Sex-Based Differences in *Smgc* Expression in the Submandibular Gland of C57BL/6 Mice

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Key Words

Sex-based differences · Salivary proteins · Estrogen · Ovariectomy · Menopause

submandibular gland. Our findings suggest that salivary proteins have potential as markers for evaluating therapies for menopausal symptoms.

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Abstract

Objective: The decrease in female hormone levels at menopause affects whole-body homeostasis. Various therapies including hormone therapy and treatment with herbal supplements are available to improve menopausal symptoms. However, a method for evaluating their effectiveness has not been established. We sought to identify useful biomarkers to assess therapy efficacy. **Methods:** We searched for salivary proteins affected by changes in female hormone levels in mouse submandibular glands. **Results:** The expression of submandibular gland protein C (*Smgc*) was decreased following ovariectomy, while the expression of the alternative splicing transcript *t-Smgc* was increased. Notably, *Smgc* expression increased following β -estradiol administration, and was barely detectable in the submandibular glands of male mice. **Conclusion:** The results suggest that *Smgc* expression may be estrogen dependent. Moreover, changes in the SMGC protein amount in the saliva were in accordance with those in mRNA expression in the

Introduction

Female hormones such as estrogen and progesterone are important for menstruation and maintaining homeostasis. Estrogen production dramatically decreases at menopause, causing symptoms including hot flushes, night sweats, dry skin and mucosa, and osteoporosis. Hormone therapy is a well-established treatment for menopausal symptoms; however, adverse effects of female hormones have limited their widespread use. Alternative treatments such as herbal supplements are available; however, there is no useful method to evaluate their effectiveness.

Biomarkers are important tools for evaluating medical therapies. Molecules with activity regulated by certain therapies are especially useful for assessing therapy efficacy; for example, various molecules have been identified as biomarkers for cancer treatments [1, 2].

Table 1. Primer sets for RT-PCR

Gene	Primer sequence	Gene bank accession No.
<i>Smgc</i>	5'-ACAGTCTCTACACTTAGGTCCCA-3' (+9 to +23) 5'-ACTTGTGATCGGTTTCGACTATT-3' (+716 to +695)	NM_198927
<i>t-Smgc</i>	5'-ACAGTCTCTACACTTAGGTCCCA-3' (+9 to +23) 5'-GGATGACCAGTCACAAACACTA-3' (+2,646 to +2,625)	NM_198927
<i>Muc19</i>	5'-GATTATGCGATTGGTTCATCCT-3' (+22,175 to +22,196) 5'-GTGCAATGTCCCTGAACTCATA-3' (+22,523 to +22,502)	NM_207243
<i>GPDH</i>	5'-ATGGCGTTTCAAAAGGCAGT-3' (+320 to +339) 5'-AATCCTCCACAACACTACGGTC-3' (+2,499 to +2,480)	NM_001145820

Estrogen regulates the expression of multiple target genes known as estrogen-responsive genes, including those encoding efp (estrogen-responsive finger protein) [3], prolactin [4], pS2 [5] and uteroglobin [6]. Estrogen binds to estrogen receptors (ERs), which are members of the nuclear receptor superfamily and act as ligand-dependent transcription factors to regulate target gene expression by directly binding to specific DNA sequences called estrogen response elements (EREs) [7]. Incomplete EREs or ERE half-sites are regulated by other transcription factors, including AP-1 (activating protein 1) [8], Sp1 (specificity protein 1) [9] and NF- κ B (nuclear factor- κ B) [10]. Most estrogen-responsive genes have been found in the brain [11], bone marrow [12], heart [13] and other tissues. These organs can only be accessed by invasive approaches, preventing the development of a method to easily evaluate the effectiveness of menopause treatments.

A potential noninvasive approach for the evaluation of hormone therapy is the use of salivary samples. Salivary glands exhibit sexual dimorphism; volumes of the parotid and submandibular glands are reportedly larger in males, as is the saliva flow rate [14]. Dry mouth often occurs in women experiencing menopause [15], and most sex hormones are present in saliva at levels that correlate with those in blood [16, 17]. Moreover, salivary glands are reported to express ERs [18, 19]. Unfortunately, there is a dearth of information regarding the specific molecules regulated by estrogen and ERs in the salivary system.

Here, we attempted to identify estrogen-responsive genes in the mouse submandibular glands and saliva. This led to the identification of the submandibular gland protein C gene (*Smgc*) as an estrogen-responsive gene that could be used to evaluate female hormone therapy and its alternatives in mice.

Materials and Methods

Experimental Animals

Seven-week-old male and female C57BL/6J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan) and fed modified AIN-93G containing corn oil instead of soybean oil to avoid isoflavones, which are phytoestrogens. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the National Food Research Institute (NARO; approval No. H24-009, H25-014, H26-019). The mice were killed by cervical dislocation, and their submandibular glands were extirpated and used for preparing RNA for reverse transcription-polymerase chain reaction (RT-PCR) and for preparing protein samples. The extirpations were carried out in the laboratory registered with the NARO IACUC.

Ovariectomy and Subsequent Estrogen Therapy

Female mice were sham-treated or ovariectomized (OVX) under isoflurane anesthesia. For one experiment, 12 OVX mice were divided into 2 groups: the first group was treated with daily subcutaneous 17 β -estradiol injections (0.6 μ g $\cdot$ kg⁻¹ $\cdot$ day⁻¹; Sigma-Aldrich, St. Louis, Mo., USA), beginning 1 week after OVX, and the other group served as a control. 17 β -estradiol was dissolved in DMSO at 2.5 μ g/ml and mixed with phosphate-buffered saline before use.

Saliva Collection

Three weeks after surgery, the mice were weighed and anesthetized with an intraperitoneal injection of pentobarbital (36 mg/kg). Pilocarpine (0.1 mg/kg) was intraperitoneally injected to stimulate salivation, and saliva secreted into the oral cavity was collected by capillary action using disposable glass capillary pipettes (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany).

RNA Extraction and cDNA Microarray Analysis

Total RNA was extracted from the submandibular gland using the illustra RNAspin kit (GE Healthcare UK Ltd., Amersham, UK) and used for cDNA microarray analysis and RT-PCR. First-strand cDNA was reverse transcribed from 500 ng total RNA from the submandibular glands using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, Calif., USA). The microarray analysis was performed with a GeneChip[®] 3' IVT Express Kit (Affymetrix Japan KK, Tokyo, Japan) as reported previously [20].

Fig. 1. Expression of the *Smgc*/*Muc19* gene in mouse submandibular glands. mRNA levels of *Smgc*, *t-Smgc* and *Muc19* were quantified by semiquantitative RT-PCR. **a** Representative results of *Smgc*, *t-Smgc* and *Muc19* mRNA in the submandibular glands of sham-treated mice (WT), OVX mice and male mice. *GPDH* served as the

internal control. **b** The bar graph shows the average relative amplicon band intensity as determined by densitometry ($n = 6-25$). * $p < 0.001$, ** $p < 0.01$ compared with sham-treated mice (one-way ANOVA followed by Dunnett's test). Values are expressed as means $\pm$ SEs.

RT-PCR

The primers were synthesized based on published sequences (table 1). RT-PCR was performed using 25 ng of total RNA, 10 μ M of each primer, and rTaq DNA polymerase (TaKaRa Bio, Otsu, Japan). After 3 min at 95°C, 20–40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min were performed, followed by a final extension at 72°C for 7 min. Amplified sequences were visualized by gel electrophoresis on a 0.8% agarose gel and quantified using the CS Analyzer software (ATTO, Tokyo, Japan).

Western Blot Analysis

Frozen submandibular glands were homogenized and lysed using CelLytic MT (Sigma-Aldrich). Saliva samples were directly mixed with NuPAGE LDS sample buffer (Life Technologies). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were performed as described previously [21]. A polyclonal antibody for SMGC (Everest Biotech Ltd., Upper Heyford, UK) was used at a dilution of 1:1,000 for saliva samples and of 1:5,000 for salivary gland samples, which were subsequently incubated with horseradish peroxidase-linked anti-

goat IgG (1:2,500; GE Healthcare UK). The signals were observed with LightCapture (ATTO) and quantitation was carried out using the CS Analyzer software.

Plasmid Construction

The different regions of the *Smgc* promoter were cloned into the luciferase reporter vector pNL1.1 (Promega, Madison, Wis., USA). XETL [22] subcloned into the pNL1.1 vector was used as a positive control.

Luciferase Assay

The HEK 293 cell line (Life Technologies) was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Life Technologies) at 37°C in a 5% CO₂ humidified incubator. For all experiments, 1.67 ng/ μ l plasmid DNA was used for transfection. The reporter plasmids were transfected using Lipofectamine LTX (reagent:DNA, 3:1; Life Technologies). 17 β -estradiol (100 nM) was added 24 h after the transfection. The luciferase assay was carried out after 24 h of 17 β -estradiol treatment using the Nano-Glo-Luciferase Reporter Assay system (Promega).

Fig. 2. SMGC protein expression in saliva and the submandibular gland. **a** Representative Western blot of SMGC expression in saliva and the submandibular gland. The arrow and arrowheads indicate SMGC protein and nonspecific bands, respectively. Total protein (lane 1, 24 µg from saliva; lane 2, 8 µg from saliva; lane 3, 4.5 µg from the submandibular gland; lane 4, 0.9 µg from the submandibular gland) was analyzed for each sample. **b** SMGC protein

expression in saliva and the submandibular glands of sham-treated mice (WT), OVX mice and male mice. Total protein from saliva (24 µg) and the submandibular gland (4.5 µg) were analyzed. The arrows indicate SMGC protein. **c** Relative protein levels of SMGC in saliva and the submandibular gland (n = 6–9). The average WT band intensity was normalized to 1. Values are expressed as means ± SEs.

Statistical Analysis

Quantitative RT-PCR, Western blot and luciferase assay data were compared with one-way analysis of variance (ANOVA) followed by Dunnett's test using the SigmaPlot software (Systat Software Inc., San Jose, Calif., USA). $p < 0.05$ was considered significant.

Results

Identification of Estrogen-Responsive Genes in the Submandibular Glands

To identify proteins with an estrogen concentration-dependent expression, we compared gene expressions in the submandibular glands of OVX and sham-treated C57BL/6J mice using cDNA microarray technology. We

selected the genes of which the average expression differed by more than 1.8-fold between the sham and OVX groups, annotated the selected 85 genes, and found the gene for SMGC to be highly expressed in the submandibular glands.

Smgc Expression Is Estrogen Dependent in the Submandibular Glands

SMGC protein is a product of the *Smgc/Muc19* gene that encodes three transcripts: *Smgc*, truncated *Smgc* (*t-Smgc*) and *mucin 19* (*Muc19*) [23, 24]. *Smgc* is expressed in terminal tubule cells, a type of transient secretory cell, in the rat submandibular glands [25, 26]. *Muc19* is a member of the mucin family [27]. Mucins are glycoproteins responsible for mucus viscosity and elasticity. *Smgc/Muc19*

is composed of 60 exons: *Smgc* is encoded by exons 1–18, *Muc19* is encoded by exons 1 and 19–60 [26], and the truncated splice variant *t-Smgc* is encoded by exons 1, 17 and 18. *Smgc* is reportedly expressed during embryonic and early postnatal stages in rats and mice [23, 25], whereas *t-Smgc* is expressed in both neonatal and adult salivary glands [28]. To verify that these transcripts are related to estrogen, we performed RT-PCR with RNA from submandibular glands excised from OVX and sham-treated female and male mice (fig. 1). RT-PCR revealed that *Smgc* expression was significantly reduced by OVX and was minimal in the submandibular glands of male mice. We used a primer pair for *t-Smgc* capable of detecting both *Smgc* and *t-Smgc* expression. RT-PCR analysis with this primer pair showed that *t-Smgc* and *Smgc* expressions were significantly increased and decreased, respectively, in the submandibular glands of OVX mice. These results suggested that OVX may produce alternative splicing. The primer pair for *t-Smgc* was not able to detect *Smgc* in male mice. *Muc19* expression was higher in male mice than in sham-treated and OVX female mice. No significant difference between WT and OVX female mice was noted, likely due to interindividual differences in *Muc19* expression.

SMGC Protein Is Expressed in Saliva and the Submandibular Glands

Next, we analyzed *Smgc* expression at the protein level by Western blotting with samples from saliva and the submandibular glands (fig. 2). First, we determined the specificity of the anti-SMGC antibody using samples from sham-treated female C57BL/6J mice. A 70-kDa band was detected in both sample types; extra bands were detected at 50 kDa in saliva and at 65 kDa in the submandibular glands (fig. 2a). Because the calculated size of SMGC is 74.4 kDa, we selected the 70-kDa bands as the SMGC-specific bands. Expression of the SMGC protein was lower in male mice than in sham-treated female mice in both saliva and the submandibular glands (fig. 2b, c). SMGC protein expression in the saliva of OVX mice was lower than in that of sham-treated mice; SMGC levels were approximately equal in the submandibular glands of both groups. Western blot analysis did not reveal significant differences among the sample types.

Smgc Expression Is Regulated by β -Estradiol in the Submandibular Glands

The RT-PCR and Western blot results suggested that *Smgc* expression is regulated by estrogen. To confirm estrogen-dependent expression, RT-PCR and Western blot analyses were carried out for OVX mice subcutaneously

Fig. 3. Effect of estradiol on *Smgc* mRNA expression in the submandibular glands of OVX mice. **a** Representative RT-PCR analysis results of *Smgc* and *t-Smgc* mRNA expression in the submandibular glands of sham-treated mice (WT), OVX mice and β -estradiol-treated OVX mice (OVX+E2). *GPDH* served as the internal control. **b** The bar graph shows the average relative amplicon band intensity determined by densitometry (n = 9–12). * p < 0.05 compared with OVX mice (one-way ANOVA followed by Dunnett's test). Values are expressed as means $\pm$ SEs.

injected with 17 β -estradiol (fig. 3, 4). *Smgc* mRNA expression in the submandibular glands significantly increased with 17 β -estradiol, while *t-Smgc* mRNA expression decreased slightly (fig. 3). The level of SMGC protein in both saliva and the submandibular gland also tended to increase, albeit insignificantly, with 17 β -estradiol (fig. 4).

To evaluate whether the expression of *Smgc* and *t-Smgc* is regulated by β -estradiol, we performed a sequence analysis of the *Smgc/Muc19* promoter region (5 kbp upstream of the transcription start site). As shown in figure 5a, the *Muc19/Smgc* promoter region contains five ERE half-sites. In the region 450 bp upstream of the transcription start site, an ERE half-site, an AP-1 site and two SP1 sites were identified; however, this region lacks full EREs (fig. 5b). Luciferase assays of this promoter region showed that the promoter activity tended to decrease by deletion of the AP-1 site (fig. 5c).

Fig. 4. Effect of β -estradiol on SMGC protein expression in the submandibular glands of ovariectomized mice. **a** Representative results for SMGC protein expression in saliva and the submandibular glands of sham-treated mice (WT), OVX mice and β -estradiol-treated OVX mice (OVX+E2) assessed by Western blot analysis. Total protein from saliva (24 μ g) and the submandibular gland (4.5 μ g) were analyzed. **b** Relative protein levels of SMGC in saliva and the submandibular glands ($n = 4-9$). The average WT band intensity was normalized to 1. Values are expressed as means $\pm$ SEs.

Discussion

In this study, we aimed to identify estrogen-responsive genes in the salivary system and found that *Smgc* expression was estrogen level dependent. In the process of annotating the selected genes, we found that cysteine-rich secretory protein 1 and demilune cell and parotid protein 3 genes are also expressed in the salivary glands. However, one-way ANOVA did not reveal any significant differences in gene expression, probably because the submandibular glands are comprised of heterogeneous cell populations.

Discrepant results have been reported for *Smgc* expression in adult mouse submandibular and sublingual glands. Zinzen et al. [23] showed that female but not male C57BL/6J adult mice express *Smgc* in the submandibular glands. Conversely, Culp et al. [24] reported that *Smgc* is expressed during the neonatal and early postnatal stages in the sublingual gland of NFS/NCr mice but not in the submandibular or sublingual glands of adult male and female mice. In the present study, we found that *Smgc* expression is affected by estrogen in female C57BL/6J mice. The differences in these results can be attributed to the different genetic backgrounds used. Some NFS/N substrains have been reported to possess an abnormal salivary system. For example, the sublingual glands of a mutant substrain, NFS/*sld*, exhibit arrested differentiation in the sublingual glands [29]. NFS/*sld* mutant mice are used as an animal model for

primary Sjögren's syndrome [30]. These studies suggest that the NFS strain may have a genetic predisposition to unusual salivary system development.

We found a clear inverse correlation between *Smgc* and *t-Smgc* mRNA expression. In addition, we found an ERE half-site, AP-1 site and SP1 site within the 450-bp region upstream of the transcription start site. However, luciferase assays showed that the role of this region in the responsiveness to β -estradiol is very limited. This result suggests that female hormones might regulate *Muc19/Smgc* gene expression through as yet unknown regulatory systems. We did not analyze *Smgc* and *t-Smgc* expression in developmental stages because high *Smgc* expression has been reported during salivary gland development in both C57BL/6J and NFS/NCr mice [25, 28]. This high expression was observed irrespective of the sex and genetic background of the mice. Estradiol levels are higher in rat embryonic tissues than in newborn tissues [31], and plasma estradiol levels are high in both male and female neonatal rats, while the levels decrease with growth. Taken together, these findings suggest that estradiol likely drives high *Smgc* expression in the embryonic and neonatal salivary glands as it does in adult female mice.

We did not observe a coordinated expression of *Smgc* and *Muc19*. Das et al. [28] showed that the expression level of *t-Smgc* correlates with that of *Muc19*. We found similar results in male mice, but OVX mice exhibited low *Muc19* expression despite high *t-Smgc* expression. More-

Fig. 5. Transcription activity of the *Smgc/Muc19* promoter region. **a** Schematic representation of the region upstream of the *Smgc/Muc19* gene showing the locations of putative transcription factor binding sites. **b** Schematic representation of the region upstream of the *Smgc/Muc19* gene used for the reporter assay in **c**. **c** Luciferase assays. The luciferase reporter vectors are shown in the left panel. Quantitation of the luciferase expression is shown in the right panel. All the transfections were repeated more than 4 times, and the results are expressed as means $\pm$ SDs. RLU = Relative light units; tk = thymidine kinase; Nluc = NanoLuc luciferase; E2 = 17 β -estradiol.

over, the individual differences in *Muc19* expression were high in our study. The inconsistent expression data could possibly be attributed to the PCR primer design. Additional studies are needed to clarify this issue.

Our study has several limitations. The most significant is that there is no human homologue of *Smgc*. Still, it may be a useful biomarker for mouse studies designed to assess menopause therapies. Furthermore, our microarray analysis did not reveal any gene clusters regulated by female hormones because individual differences were observed in the expression levels of most genes. If such clusters exist, there might be additional potential marker genes that are common to both mice and humans.

In our study, estrogen-based differences in SMGC expression between male and female mice were detectable at the protein level in saliva and the submandibular glands. We found a similar difference at the protein level in saliva between OVX and sham-treated female mice, but it was not significant. Moreover, the amount of

SMGC protein in the submandibular gland of OVX mice was higher than in sham-treated female mice, although not significantly. The SMGC protein level in the submandibular gland of sham-treated female mice is not necessarily higher than that in OVX mice because SMGC in the submandibular gland is constantly secreted into the oral cavity along with saliva, while *Smgc* mRNA is not. The anti-SMGC antibody used in our study had low sensitivity and specificity for quantitatively detecting SMGC protein, so further studies are needed to clarify its expression.

Estrogen regulates the expression of a wide array of genes and affects various organs. However, the effects of estrogen in most organs cannot be easily evaluated because it is difficult to sample tissues. Saliva is a biological sample that is relatively easily and painlessly obtained. Interestingly, estrogen-regulated gene expression reportedly occurs in the salivary gland [32]. In this study, we demonstrated estrogen-dependent SMGC protein

expression in saliva samples and *Smgc* expression in the mouse submandibular gland, with sex-dependent differences. Our results suggest the existence of female-specific salivary proteins, the levels of which could be measured to evaluate the effects of estrogen or phytoestrogen.

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