of SLGs in relation to gene expression and thus may serve as a functional food ingredient.

Keywords:

Whey, Aging, DNA microarray, Salivary glands, Atrophy, Dysfunction

Abbreviations: AR, androgen receptor; AP-2, activating protein-2; FDR, false discovery rate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SLGs, sublingual glands; OC, water-drinking elderly rat; OJ, whey-drinking elderly rat; PRP, proline-rich protein; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

1. Introduction

Xerostomia, which is also referred to as dry mouth, is a common problem among the elderly (Gonsalves *et al.*, 2008; Sun *et al.*, 2013). Xerostomia leads to infectious conditions, such as periodontal disease and caries, in the oral cavity (Sreebny, 2000; Guggenheimer & Moore, 2003); these, in turn, lead to an increased risk of atherosclerosis, cardiovascular disease (Meurman *et al.*, 2004) and diabetes (Darre *et al.*, 2008). Thus, xerostomia can result in not only oral diseases, but also systemic diseases. In addition, xerostomia causes dysphagia (Guggenheimer & Moore, 2003), resulting in a reduced quality of life, and increases susceptibility to aspiration pneumonia (Kikutani *et al.*, 2015), which can cause mortality.

Salivary glands consist of three major glands, i.e., the parotid glands, submandibular glands and sublingual glands (SLGs), as well as numerous minor glands. In the unstimulated condition, the parotid glands have a very low secretion rate. In contrast, the submandibular glands and SLGs secrete relatively more saliva under unstimulated conditions (Proctor & Carpenter, 2007). The glands are innervated by autonomic nerves (Proctor & Carpenter, 2007). Noradrenaline released from sympathetic nerves stimulates salivary secretion through α_1 - and β -adrenoceptors. Activation of α_1 -adrenoceptors induces fluid secretion from the salivary glands via an increase in the intracellular concentration of calcium, while activation of β -adrenoceptors induces protein secretion from the glands via the activation of protein kinase A. Acetylcholine released from parasympathetic nerves interacts with

the M₁- and M₃-muscarinic cholinergic receptors to induce fluid secretion from the salivary glands via activation of the calcium signaling pathway (Proctor & Carpenter, 2007). An age-related decrease in salivary secretion has been recognized during both the unstimulated condition (Ship *et al.*, 2002; Pan *et al.*, 2009) and the stimulated condition (Inoue *et al.*, 2003; Choi *et al.*, 2013).

Whey, a co-product of cheese manufacturing, can supply not only nourishment, but also many biologically active components to humans. For example, whey protein stimulates protein synthesis in relation to gene expression in skeletal muscle after exercise (Kanda *et al.*, 2013; Kanda *et al.*, 2014) as well as the accretion of muscle protein in the elderly (Pennings *et al.*, 2011). In addition, it has been shown to reduce tumorigenesis in the rat colon (Xiao *et al.*, 2005). Whey protein also has an insulinotropic effect in both type 1 (Hwang *et al.*, 2012) and type 2 (Pasin & Comerford, 2015) diabetes. Beta-lactoglobulin, a major component of whey, acts as a molecular carrier and alters the bioaccessibility of linoleate/linoleic acid, reducing the risk of cardiovascular disease (Le Maux *et al.*, 2012). Thus, whey is a functional food with possible therapeutic applications.

To the best of our knowledge, no study has assessed the impact of whey on the aging process in the salivary glands; thus, in the present study, we examined the effects of whey supplementation on age-related changes in morphology, gene expression and function of rat salivary glands. We found a new function of whey that recover age-dependent atrophy and functional decline of the SLGs in relation to gene expression.

2. Materials and methods

2.1. Experimental animals and collection of saliva

Eight-week-old male Wistar rats (body weight: 211 ± 18 g) were purchased from SLC, Inc. (Shizuoka, Japan) and given a standard laboratory chow (MF; Oriental Yeast,

Tokyo, Japan). At the age of 12-week (young, body weight: 294 ± 6 g) and 84-week (elderly, body weight: 491 ± 5 g), rats were divided into water-drinking group and whey-drinking group. One young group and one elderly group were provided water ad libitum for 4 weeks and one young group and one elderly group were provided cheese whey from Jersey cattle (donated by Ooyama Ranch Inc., Kagawa, Japan) ad libitum for 4 weeks. Compositional characteristics of whey are in Supplementary table1. They were maintained in a temperature-controlled environment ($22 \pm 2^{\circ}\text{C}$) with a 12-h-light/12-h-dark cycle in accordance with the guidelines established by the Animal Care Committee of Tokushima University Graduate School. Salivary glands were rapidly removed from rats sacrificed by a blow to the head under chloroform-inhalation. Cevimeline (10.0 mg/kg)(Daiichi-Sankyo Pharmaceutical Co. Tokyo, Japan) was injected intraperitoneally into rats. During the first 30 min after cevimeline-injection, saliva was collected by pipette.

2.2. Histological analysis

Salivary glands for light microscopic examination were fixed by immersion in 10% phosphate-buffered formalin and then processed for paraffin sections. Routinely, 5- μm sections were cut and stained with hematoxylin and eosin.

2.3. DNA microarray experiments

Four rats were randomly selected from each of the water-drinking and whey-drinking groups. The total RNA was isolated from the SLGs of the rats using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and QIAcube (Qiagen GmbH). RNA purity was assessed by the ratio of the absorbance at 260 and 280 nm measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and

was examined using an Agilent2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized from 400 ng of total RNA using the Whole Transcript (WT) Expression Kit (Ambion, Austin, TX, USA) according to the supplier's protocols. The resultant cDNA was fragmented and end-labeled with the GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Then, 5.5 µg of the fragmented and labeled DNA target was hybridized to the Affymetrix GeneChip® Rat Gene 1.0 ST Array (Affymetrix) at 45°C for 17 h in a GeneChip® Hybridization Oven 640 (Affymetrix) according to the recommended experimental protocols provided by the supplier. This array comprised more than 722,254 unique 25-mer oligonucleotide features constituting more than 27,342 probe sets for known and unknown genes. The hybridized arrays were washed and stained in a GeneChip® Fluidics Station 450 and scanned with a GeneChip® Scanner 3000 7G (Affymetrix), and GEL files were generated for each array. In each comparison experiment, the intensity levels for the two chips were normalized by dye swap and flag treatments using Affymetrix Expression Console Software (Affymetrix).

2.4. Analysis of microarray data

Microarray data were imported into GeneSpring GX version 12 (Agilent, Santa Clara, CA, USA). This software package allows multi-filter comparison using data from different experiments to perform normalization and the generation of restriction lists and functional classifications of the differentially expressed gene. The signal intensities of the selected genes that were up-regulated or down-regulated by at least 1.5-fold when compared to a control group were extracted by this software and a false discovery rate (FDR) value was calculated using the Significant Analysis of Microarrays method (Tusher *et al.*, 2001). FDR value was rounded off a number of the decimal point third position and the expression change was taken as informative when FDR value was $\leq$ 0.1.

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153 2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

154 The differential expression of selected genes was assessed by qRT-PCR. cDNA was
155 synthesized from 500 ng of RNA extracted from the SLGs using the High Capacity
156 cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). cDNA was
157 amplified using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The
158 primers listed in Supplementary table 2 were designed using Primer-BLAST software
159 (NCBI). Amplified cDNA was detected using the Power SYBR Green PCR Master Mix
160 Reagent kit (Applied Biosystems). The PCR conditions were as follows: incubation for
161 10 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 15 s
162 at 60°C and extension for 15 s at 72°C. The target and reference genes were amplified
163 on the same plate. A non-template control was included for all of the primer pairs in
164 each run. Data were analyzed using StepOnePlus™ software (version 2.3; Applied
165 Biosystems). The expression values were normalized to that of β -actin as an endogenous
166 control as it showed little variation in expression across the sample sets. The mean fold
167 change of expression in the SLGs of whey-drinking rats compared with that of
168 water-drinking rats using the $2^{-\Delta\Delta CT}$ method. Analyses were performed in triplicate
169 using RNA samples from at least three different rats.

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171 2.6. Gel electrophoresis and Western blot analysis.

172 SLGs and saliva samples were dissolved with Laemmli sample buffer (Bio-Rad,
173 Hercules, CA, USA) and the samples with 15 μ g of protein or 15 μ l were subjected to
174 SDS-polyacrylamide gel electrophoresis (PAGE) on 10%, 12.5% and 15% gradient gels.
175 For immunoblot analysis, the separated proteins were transferred onto a nitrocellulose
176 transfer membrane (Hybond ECL; Amersham Biosciences, Little Chalfont, UK).
177 Membranes were blocked overnight with 0.1% Tween 20 in Tris-buffered saline pH 7.4.

The blots were then probed with rabbit anti-basic proline-rich protein (PRP, 1:10 000; a kind gift from Prof. David Castle, University of Virginia (Castle & Castle, 1993)), rabbit anti-cystatin S (1:500, Sino Biological Inc., Beijing, China), or rabbit anti-amylase (1:1500, Calbiochem, Darmstadt, Germany) antibody. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and incubated with horseradish peroxidase-linked donkey anti-rabbit IgG, whole Ab (1:5000, GE Healthcare, Piscataway, NJ, USA) for 1 h. Immunoblots were processed using enhanced chemiluminescence (ECL) (Amersham) and the signals were visualized on a Chemi Doc apparatus (Bio-Rad, Hercules, CA, USA). The bands were quantified using Quantity One software (Bio-Rad).

2.6. Statistical analysis

Data are expressed as the mean value $\pm$ standard error. To test for statistically significant differences between two groups, a paired Student's *t*-test was used for biochemical tests, qRT-PCR and Western blot analysis. A *p* value of less than 0.05 was considered to be statistically significant. In microarray analysis, Moderated *t*-test was used.

3. Results

3.1. Animals

Body weight at the beginning and end of the study, drinking volume, salivary gland weight and salivary protein concentration were not significantly different between the water-drinking (control) young rats and the whey-drinking young rats (Table 1). In elderly rats, body weight at the beginning and end of the study, drinking volume, parotid and submandibular gland weight were not also significantly different between the water-drinking group and the whey-drinking group. SGL weight, salivary volume and salivary protein concentration were significantly higher in whey-drinking elderly rats compared to water-drinking elderly rats.

3.2. Morphology

To evaluate the effect of whey-administration on the morphology of the SLGs, histology was performed on SLGs tissue sections using hematoxylin and eosin staining (Fig. 1A-D). Atrophy was detected in the SLGs of elderly water-drinking rats (Fig. 1C), but not in the SLGs of the younger water-drinking rats (Fig. 1A). Whey-drinking did not affect the morphology of the SLGs in the younger whey-drinking rats (Fig. 1B), and age-dependent atrophy of the SLGs was not detected in the elderly whey-drinking rats (Fig. 1D). These results suggested that whey-drinking prevented and restored age-dependent atrophy of the SLGs.

3.3. Microarray

Microarray chips are a powerful technology that allows the simultaneous measurement of the expression levels of thousands of genes. The resulting expression profiles have led to dramatic advances in the understanding of cellular processes at the molecular level. Total RNA was isolated from the SLGs of eight rats in the 84-week-old group; samples from the rats in the water-drinking group and the whey-drinking group ($n = 4$ each) were compared. Of 27,342 genes, 42 genes were up-regulated by at least 1.5-fold change with $FDR \leq 0.1$ in the whey-drinking group when compared to the water-drinking group (Table 2). The up-regulated genes included those encoding major salivary families of specific secretory proteins (Scarano *et al.*, 2010), i.e., PRP genes (*Prp2*, *Prol1*, *Prog1*, *Prp15*), cystatin genes (*Cyss*, *Vegp2*), and amylase gene (*Amy1a*). The expression of tissue-specific genes, i.e., kallikreins (*Klks3*, *Klk1c10*, *Klk1*) and kallikrein-related genes (*Ton*, *Klk11*, *Klk1c10l2*), was induced in the whey-drinking group. The levels of androgen-binding protein gene (*Abpa*) and androgen-related protein genes (*Smr3a*) were also increased in the whey-drinking group when compared to the water-drinking group.

Of 27,342 genes, 7 genes were down-regulated by at least 1.5-fold change with FDR ≤ 0.1 in the whey-drinking group when compared to the water-drinking group (Table 3). The down-regulated genes included those associated with lipid metabolism, transcription regulation, cancer and senescence.

In order to investigate the common genes showing fluctuating expression levels in the four sets, Venn diagram analysis was performed (Fig. 2 A). Each set comprised microarray data from the whey-drinking group vs. those from the water-drinking group. In the first (Fig. 2 B-a), second (Fig. 2 B-b), third (Fig. 2 B-c) and fourth (Fig. 2 B-d) sets, the whey-drinking group showed 512, 238, 473, and 938 entities with more than a 1.5-fold difference when compared to the water-drinking group, respectively. Among the four sets, 21 entities were overlapped (Fig. 2A). Because this array comprised more than 722,254 unique 25-mer oligonucleotide features constituting more than 27,342 probe sets, 21 entities represent 13 genes. The 13 genes are listed in Table 4. Transcription factor AP-2 gene (*Tcfap2b*), which is a salivary gland-specific transcription factor gene (Hu & Gallo, 2010), and androgen-binding protein alpha (*Abpa*), which is a pheromone gene (Karn & Laukaitis, 2009), are induced by whey-administration, suggesting that androgen-dependent transcription programs functioned together with a tissue-specific collaborating factor, AP-2, in whey-drinking rats.

3.4. qRT-PCR

In order to verify the results of the microarray analysis, selected genes with an expression level that differed by at least 1.5-fold between the whey-drinking group and the water-drinking group were confirmed using qRT-PCR. Whey administration induced an approximately 15-fold up-regulation of the *Cyss* and *Prb1* genes and an approximately 4-fold up-regulation of the *Tcfap2b* and *Abpa*. In addition, whey administration induced a 2-fold up-regulation of the *Amy1a* and *Lyz2* genes. All of the results of qRT-PCR analysis were consistent with the microarray expression profiles (Fig. 3).

3.5. Western blotting

Western blot analysis was used to quantify the changes in levels of selected proteins in the SLGs (Fig. 4 A) and saliva (Fig. 4 B) in response to whey-drinking. PRP, cystatin S and amylase levels in the SLGs were increased by 3-, 4.5- and 5-fold, respectively, in the whey-drinking group in comparison with the water-drinking group (Fig. 4A). Salivary PRP, cystatin S and amylase levels were increased by 2.5- to 3-fold in the whey-drinking group in comparison with the water-drinking group (Fig. 4B). These results suggested that whey-drinking prevented and/or restored the age-dependent functional decline in SLGs and saliva.

4. Discussion

This is the first study to report that whey prevented and recovered age-dependent atrophy and functional decline of the salivary glands in relation to gene expression. Saliva is a unique body fluid that continually bathes the oral cavity and larynx to aid in essential functions, such as mastication, oral microbial defense, gustation, lubrication, speech, deglutition, digestion, mineralization of teeth, and the protection of mucosal tissues (Humphrey & Williamson, 2001; Amerongen & Veerman, 2002). The fundamental functions of saliva are strictly connected to its composition that consists in a complex hypotonic aqueous solution containing proteins, peptides, enzymes, hormones, sugars, lipids and others (Messana *et al.*, 2008). Recent proteomic approaches have revealed that more than 2,400 proteins are contained in saliva (Castagnola *et al.*, 2011).

PRPs account for more than 60% of the weight of the total salivary proteome (Messana *et al.*, 2008). They have been divided into three classes: basic PRP, acidic PRP, and glycosylated PRP. Among the PRP genes induced by whey-drinking, *Prb1* belongs to the basic PRP group, *Prp2* and *Prp15* belong to the acidic PRP group and *Prpg1* belongs to the glycosylated PRP group. According to our results, the levels of PRPs were also increased in the SLGs and saliva by whey-drinking. Packaging of PRPs

into the secretory granules of salivary glands may be important in the reduction of osmotic activity during granule maturation (Castle & Castle, 1993). One of the biological roles of salivary PRPs is to protect epithelial cells against the toxic effects of food (Messana *et al.*, 2008).

Cystatins act as an inhibitor of cysteine proteinase and belong to an evolutionally related superfamily (Shaw & Chaparro, 1999). Cystatin S accounts for 8% of the weight of salivary proteins (Messana *et al.*, 2008). Von Ebner gland proteins (*Vegp2*) also act as an inhibitor of cysteine proteinase (Amerongen & Veerman, 2002). Coupled with the high level of *Cyss* gene expression in the whey-drinking group, the expression of cystatin S protein also increased in the SLGs and saliva due to whey administration. Salivary cystatin S plays a role in controlling proteolytic activity, either from the host or from microorganisms, to protect the oral cavity.

Salivary amylase accounts for approximately 20% of the weight of salivary proteins (Messana *et al.*, 2008). Amylase (*Amy1a*, salivary type) was induced in the SLGs of the whey-drinking group when compared to those of the water-drinking group.

Kallikreins, which are serine proteases, are secreted as inactive zymogen granules and are activated by the cleavage of an N-terminal peptide (Yousef & Diamandis, 2003). Approximately 13 kallikreins have been identified in rat. The expression of kallikrein genes is tissue-specific. *Klk1*, which is mainly expressed in the salivary glands, kidney and pancreas, plays a constitutive and/or developmental role in glands or renal physiology (Swift *et al.*, 1982; Clements *et al.*, 1990). *Klk1* and *Klk1*-related peptidase genes (*Klks3*, *Klk1c10*, *Klk1l*, *GK11*, *Klk1b21*, *Klk1c10l2*) were up-regulated by 1.6- to 11.5-fold in the SLGs of the whey-drinking group when compared to those of the water-drinking group. Tonin (*Ton*) is also a serine proteinase from the kallikrein family; it cleaves angiotensin II from angiotensin I, from the synthetic tetradecapeptide corresponding to the N-terminal segment of angiotensinogen and directly from angiotensinogen (Boucher *et al.*, 1977; Pacheco Dda *et al.*, 2013). There is 75% homology between the sequence of the γ -subunit of nerve growth factor and tonin (Lazure *et al.*,

1981). Tonin isolated from rat submandibular glands has also been reported to be very closely related to the epidermal growth factor-binding protein (Lazure *et al.*, 1981). These results suggest that kallikrein family proteins induced by whey-administration may prevent and/or restore age-dependent atrophy of SLGs.

Androgen-binding protein (*Abpa*), which is a member of the secretoglobin family (Jackson *et al.*, 2011), is expressed in the SLGs as well as the submandibular and parotid glands, and is then secreted into the saliva (Vandewege *et al.*, 2013). The secretoglobin family has an important role in modulating anti-inflammation, tissue repair, and tumor suppression and activation (Jackson *et al.*, 2011). This family of proteins has also been reported to repair dry eye (Versura *et al.*, 2010).

The AP-2 family of transcription factors consists of five members, *AP-2 α* , *AP-2 β* , *AP-2 γ* , *AP-2 δ* and *AP-2 ϵ* , each encoded by a separate gene (Eckert *et al.*, 2005). Administration of whey induced expression of the *AP-2 β* gene, but not the *AP-2 α* , *AP-2 γ* , *AP-2 δ* or *AP-2 ϵ* genes (Supplementary table 3). AP-2 is known as a tissue-specific collaborating factor and plays a critical role in the androgen-dependent transcription program (Hu & Gallo, 2010; Pihlajamaa *et al.*, 2014).

Cyss, one of the up-regulated genes in this study, has a potential AP-2 binding site (–194, TGGGGA) and androgen receptor (AR) response element which is the same as the glucocorticoid/progesterone response element in 5'-flanking region (Shaw & Chaparro, 1999). In androgen-dependent transcription programs, AR-binding is not sufficient to activate or repress the transcription and collaborating factors are necessary as AR modulators. More than 90% of up- or down-regulated genes by whey-administration in this experiment have an AR response element and AP-2 binding site in their promoter regions, suggesting that enhancement of AR-binding and promoter binding by AP-2 regulated the expression of genes associated with salivary proteins or lipid metabolism in salivary glands.

Dietary caloric restriction manipulates aging, maintaining health, and function (Roth & Ingram, 2015). Regardless of a caloric restriction, administration of whey alters

gene expression in elderly salivary glands and then prevents and/or restores age-dependent dysfunction of salivary glands, leading to maintain systemic health.

5. Conclusion

Comprehensive analysis of genetic profiles revealed that oral administration of whey induced the expression of *Abpa* and *Tcfap2b* in SLGs of elderly rats. Androgens transported through the cytoplasm by androgen binding proteins interact with AR in nuclei. AR and AP-2 beta binding to promoter of genes encoding salivary proteins and salivary gland homeostasis initiates the transcriptional responses. Through this process, bioactive components of whey restore atrophy and dysfunction of salivary glands in aged rats. Kallikrein family including tonin may prevent and/or restore age-dependent atrophy of SLGs. To our knowledge, this is the first study to report that whey-administration alters gene expression, thereby preventing and/or restoring age-dependent atrophy and functional decline in the salivary glands of aged rats.

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References

- Amerongen, A.V. & Veerman, E.C. (2002). Saliva--the defender of the oral cavity. *Oral diseases*, 8, 12-22.
- Boucher, R., Demassieux, S., Garcia, R. & Genest, J. (1977). Tonin, angiotensin II system. A review. *Circulation research*, 41, 26-29.

372 Castagnola, M., Picciotti, P.M., Messana, I., Fanali, C., Fiorita, A., Cabras, T., Calo, L.,
 373 Pisano, E., Passali, G.C., Iavarone, F., Paludetti, G. & Scarano, E. (2011.) Potential
 374 applications of human saliva as diagnostic fluid. *Acta otorhinolaryngologica*
 375 *Italica : organo ufficiale della Societa italiana di otorinolaringologia e chirurgia*
 376 *cervico-facciale*, 31, 347-357.
 377 Castle, A.M. & Castle, J.D. (1993). Novel secretory proline-rich proteoglycans from rat
 378 parotid. Cloning and characterization by expression in AtT-20 cells. *The Journal of*
 379 *biological chemistry*, 268, 20490-20496.
 380 Choi, J.S., Park, I.S., Kim, S.K., Lim, J.Y. & Kim, Y.M. (2013). Analysis of age-related
 381 changes in the functional morphologies of salivary glands in mice. *Archives of oral*
 382 *biology*, 58, 1635-1642.
 383 Clements, J.A., Matheson, B.A. & Funder, J.W. (1990). Tissue-specific developmental
 384 expression of the kallikrein gene family in the rat. *The Journal of biological*
 385 *chemistry*, 265, 1077-1081.
 386 Darre, L., Vergnes, J.N., Gourdy, P. & Sixou, M. (2008). Efficacy of periodontal
 387 treatment on glycaemic control in diabetic patients: A meta-analysis of
 388 interventional studies. *Diabetes & metabolism*, 34, 497-506.
 389 Eckert, D., Buhl, S., Weber, S., Jager, R. & Schorle, H. (2005). The AP-2 family of
 390 transcription factors. *Genome biology*, 6, 246.
 391 Gonsalves, W.C., Wrightson, A.S. & Henry, R.G. (2008). Common oral conditions in
 392 older persons. *American family physician*, 78, 845-852.
 393 Guggenheimer, J. & Moore, P.A. (2003). Xerostomia: etiology, recognition and
 394 treatment. *Journal of the American Dental Association*, 134, 61-69; quiz 118-119.
 395 Hu, Z. & Gallo, S.M. (2010). Identification of interacting transcription factors regulating
 396 tissue gene expression in human. *BMC genomics*, 11, 49.
 397 Humphrey, S.P. & Williamson, R.T. (2001). A review of saliva: normal composition, flow,
 398 and function. *The Journal of prosthetic dentistry*, 85, 162-169.

399 Hwang, K.A., Hwang, Y.J., Ha, W., Choo, Y.K. & Ko, K. (2012). Oral administration of
400 insulin-like growth factor-I from colostral whey reduces blood glucose in
401 streptozotocin-induced diabetic mice. *The British journal of nutrition*, 108, 39-45.

402 Inoue, N., Iida, H., Yuan, Z., Ishikawa, Y. & Ishida, H. (2003). Age-related decreases in
403 the response of aquaporin-5 to acetylcholine in rat parotid glands. *Journal of dental
404 research*, 82, 476-480.

405 Jackson, B.C., Thompson, D.C., Wright, M.W., McAndrews, M., Bernard, A., Nebert,
406 D.W. & Vasiliou, V. (2011). Update of the human secretoglobin (SCGB) gene
407 superfamily and an example of 'evolutionary bloom' of androgen-binding protein
408 genes within the mouse Scgb gene superfamily. *Human genomics*, 5, 691-702.

409 Kanda, A., Ishijima, T., Shinozaki, F., Nakayama, K., Fukasawa, T., Nakai, Y., Abe, K.,
410 Kawahata, K. & Ikegami, S. (2014). Post-exercise impact of ingested whey protein
411 hydrolysate on gene expression profiles in rat skeletal muscle: activation of
412 extracellular signal-regulated kinase 1/2 and hypoxia-inducible factor-1alpha. *The
413 British journal of nutrition*, 111, 2067-2078.

414 Kanda, A., Nakayama, K., Fukasawa, T., Koga, J., Kanegae, M., Kawanaka, K. &
415 Higuchi, M. (2013). Post-exercise whey protein hydrolysate supplementation
416 induces a greater increase in muscle protein synthesis than its constituent amino
417 acid content. *The British journal of nutrition*, 110, 981-987.

418 Karn, R.C. & Laukaitis, C.M. (2009). The mechanism of expansion and the volatility it
419 created in three pheromone gene clusters in the mouse (*Mus musculus*) genome.
420 *Genome biology and evolution*, 1, 494-503.

421 Kikutani, T., Tamura, F., Tashiro, H., Yoshida, M., Konishi, K. & Hamada, R. (2015).
422 Relationship between oral bacteria count and pneumonia onset in elderly nursing
423 home residents. *Geriatrics & gerontology international*, 15, 417-421.

424 Lazure, C., Seidah, N.G., Thibault, G., Boucher, R., Genest, J. & Chretien, M. (1981).
425 Sequence homologies between tonin, nerve growth factor gamma-subunit,

426 epidermal growth factor-binding protein and serine proteases. *Nature*, 292,
 427 383-384.

428 Le Maux, S., Giblin, L., Croguennec, T., Bouhallab, S. & Brodkorb, A. (2012).
 429 beta-Lactoglobulin as a molecular carrier of linoleate: characterization and effects
 430 on intestinal epithelial cells in vitro. *Journal of agricultural and food chemistry*, 60,
 431 9476-9483.

432 Messana, I., Inzitari, R., Fanali, C., Cabras, T. & Castagnola, M. (2008). Facts and
 433 artifacts in proteomics of body fluids. What proteomics of saliva is telling us?
 434 *Journal of separation science*, 31, 1948-1963.

435 Meurman, J.H., Sanz, M. & Janket, S.J. (2004). Oral health, atherosclerosis, and
 436 cardiovascular disease. Critical reviews in oral biology and medicine : an official
 437 publication of *the American Association of Oral Biologists*, 15, 403-413.

438 Pacheco Dda, F., Pacheco, C.M., Lima Mde, P., Bader, M., Souza Ade, L., Pesquero, J.L.,
 439 Castro Perez, A. & Duarte, I.D. (2013). Antinociceptive response in transgenic mice
 440 expressing rat tonin. *European journal of pharmacology*, 713, 1-5.

441 Pan, Y., Iwata, F., Wang, D., Muraguchi, M., Ooga, K., Ohmoto, Y., Takai, M., Cho, G.,
 442 Kang, J., Shono, M., Li, X.J., Okamura, K., Mori, T. & Ishikawa, Y. (2009).
 443 Identification of aquaporin-5 and lipid rafts in human resting saliva and their
 444 release into cevimeline-stimulated saliva. *Biochimica et biophysica acta*, 1790,
 445 49-56.

446 Pasin, G. & Comerford, K.B. (2015). Dairy Foods and Dairy Proteins in the
 447 Management of Type 2 Diabetes: A Systematic Review of the Clinical Evidence.
 448 *Advances in nutrition (Bethesda, Md.)*, 6, 245-259.

449 Pennings, B., Boirie, Y., Senden, J.M., Gijsen, A.P., Kuipers, H. & van Loon, L.J. (2011).
 450 Whey protein stimulates postprandial muscle protein accretion more effectively
 451 than do casein and casein hydrolysate in older men. *The American journal of*
 452 *clinical nutrition*, 93, 997-1005.

453 Pihlajamaa, P., Sahu, B., Lyly, L., Aittomaki, V., Hautaniemi, S. & Janne, O.A. (2014).
454 Tissue-specific pioneer factors associate with androgen receptor cistromes and
455 transcription programs. *The EMBO journal*, 33, 312-326.

456 Proctor, G.B. & Carpenter, G.H. (2007). Regulation of salivary gland function by
457 autonomic nerves. *Autonomic neuroscience : basic & clinical*, 133, 3-18.

458 Roth, G.S. & Ingram, D.K. (2015). Manipulation of health span and function by dietary
459 caloric restriction mimetics. *Annals of the New York Academy of Sciences*. 1-5.

460 Scarano, E., Fiorita, A., Picciotti, P.M., Passali, G.C., Calo, L., Cabras, T., Inzitari, R.,
461 Fanali, C., Messana, I., Castagnola, M. & Paludetti, G. (2010). Proteomics of saliva:
462 personal experience. *Acta otorhinolaryngologica*, 30, 125-130.

463 Shaw, P.A. & Chaparro, O. (1999). The 5'-flanking sequence and regulatory elements of
464 the cystatin S gene. *Biochemical and biophysical research communications*, 261,
465 705-711.

466 Ship, J.A., Pillemer, S.R. & Baum, B.J. (2002). Xerostomia and the geriatric patient.
467 *Journal of the American Geriatrics Society*, 50, 535-543.

468 Sreebny, L.M. (2000). Saliva in health and disease: an appraisal and update.
469 *International dental journal*, 50, 140-161.

470 Sun, A., Wu, K.M., Wang, Y.P., Lin, H.P., Chen, H.M. & Chiang, C.P. (2013). Burning
471 mouth syndrome: a review and update. *Journal of oral pathology & medicine :*
472 official publication of *the International Association of Oral Pathologists and the*
473 *American Academy of Oral Pathology*, 42, 649-655.

474 Swift, G.H., Dagorn, J.C., Ashley, P.L., Cummings, S.W. & MacDonald, R.J. (1982). Rat
475 pancreatic kallikrein mRNA: nucleotide sequence and amino acid sequence of the
476 encoded preproenzyme. *Proceedings of the National Academy of Sciences of the*
477 *United States of America*, 79, 7263-7267.

478 Tusher, V.G., Tibshirani, R. & Chu, G. (2001) Significance analysis of microarrays
479 applied to the ionizing radiation response. *Proceedings of the National Academy of*
480 *Sciences of the United States of America*, 98, 5116-5121.

Vandewege, M.W., Phillips, C.J., Wickliffe, J.K. & Hoffmann, F.G. (2013). Evolution of the ABPA subunit of androgen-binding protein expressed in the submaxillary glands in New and Old World rodent taxa. *Journal of molecular evolution*, 76, 324-331.

Versura, P., Nanni, P., Bavelloni, A., Blalock, W.L., Piazzzi, M., Roda, A. & Campos, E.C. (2010). Tear proteomics in evaporative dry eye disease. *Eye* (London, England), 24, 1396-1402.

Xiao, R., Badger, T.M. & Simmen, F.A. (2005). Dietary exposure to soy or whey proteins alters colonic global gene expression profiles during rat colon tumorigenesis. *Molecular cancer*, 4, 1.

Yousef, G.M. & Diamandis, E.P. (2003). Tissue kallikreins: new players in normal and abnormal cell growth? *Thrombosis and haemostasis*, 90, 7-16.

Figure Legends

Fig. 1. Effect of whey- or water-drinking on the morphology of the sublingual glands (SLGs). Eight-week-old rats were given water (A) or whey (B) for 1 month. Eighty-four-week-old rats were given water (C) or whey (D) for 1 month. Bar, 10 μ m.

Fig. 2. Venn diagram and scatterplot analysis. Using a Venn diagram (A), overlapping genes were analyzed among four sets. One set comprised microarray data from a whey-drinking elderly rat (OJ) vs. those from a water-drinking elderly rat (OC). Scatterplot analysis (B) of genes that were differentially expressed by more than 1.5-fold between the whey-drinking group and the water-drinking group. Four separate experiments were performed. In B-a, B-b, B-c, and B-d there were 512, 238, 473, and 938 entities, respectively, with more than a 1.5-fold difference between the whey-drinking group and the water-drinking group.

Fig.3. Effects of whey-drinking on the mRNA levels analyzed by qRT-PCR. The total RNA obtained from SLGs from 84-week-old rats given water or whey. Mean values with standard errors of relative mRNA expression compared to water-drinking group are shown. n=3, *p<0.05, **p<0.01, ***p<0.001.

Fig.4. Effects of whey-drinking on proline-rich protein (PRP), cystatin S, and amylase levels in SLGs and saliva. SLGs (A) and saliva (B) samples were treated with Laemmli sample buffer and SLGs sample with 15 μ g of protein or 15 μ l saliva were subjected to SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-PRP antibody (a), anti-cystatin S antibody (b), and anti-amylase antibody (c). A typical western blot is shown in photography. Densitometric analysis was expressed in graphs as the relative amount of chemiluminescence in whey-drinking group vs. water-drinking. Mean values with

535 of three separate experiments standard error were shown. $n=3$, $*p<0.05$, $**p<0.01$,
536 $***p<0.001$.
537

Table 1. Body weight, drinking volume, salivary gland weight, salivary volume and salivary protein concentration levels in water-drinking group and whey-drinking group.

		Young rats		Elderly rats	
		Water	Whey	Water	Whey
Body weight	Initial	293±3 (12-week)	294±6 (12-week)	494±5 (84-week)	489±5 (84-week)
	Final	327±17 (16-week)	315±1 (16-week)	489±5 (88-week)	479±2 (88-week)
Drinking volume (g/day)		22.3±0.5	24.1±1.4	25.7±0.8	27.1±0.5
Salivary gland weight (mg)	Parotid gland	196±4	197±8 **	152±7	149±7
	Submandibular gland	255±5	252±8	252±10	273±6
	Sublingual gland	48.4±4.5	48.5±0.8	46.0±1.2	54.9±1.7 **
Salivary volume (mg/10-20min)		296±13	345±15 *	180±12	313±6 **
Salivary protein concentration (mg/ml)		9.67±0.11	9.50±0.09	7.64±0.68	9.99±0.46 *

Data represent means ± SE (n=6).

*p<0.05, **p<0.01

Table 2. Genes up-regulated by >1.5-fold in SLGs from whey-drinking rats compared to those from water-drinking rats

Genedescription	Genesymbol	Fold change	Accession no.	p-value	FDR
Major salivary protein genes					
Proline-rich proteins					
proline rich, lacrimal 1	Prol1	8.66	NM_133513	0.035	0.102 [#]
proline rich protein 2	Prp2	7.20	NM_001013211	0.060	0.102 [#]
proline-rich proteoglycan 1	Prpg1	3.06	NM_172064	0.017	0.102 [#]
proline-rich protein 15	Prp15	2.81	NM_012632	0.033	0.102 [#]
proline-rich protein BstNI subfamily 1	Prb1	1.72	M83567	0.175	0.196
Cystatin proteinase inhibitor genes					
cystatin S	Cyss	25.85	NM_198685	0.003	0.054 [#]
von Ebners gland protein 2	Vegp2	2.16	NM_053574	0.075	0.103 [#]
Amylase					
amylase, alpha 1A (salivary)	Amy1a	3.27	NM_001010970	0.056	0.102 [#]
Salivary gland homeostasis protein genes					
Serine endopeptidases					
tonin	Ton	15.66	NM_012677	0.052	0.102 [#]
kallikrein, submaxillary gland S3	Klks3	11.44	NM_175759	0.060	0.102 [#]
T-kininogenase	Klk1c10	10.63	NM_001135173	0.071	0.102 [#]
kallikrein 1	Klk1	6.90	NM_001005382	0.062	0.102 [#]
kallikrein 1-like peptidase	Klk1l	3.92	NM_012593	0.041	0.102 [#]
glandular kallikrein 11	Gk11	2.81	NM_001003977	0.142	0.162
kallikrein 1-related peptidase b21	Klk1b21	2.17	NM_001013067	0.050	0.102 [#]
kallikrein 1-related peptidase c10-like 2	Klk1c10l2	1.62	L33840	0.069	0.102 [#]
Androgen related protein genes					
submaxillary gland androgen regulated protein 3A	Smr3a	10.68	NM_001017497	0.071	0.102 [#]
androgen binding protein, alpha	Abpa	4.98	NM_001100859	0.035	0.102 [#]
ABP beta	LOC494538	4.71	GU269241	0.033	0.102 [#]
similar to androgen-binding protein	LOC689199	1.73	NM_001170457	0.181	0.196

eta					
androgen binding protein zeta	Abpz	1.68	GU269242	0.190	0.200
Transcriptional regulator genes					
transcription factor AP-2 beta	Tcfap2b	2.13	NM_001106896	0.003	0.054 [#]
Signal transduction genes					
phosphodiesterase 3A, cGMP inhibited	Pde3a	1.67	NM_017337	0.002	0.054 [#]
Minor salivary protein genes					
prolactin induced protein	Pip	6.38	NM_022708	0.035	0.102 [#]
glutamine/glutamic acid-rich protein A	Grpca	5.91	NM_181440	0.055	0.102 [#]
cytidine monophosphate-N-acetylneuraminic acid hydroxylase	Cmah	4.96	NM_001024273	0.079	0.105
alpha-2u globulin PGCL4	Obp3	4.59	NM_147215	0.050	0.102 [#]
alpha-2u globulin PGCL2	LOC298116	4.45	NM_001033959	0.051	0.102 [#]
alpha-2u globulin PGCL3	LOC259244	4.37	NM_147212	0.057	0.102 [#]
salivary protein 1	Spt1	4.35	NM_019171	0.069	0.102 [#]
major urinary protein 5	Mup5	4.35	NM_203325	0.044	0.102 [#]
major urinary protein 4	Mup4	4.24	NM_198784	0.050	0.102 [#]
alpha-2u globulin PGCL5	LOC259245	4.08	NM_147213	0.049	0.102 [#]
variable coding sequence A1	Vcsa1	3.83	NM_012684	0.102	0.121
alpha2u globulin	LOC298111	3.73	NM_001024248	0.051	0.102 [#]
variable coding sequence A2	Vcsa2	3.54	NM_198729	0.021	0.102 [#]
alpha-2u globulin PGCL1	LOC259246	3.30	NM_147214	0.069	0.102 [#]
ABO blood group	Abo	3.04	NM_023094	0.088	0.112
chemokine (C-X-C motif) ligand 13	Cxcl13	1.63	NM_001017496	0.293	0.293
lactoperoxidase	Lpo	1.60	NM_001105829	0.010	0.102 [#]
Other genes					
RGD1559532	RGD1559532	3.43	NM_001024983	0.036	0.102 [#]
carbonic anhydrase 6	Car6	2.77	NM_001134841	0.020	0.102 [#]
claudin 22	Cldn22	2.66	NM_001110143	0.081	0.106
triadin	Trdn	2.27	NM_021666	0.100	0.122
glycosylation dependent cell adhesion molecule 1	Glycam1	2.09	NM_012794	0.265	0.270

epidermal growth factor	Egf	1.91	NM_012842	0.038	0.102 [#]
angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Agt	1.87	NM_134432	0.194	0.201
similar to lipase-like, ab-hydrolase domain containing 2	RGD1565682	1.83	XM_008760354	0.033	0.102 [#]
carbonic anhydrase II	Car2	1.77	NM_019291	0.051	0.102 [#]
similar to 9530008L14Rik protein	RGD1305679	1.72	BC088287	0.023	0.102 [#]
similar to protein of unknown function	RGD1566243	1.69	NM_001134635	0.037	0.102 [#]
melanoma inhibitory activity	Mia	1.64	NM_030852	0.039	0.102 [#]
solute carrier family 6 (neurotransmitter transporter)	Slc6a14	1.63	NM_001037544	0.096	0.119
solute carrier family 37 (glycerol-3-phosphate transporter)	Slc37a2	1.55	NM_001191994	0.073	0.103 [#]
coiled-coil domain containing 129	Ccdc129	1.53	NM_001191973	0.042	0.102 [#]
scavenger receptor class A, member 3	Scara3	1.50	NM_001108870	0.135	0.157

[#]FDR $\leq$ 0.1

Table 3. Genes down-regulated by >1.5-fold in SLGs from whey-drinking rats compared to those from water-drinking rats

Genedescription	Genesymbol	Fold change	Accession no.	p-value	FDR
Lipid metabolism related genes					
similar to fatty aldehyde dehydrogenase-like	LOC688778	-2.85	XM_008760188	0.002	0.000 [#]
prostaglandin D receptor-like	Ptgdr1	-1.51	NM_022241	0.019	0.037 [#]
Transcriptional regulator related genes					
forkhead box P2	Foxp2	-1.55	XM_002729284	0.003	0.013 [#]
Cancer related genes					
hemopexin	Hpx	-1.58	NM_053318	0.072	0.090 [#]
protease, serine, 37	Prss37	-1.52	NM_001108625	0.005	0.016 [#]
Signal transduction genes					
synaptosomal-associated protein 25	Snap25	-2.18	NM_030991	0.118	0.131
immediate early response 3	Ier3	-1.53	NM_212505	0.007	0.016 [#]
Immune response					
mCG127631-like	LOC366766	-1.71		0.021	0.041 [#]
pre-B lymphocyte 3-like	LOC682821	-1.51	NW_001084890	0.301	0.301
Senescence related genes					
cysteine-rich, angiogenic inducer, 61	Cyr61	-1.96	NM_031327	0.029	0.408

[#]FDR ≤ 0.1

Table 4. List of overlapping genes in a Venn diagram

Genedescription	Genesymbol	Fold change	Accession no.
Secretory proteins			
cystatin S	Cyss	25.85	NM_198685
alpha-2u globulin PGCL1	LOC259246	3.30	NM_147214
alpha-2u globulin PGCL2	LOC298116	4.45	NM_001033959
alpha-2u globulin PGCL3	LOC259244	4.37	NM_147212
alpha-2u globulin PGCL4	Obp3	4.59	NM_147215
alpha-2u globulin PGCL5	LOC259245	4.08	NM_147213
major urinary protein 4	Mup4	4.24	NM_198784
major urinary protein 5	Mup5	4.35	NM_203325
Homeostasis proteins			
kallikrein 1	Klk1	6.90	NM_001005382
androgen binding protein, alpha	Abpa	4.98	NM_001100859
transcription factor AP-2 beta	Tcfap2b	2.13	NM_001106896
similar to fatty aldehyde dehydrogenase-like	LOC688778	-2.85	XM_008760188
pre-B lymphocyte 3-like	LOC682821	-1.51	NW_001084890

Fig.1

Fig. 2

A

Entity List 1 : [OJ1] vs [OC1]
Fold change ≥ 1.5
512 entities

Entity List 4 : [OJ4] vs [OC4]
Fold change ≥ 1.5
938 entities

Entity List 2 : [OJ2] vs [OC2]
Fold change ≥ 1.5
238 entities

Entity List 3 : [OJ3] vs [OC3]
Fold change ≥ 1.5
473 entities

B

Fig.3

FIG. 4.