Localization of the Candidate Genes \textit{ELOVL5} and \textit{SCD1} for ‘Male Effect’ Pheromone Synthesis in Goats (\textit{Capra hircus})

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Abstract. The ‘male effect’ is a well-known phenomenon in female sheep and goats, whereby pheromone-induced activation of reproductive function occurs. In a previous study, we showed that the genes for elongation of long-chain fatty acids family member 5 (\textit{ELOVL5}) and stearoyl-CoA desaturase 1 (\textit{SCD1}) increased their expression significantly, concomitant with induction of pheromone synthesis. Therefore, these genes were considered to be prime candidate genes for pheromone synthesis. In the present study, we performed \textit{in situ} hybridization to investigate where these two genes are expressed in goat skin. Strong positive signals were detected for both genes in the head skin of the male goat, which is the main site of pheromone production, and were mainly in the basal layer of the sebaceous gland cells, with the remaining cells showing negligible signals. None of the cells in the rump skin of the male goat or the head skin of the orchidectomized goat, neither of which produce pheromone, exhibited strong positive signals. The present study demonstrates that expression of these two candidate genes for pheromone synthesis is primarily localized in the sebaceous glands of the pheromone-producing skin region.

Key words: Goat, \textit{in situ} hybridization, Male effect, Pheromone, Sebaceous gland


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Pheromones are used by some mammalian species as chemical signals for social communication among individuals of the same species [1]. In sheep [2] and goats [3], the ‘male effect’ is well known as a pheromone-induced phenomenon in which seasonally anovulatory females resume ovarian cyclicity in response to introduction of a male. In goats, we have previously shown that the ‘male effect’ pheromone is produced in a testosterone (T)-dependent manner in the skin of the head region, but not in the skin of the rump region [4, 5], using a bioassay for pheromone activity that we had developed previously [6, 7]. Dihydrotestosterone (DHT) treatment induces pheromone production in the skin of the rump region, where pheromones are not produced constitutively [8]. Interestingly, ‘male effect’ pheromone activity is also induced in female skin by androgens [9]. We found that expression of the genes for elongation of long-chain fatty acids family member 5 (\textit{ELOVL5}) and stearoyl-CoA desaturase 1 (\textit{SCD1}) increased significantly, concomitant with induction of pheromone synthesis, in three types of skin models, i.e., the head skin of T-treated orchidectomized (ODX) male goats, rump skin of DHT-treated ODX male goats and head skin of DHT-treated ovariectomized female goats, thereby implicating these two genes as prime can-
didates for pheromone synthesis [10].

In the present study, we used in situ hybridization to localize the expression of the ELOVL5 and SCD1 genes in goat skin and attempted to discern the association between these genes and pheromone synthesis with regard to the site of expression. We examined histologically three skin types: 1) pheromone-positive type skin from the head regions of male goats; 2) pheromone-negative type skin from the rump regions of male goats; and 3) pheromone-negative type skin from the head regions of ODX goats.

**Materials and Methods**

*Animals*

Three adult male and two long-term (> 6 months) ODX Shiba goats that were 1–3 years of age and maintained in a closed colony at the experimental station of the University of Tokyo (Kasama, Japan) were used in this study. The Shiba goat is a non-seasonal breeder and reproduces year-round. They were housed under conditions of natural day length and temperature and were fed daily with sufficient amounts of dry hay and formula feed. Water was available ad libitum.

*Tissue preparation for in situ hybridization*

The goats were anesthetized using ketamine HCl and xylazine HCl, and squares of skin (1 × 1 cm) were removed with a scalpel from the head regions of the male and ODX goats; squares of skin were also removed from the rump regions of the male goats. The segments of skin used for in situ hybridization were torn into narrow strips (1 × 5 mm). These strips were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight and then soaked in 30% sucrose and 0.01% diethylpyrocarbonate (DEPC) in PBS for 2–3 h. The strips were embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and frozen at −80 C. Sections (12-μm thick) were cut in a cryostat, attached to silanized glass slides and dried for 1 h.

This study was approved by the Animal Care and Use Committee of the Faculty of Agriculture of the University of Tokyo.

*Preparation of the cRNA probes*

Total RNA was extracted from the head skin of the male goats. The total RNA was used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and the cDNA was used as templates for RT-PCR. The specific PCR primers used were 5’-AGCGGACAT-GAAGATCATCC-3’ and 5’-AAGAGAAGCTGCG-TGGTGTGG-3’ for ELOVL5 and 5’-ACTTGGAAGAGACATCCGC-3’ and 5’-TCTCTTGGAAACTCACCAGC-3’ for SCD1, respectively; these were designed based on sequences in the open reading frame. PCR amplifications were carried out for 35 cycles of 30 sec at 95 C, 30 sec at 58 C and 1 min at 72 C. The 545-bp and 525-bp PCR products corresponding to the ELOVL5 and SCD1 cDNA fragments, respectively, were subcloned into pBluescript II (Stratagene, La Jolla, CA, USA). The plasmids were linearized by digestion with EcoRI or SalI, and the antisense and sense RNA probes were synthesized using T3 or T7 polymerase (Roche Diagnostics, Indianapolis, IN, USA). They were then labeled with digoxigenin (DIG) using DIG RNA labeling mix (Roche Diagnostics) according to the manufacturer’s instructions. The reaction was stopped by addition of ethylenediaminetetraacetic acid (EDTA), and the transcripts were precipitated with ethanol and sodium acetate. The cRNA probes were dried and then dissolved in DEPC-treated water.

*In situ hybridization*

Skin sections were fixed in 4% paraformaldehyde in PBS for 5 min and then treated with 1 μg/ml proteinase K in PBS at 37 C for 2 min. The sections were refixed with 4% paraformaldehyde in PBS for 10 min and neutralized with 0.2% glycine in PBS for 5 min. They were then placed in 0.2 M HCl for 20 min. After washing, they were hybridized with 2.5 μg/ml cRNA probes, which were diluted with hybridization solution [50% formamide, 10 mM Tris-HCl (pH 7.6), 0.2 mg/ml yeast tRNA, 1 × Denhardt’s solution (pH 7.9), 0.6 M NaCl, 0.25% sodium dodecylsulphate (SDS), 1 mM EDTA] at 58 C overnight. After hybridization, the samples were washed twice with 5 × standard saline citrate (SSC)/50% formamide at 50 C for 20 min and treated with 20 μg/ml RNase at 37 C for 30 min. They were then washed twice with 2 × SSC and twice with 0.2 × SSC at 50 C. After treatment with 1.0% blocking reagent (Roche Diagnostics) in Tris-buffered saline at room temperature for 30 min, they were incubated with alkaline phosphatase-con-
jugated anti-DIG antibody (Roche Diagnostics) according to the manufacturer’s instructions. The positive signals were visualized with 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) as chromogenic substrates.

Results

The sebaceous glands of the head region were larger in size and more in number compared with those of the rump region in the male goats, and the size of sebaceous glands in the head region skin of male goats was larger than that of the ODX goats as shown in our previous study [5]. Except for the sebaceous glands, there was no appreciable difference in morphology among these skin samples.

In situ hybridization revealed strong expression of ELOVL5 mRNA, mainly in the basal layer of the sebaceous glands of the head skin of the male goat (arrows in A). This positive signal was not observed in the rump skin of the male goat (B) or the head skin of the ODX goat (C). No signal was detected in the head skin of the male goat using the sense probe (D). The insert box shows a high magnification view. The bars represent 500 µm.

Fig. 1. In situ hybridization analysis using DIG-labeled probes for the ELOVL5 gene in goat skin. In situ hybridization shows strong expression of ELOVL5 mRNA that is localized mainly in the basal layer of the sebaceous glands of the head skin of the male goat (arrows in A). This positive signal was not observed in the rump skin of the male goat (B) or the head skin of the ODX goat (C). No signal was detected in the head skin of the male goat using the sense probe (D). The insert box shows a high magnification view. The bars represent 500 µm.

the skin samples from the rump regions of the male goats (Fig. 1B) or those from the head regions of the ODX goats (Fig. 1C). Strong expression of SCD1 mRNA was detected in the basal layer and inner cells of the sebaceous acinus of the head skin of the male goats (arrows in Fig. 2A) but was not detected in the rump skin of the male goats (Fig. 2B) or the head skin of the ODX goats (Fig 2C). No sections exhibited staining signals for the sense probe of either gene (Fig. 1D and 2D). Similar expression patterns were observed for both genes in the respective skin regions of the other two male goats and another ODX goat.

Discussion

The present study demonstrates that two candidate genes for pheromone synthesis, ELOVL5 and SCD1, are expressed in the basal layer of the sebaceous glands of pheromone-positive skin. Our previous results suggest that pheromone molecules are emitted from the sebaceous gland of the head skin of the male goat; 1) An extract of the upper dermal skin layer, which contains the sebaceous
glands without the epidermal subcutaneous layer, and the lower part of the dermal layer, which contains sweat glands, has been shown to have pheromone activity [4, 5, 8]. 2) A previous study did not detect pheromone activity in the rump skin of the male goat [5]. Treatment with DHT, but not T, induces pheromone production in the rump region [8]. The regional difference in reactivity to androgens of head and rump skin may reflect the fact that 5α-reductase, which converts T to DHT, is expressed at a lower level in rump skin than in head skin and that expression of this enzyme is localized to the sebaceous glands in head skin [5].

It is normally thought that as the peripheral cells of the sebaceous gland develop and produce lipids, they are pushed toward the center of the acinus [11]. It is also thought that the secretion of the sebaceous gland, sebum, is formed when the fully mature, lipid-rich cells die and disintegrate [12]. It has been reported that the components of sebum contain squalene and wax esters, as well as cholesterol esters, triglycerides and possibly some free fatty acids [13, 14]. ELOVL5 is involved in elongation of various polyunsaturated long-chain fatty acids of C_{18}–C_{20} [15], and SCD1 catalyses delta-9 desaturation of various fatty acyl-CoA substrates [16]. Therefore, it appears likely that these genes play some roles in the synthetic pathway of free fatty acids and other substances contained in the sebaceous glands that could induce the ‘male effect’ pheromone.

In the present study, ELOVL5 and SCD1 were expressed exclusively in the sebaceous gland cells of the head skin of the male goat. This result supports the notion that expression of these genes is associated with pheromone production and regulation. However, further investigations are needed, including establishment of a primary culture of sebaceous gland cells [17, 18] and functional analysis of these genes using cultured cells [19], in order to provide conclusive evidence that the pheromone is produced in this gland and that these genes play important roles in pheromone synthesis and regulation. These in vitro approaches, in conjunction with in vivo studies, will also help to elucidate the mechanism underlying production of the ‘male effect’ pheromone.

**Fig. 2.** In situ hybridization analysis using DIG-labeled probes for the SCD1 gene in goat skin. In situ hybridization shows strong expression of SCD1 mRNA that is localized mainly in the basal layer of the sebaceous glands of the head skin of the male goat (arrows in A). This positive signal was not observed in the rump skin of the male goat (B) or the head skin of the ODX goat (C). No signal was detected in the head skin of the male goat using the sense probe (D). The insert box shows a high magnification view. The bars represent 500 µm.
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