concentration and amylase activity between the mice. The protein concentration per min in 1 ml saliva was significantly lower in NOD/SCID.*e2f1*$^{-/-}$ mice as compared to NOD/SCID.*e2f1*$^{+/+}$ mice. Here we did not measure the activities of lysozyme, lactoferrin, or histatins in saliva from NOD/SCID.*e2f1*$^{+/+}$ mice and NOD/SCID.*e2f1*$^{-/-}$ mice, but the activities of the innate immunity and the receptors to *C. albicans* attachment may not be sufficiently provided in oral cavity from NOD/SCID.*e2f1*$^{-/-}$ as compared with NOD/SCID.*e2f1*$^{+/+}$ mice. Mouse salivary proteins are poorly existed in NOD/SCID.*e2f1*$^{-/-}$ mice. Taken together, mouse saliva works positively for the initial colonization of *C. albicans* rather than protecting by innate immunity, an opposite function to *S. mutans* colonization [24].

The colonization decreased in a time-dependent manner without the effects of saliva volume difference in NOD/SCID.*e2f1*$^{+/+}$ and NOD/SCID.*e2f1*$^{-/-}$ mice. The colonization may be decreased by washing effects with an appropriate volume of saliva. Moreover, cell-mediated immunity plays an important role in the resistance to mucosal candidiasis [40,41]. The mucosal surface provides a protective barrier against bacterial and fungal infections in the oral cavity. The β-defensins are small cationic amphipathic peptides that exhibit a broad spectrum of activity against gram-positive and gram-negative bacteria and fungal species [42]. NOD/SCID.*e2f1*$^{+/+}$ and NOD/SCID.*e2f1*$^{-/-}$ mice do not have mature T and B cells but have a protective barrier with cell-mediated immunity. Therefore, cell-mediated immunity may not be associate with the difference of *C. albicans* colonization between NOD/SCID.*e2f1*$^{+/+}$ and NOD/SCID.*e2f1*$^{-/-}$ mice. The positive function for *C. albicans* in the oral cavity indicates a useful animal model for initial colonization of *C. albicans* in NOD/SCID wild type mice as compared to NOD/SCID.*e2f1*$^{-/-}$ mice.

Previous report suggested that the indigenous bacterial flora could suppress the extent of colonization of *C. albicans* by interfering with its ability to attach to mucosal surface in comparison with germ-free rats and conventional rat [43]. This was explained by competing for epithelial receptor sites required for Candida attachment or by enzymatically altering the surfaces of the yeast cells. Our mouse model system using oral inoculation of *C. albicans* is different from previous report using germ free and conventional rat and indicates better model to explore effects of salivary components and indigenous microorganisms on the colonization under the natural background condition than previous model system. Sucrose works as a substrate for production of glucan by oral streptococci. The drinking of sucrose water before inoculation provides a source of glucan for the restoration of indigenous microorganisms. CHX was uniformly effective against strains of common borne microorganisms. The treatment with CHX disinfected the indigenous microorganism before inoculation in both no-sucrose and sucrose drinking water [44,45]. However, the indigenous microorganisms contaminated the sample of *C. albicans* in the mice fed 1% sucrose water. The colonization of *C. albicans* was not affected by restoration of indigenous microorganisms. Therefore, the initial colonization of *C. albicans* is not affected by later colonization of indigenous microorganisms in the oral cavity. It is suggested that the colonized cells of *C. albicans* are not removed by the enzymatical or physical effect by indigenous microorganisms.

Yeast-form cells adhere more effectively than hypal cells to endothelial cells under conditions of flow [46]. Although cells locked in the filamentous state also display reduced virulence [47-49], the ability to form hyphae is important for *C. albicans* to cause disease after dissemination: cells locked in the yeast form remain avirulent until they are permitted to form hyphae, after which, mice succumb to the infection [50]. *C. albicans* hyphae are impaired in their ability to adhere to the human oral cavity by the bacterium *Streptococcus gordonii* [51]. To colonize and infect the oral environment, yeast cells must first adhere to host cells and tissues or prosthetic materials within the oral cavity or co-aggregate with the oral microbiota [52-54]. In this study, the hyphae form of *C. albicans* showed similar results as the yeast form but the restoration of the indigenous microorganisms was weaker with the hyphal form than that with the yeast form. This may indicate restriction effects by the hyphal form on the restoration.

We provide here an original new animal model system, which may be a useful model to explore oral initial colonization of *C. albicans.* The salivary protein flow may play important roles in maintaining the commensal behavior of *C. albicans* and becoming an opportunistic pathogen under the immune deficiency condition such as SCID. These mice are required for these investigations to determine several factors that contribute to the susceptibility for candidal infections.

Conclusions

In conclusion, the saliva protein flow may be very important for *C. albicans* initial colonization, where the indigenous microorganisms do not affect *C. albicans* initial colonization in the oral cavity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NK carried out animal experiments and attend to draft the manuscript. NN helped animal experiments and to draft the manuscript. TI helped to produce mouse. YK helped to maintain and produce mouse. YK participated in the design of the study. OS participated in the design of study and coordination. HS designed the study, carried out production of mouse and described manuscript. All authors read and approved the final manuscript.

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Original Article

Dynamic Changes in the Initial Colonization of *Actinomyces naeslundii* and *Streptococcus gordonii* Using a New Animal Model

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SUMMARY: *Actinomyces naeslundii* and *Streptococcus gordonii* are the predominant bacteria and initial colonizers of oral microflora. The binding of *A. naeslundii* and *S. gordonii* and the interaction between them on the salivary pellicle-coated tooth surface play an important role in the biofilm development. Recently, we reported that NOD/SCID.*e2f1*$^-$ mice are a useful model for studying oral biofilm formation by *Streptococcus mutans* on the tooth surface. In this study, we aimed to determine whether NOD/SCID.*e2f1*$^-$ mice can be used for studying oral colonization of *A. naeslundii* and *S. gordonii*. Colonization of *A. naeslundii* in mice fed with 1% sucrose water for 24 h before inoculation was higher than that among mice fed with sucrose water for 1 h. *A. naeslundii* colonization using mixed species-inoculation was lower than that using single-species inoculation 30–90 min after inoculation; however, the colonization was higher 120–180 min after inoculation. The mixed inoculation induced better colonization of *S. gordonii* than single-species inoculation 60–180 min after inoculation. Polyclonal and fluorescein isothiocyanate-labeled antibody stained bacteria showed better colonization of *S. gordonii* when a mixed culture is used in vivo. NOD/SCID.*e2f1*$^-$ mice were useful for studying the initial colonization of *A. naeslundii* and *S. gordonii*. Long-term supply of sucrose water creates a favorable environment for the initial colonization of *A. naeslundii* that, in turn, supports the colonization of *S. gordonii*.

INTRODUCTION

Dental plaque is a complex biofilm community of diverse groups of bacteria comprising more than 750 different species (1). Previous culture-based studies suggest that streptococci are prominent during the initial stages of biofilm formation on tooth surfaces (2,3). *Streptococcus gordonii* is an early colonizer of the oral cavity of infants and can comprise a substantial proportion of the biofilm on a healthy dental surface (4–6). Other genera such as *Actinomyces* spp. are also among the earliest colonizers of dental surfaces (2,3,7). These bacteria are present from infancy to adulthood and are involved in the initial stage of infectious disease development (3,8–11). The colonization of tooth or mucosal surfaces by *Actinomyces* spp. provides a substrate for the adherence of other oral bacteria including streptococci (12), resulting in the development of the plaque community. Therefore, these early colonizers play a vital role in biofilm formation in the oral environment. Unfortunately, little attention has been devoted to the dynamic changes in these early colonizers. Previous studies reported the coaggregation of *Actinomyces naeslundii* and *S. gordonii* in vitro (13–16); however, to our knowledge, there are no studies that used an animal model.

Recently, we reported a model for decreased saliva volume in NOD/SCID.*e2f1*$^{-/-}$ mice (17). Further, NOD/SCID.*e2f1*$^{-/-}$ mice lack sIgA and IgG in the saliva and have decreased NK cells. We demonstrated that NOD/SCID.*e2f1*$^{-/-}$ mice are highly susceptible to *Streptococcus mutans* colonization when NOD/SCID.*e2f1*$^{-/-}$ mice are pretreated with human saliva or sIgA using a low concentration (1%) sucrose supplement (18). This suggests that there are multiple effects exerted by sIgA on *S. mutans* during its colonization; the synergistic effects are evident when a combination of sIgA and limited nutrients is used during colonization in NOD/SCID.*e2f1*$^{-/-}$ mice. This further suggests that this mouse can be used as a new animal model to assess prevention methods for dental biofilm-dependent diseases such as dental caries. We also used this mouse for oral infection by *Candida albicans*, to demonstrate the interaction between the indigenous microorganisms and colonization and saliva volume (19). Therefore, the NOD/SCID.*e2f1*$^-$ mouse may be a useful animal model for the investigation of oral infections by various oral microorganisms.

However, very little is known about the colonization of *A. naeslundii* and *S. gordonii* separately or as mixed infections in the oral cavity using a mouse model. In this

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study, we describe the dynamic changes in the colonization of *A. naeslundii* and *S. gordonii* using mixed-species inoculation in the new NOD/SCID.*e2f1*$^-$ mouse model. The results of our in vivo study on oral biofilm formation agree with those of in vitro studies.

MATERIALS AND METHODS

Bacteria strains and culture conditions: *A. naeslundii* X600 and *S. gordonii* ATCC 10558 were cultured in brain-heart infusion (BHI) broth (Difco Laboratory, Detroit, Mich., USA) overnight in an atmosphere of H_2 and CO_2 (GasPack; Becton/Dickinson, Sparks, Md., USA) at 37°C.

Animals: Heterozygous NOD/SCID.*e2f1*$^{+/-}$ mice were bred to produce NOD/SCID.*e2f1*$^{-/-}$ and NOD/SCID.*e2f1*$^{+/-}$ (1:2, bone rate). NOD/SCID.*e2f1*$^-$ mice genotypes (NOD/SCID.*e2f1*$^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice) were identified using PCR (17). Compared with the NOD/SCID.*e2f1*$^-$ mice, NOD.B10.D2 mice have decreased saliva volume, which is higher in level than that in NOD/SCID.*e2f1*$^{-/-}$ and *e2f1*$^{+/-}$ mice; they also have IgA, IgG, and cellular immunity, and they served as the control group in nonobese diabetogenic (NOD) background mice (20). All mice used in this experiment were female mice aged 4–6 months. The experimental mice were given sterile 1% sucrose drinking water for 1 h or 24 h prior to infection (less than the concentration in juice), and were fed a commercial diet (CMF; water, 8.8%; protein, 27.4%, fat, 8.0%; mineral, 6.1%; fiber, 2.9%; nitrogen, 46.8%; Oriental Yeast Co., Tokyo, Japan); whereas, the control mice in each group were provided with sterile water and CMF. Food and fluids were withheld during the experiment following inoculation. Test animals were maintained in accordance with the guidelines of the National Institute of Infectious Diseases (Tokyo, Japan). Experimental protocols (#210110 and 210111) were approved by the National Institute of Infectious Diseases Animal Resource Committee.

Measurement of carbohydrates in the oral cavity: To detect the carbohydrates, a phenol sulfuric acid method was applied to samples from the oral cavity after the feeding of 1% sucrose water for 1 h or 24 h. Samples were collected from the oral cavities by washing with sterilized phosphate-buffered saline (PBS) and by swabbing with a sterilized cotton ball at 1 h or 24 h after finishing the sucrose supply. After sonication at 120 W for 1 min, samples were centrifuged at 10,000 × *g* for 10 min at 4°C for the supernatant. The samples may have included polysaccharides converted from sucrose by commensal oral bacteria, and sucrose remaining in the oral cavity. A special dose of 2 ml glucose (0.0025 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.03 mg/ml, 0.04 mg/ml, or 0.05 mg/ml) was prepared to establish the standard curve. After addition of 1-ml 5% phenol solution to the samples, 5 ml of 5% sulfuric acid solution was mixed with each sample and shaken immediately. The samples were placed in a water bath at 25°C for 20 min, and absorbance was read at 490 nm. Carbohydrate concentrations in the samples were calculated under the glucose standard curve.

Sampling and colony-forming unit (CFU) determination: Bacterial inoculation, sampling, and CFU counts were performed using procedures and conditions described previously (18,20). *A. naeslundii* and *S. gordonii* were cultured in BHI broth overnight and then washed twice with sterile PBS. Prior to the animal experiment, the ability of disinfect of chlorhexidine (0.2%) was evaluated using the same kind of mice for 180 min. Bacteria were inoculated into the oral cavities of mice at a final concentration of 5×10^7 CFU/ml in 0.25 ml of PBS during 2.5 min. When using the mixed bacteria, the bacteria were concentrated using centrifugation before mixing. Following inoculation, samples were collected from the labial surfaces of the maxillary incisor teeth using a sterile cotton ball every 30 min for 180 min (each time interval using a different mouse from the same cage) and dispersed in 2 ml of PBS. The samples in PBS were sonicated using ultrasonic dispersion (power output, 60 W) for 10 s, and then poured onto BHI, 5% blood, and MS agar plates to determine colony numbers. To avoid confusion with different bacterial colony, colony count number was obtained using microscopy (Olympus TL3-100; Olympus Co., Tokyo, Japan) and compared with the sample colony for each sample.

***S. gordonii* observation using mixed-species inoculation:** After sonication, the samples from the animal experiment at 150 min were observed using fluorescent antibody staining. All the samples were washed using PBS once after centrifugation at 1,380 × *g* and then incubated with anti-rPAc polyclonal antibodies (PAc; surface protein antigen from *S. mutans* [21]), which cross-react with surface protein antigens from *S. gordonii*, for 1 h at 37°C for the identification of *S. gordonii*. The samples were washed 3 times using PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (IgG-FITC, Sc-2010; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 1 h at 37°C. The cells were washed 3 times using PBS and then observed using laser microscopy (DP70; Epson, Suwa, Japan). To compare studies, the concentrations of bacteria in the animal experiment were the same as that in the in vitro studies for antibody staining. The in vitro samples were cultured for 150 min at 37°C, and then treated with the same steps used in the in vivo samples, as described above. Finally, samples were observed using laser microscopy and photographed.

Statistics: CFU data were expressed as mean ± standard deviation. All animal studies were performed independently in triplicates. The independent sample *t* test was used to compare the colony numbers using SPSS with *P* values of <0.01 and <0.05 representing statistical significance.

RESULTS

***A. naeslundii* colonization on the tooth surface of NOD/SCID.*e2f1*$^-$ mice:** In comparison to NOD.B10.D2 mice, *A. naeslundii* colonized better on the tooth surfaces of NOD/SCID.*e2f1*$^-$ mice at all time points (Fig. 1). All 3 genotypes of NOD/SCID.*e2f1* mice were susceptible to *A. naeslundii* colonization. The colony number of NOD/SCID.*e2f1*$^{+/-}$ mice was almost 30-fold higher than that of NOD.B10.D2 mice using water only at 30 min and colony counts on 5% blood medium agar (Fig. 1A). At 60 min, the colony

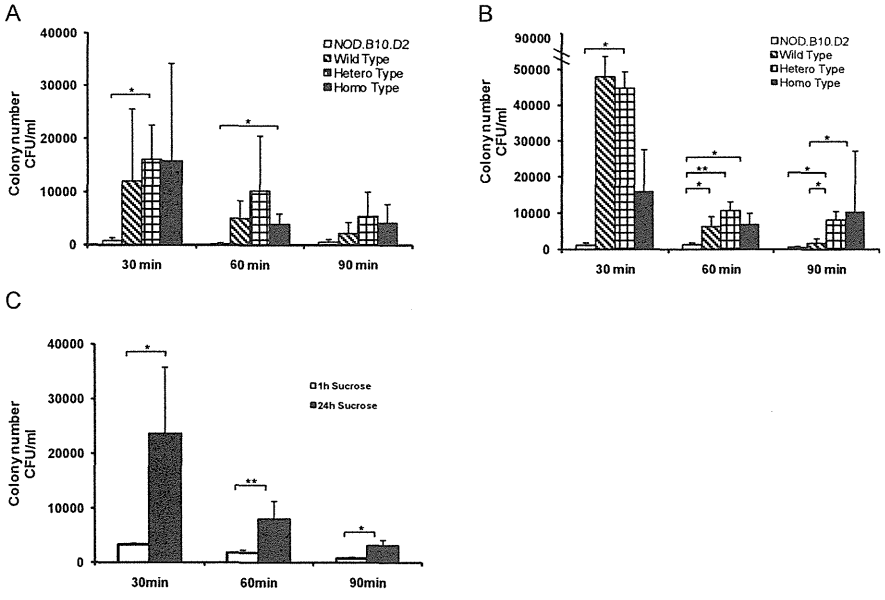

Fig. 1. Colonization of *Actinomyces naeslundii* on the tooth surfaces in mice. *A. naeslundii* colonization on the tooth surfaces of NOD.B10.D2 mice using 3 *e2f1* types ((−/−), (+/−), (+/+)). Samples were poured on 5% blood medium agar at 30 min, 60 min, and 90 min after inoculation, supplied with only water (A) and 1% sucrose water overnight (B) before the experiment. *A. naeslundii* colonization in NOD/SCID.*e2f1*$^{+/-}$ mice (C) after 1% sucrose water for 1 h was compared with that after 24 h supply. Results of the 3 independent assays are expressed as mean ± SD. One mouse was used each time, and a total of 3 mice were used at 3 time points in 1 experiment carried out for each strain. The animal experiment was independently repeated 3 times. The colony numbers in the culture samples from oral cavities were counted. One asterisk represents $P < 0.05$, and double asterisks represent $P < 0.01$.

number of NOD/SCID.*e2f1*$^{-/-}$ mice was almost 25-fold higher than that of NOD.B10.D2 mice. At 30 min, the colony number on the tooth surfaces was higher than that at 60 min and 90 min. However, there were no significant differences among NOD.B10.D2, NOD/SCID.wild-type and NOD/SCID.*e2f1*$^{-}$ mice at 90 min. The decrease in the colony number between 30 min and 60 min after inoculation was much higher than the decrease between 60 min and 90 min after inoculation. *A. naeslundii* remained on the tooth surfaces of the mice throughout the experiment. This was also observed using 1% sucrose water (Fig. 1B) where there were significant differences between the NOD.B10.D2 and NOD/SCID.*e2f1* mice. The colony number of *A. naeslundii* with 1% sucrose water overnight was higher than that without sucrose water, although not significant (Figs. 1A and 1B). The colony numbers in NOD/SCID.*e2f-1*$^{+/-}$ mice with 1% sucrose water was almost 60-fold higher than that in NOD.B10.D2 mice at 30 min (Fig. 1B). At 60 min, the colony numbers in NOD/SCID.wild-type, NOD/SCID.*e2f1*$^{+/-}$, and NOD/SCID.*e2f1*$^{-/-}$ mice were significantly higher than that in NOD.B10.D2 mice. At 90 min, the colony number in NOD/SCID.*e2f1*$^{+/-}$ mice was higher in the NOD.B10.D2 mice and NOD/SCID.wild-type mice. These data suggest that NOD/SCID.*e2f1*$^{+/-}$ mice were more suitable for studying the *A. naeslundii* initial attachment because the production of NOD/SCID.*e2f1*$^{+/-}$ mice through the mating between NOD/SCID.*e2f1*$^{+/-}$ mice is easier than that between NOD/SCID.*e2f1*$^{-/-}$ mice, where the birth rate of NOD/SCID.*e2f1*$^{+/-}$ mice and NOD/SCID.*e2f1*$^{-/-}$ mice is theoretically 50% and 25%, respectively. Hence, the following experiments used the heterozygous NOD/SCID.*e2f1* mice.

Overnight sucrose feeding enhanced *A. naeslundii* colonization: The colony number of *A. naeslundii* of mice fed 1% sucrose water for 1 h and 24 h before the experiment was compared (Fig. 1C). *A. naeslundii* colonization at all the time points after long-term (24 h) sucrose water feeding was significantly better than that after 1 h feeding of 1% sucrose water. To determine the mechanisms contributing to the increased colony numbers, carbohydrate levels in the oral cavity was measured as it is favored as a substrate for growth and biofilm formation of oral bacteria. A higher concentration was detected in mice fed with 1% sucrose water for 24 h (13.9 ± 2.7 μg/ml) than that in those fed for 1 h (0.8 ± 0.1 μg/ml).

***A. naeslundii* colonization in mixed-species inoculation with *A. naeslundii* and *S. gordonii*:** Colony numbers of *A. naeslundii* gradually decreased from 30 min to 120 min (Fig. 2). This decrease is dependent on the number of physically remaining and non-adhered bacteria at 30 min and 60 min after inoculation on the tooth surface because a high concentration of *A. naeslundii* was inoculated. After inoculation, bacteria remained physically and non-adhered bacteria decreased immediately by saliva flow. After 120 min, the colony numbers were stable and the bacteria remained on the tooth surface. At 150 min and 180 min after inoculation of the single and mixed species, non-adhered bacteria were almost removed, and adhered and colonized cells remained on the tooth surface. *A. naeslundii* colonization with single-species inoculation was better than that with mixed inoculation with *S. gordonii* 30–90 min after inoculation (Fig. 2). However, from 120 min after inoculation, the colony number of *A. naeslundii* with mix-

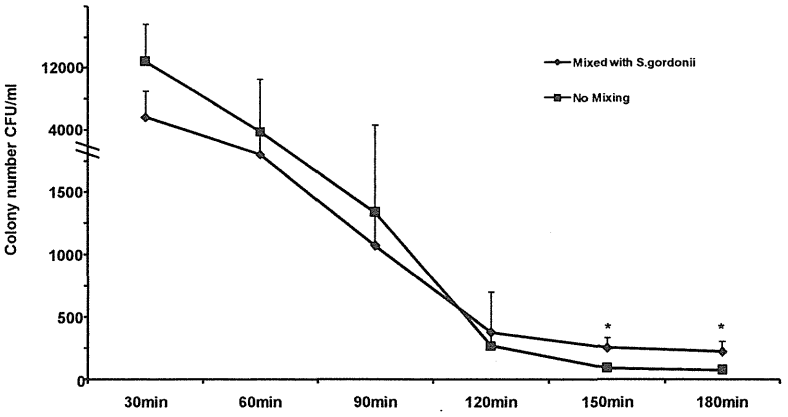

Fig. 2. *A. naeslundii* mixed with *S. gordonii* compared to single species initial attachment on tooth surfaces. NOD/SCID.*e2f-1*$^{+/-}$ mice were used; for *A. naeslundii* colonization, samples were poured on 5% blood agar plates every 30 min after inoculation and this was continued till 180 min. Data are expressed as the mean ± SD of 3 independent assays. One mouse was used each time, and a total of 6 mice were used at 6 time points in 1 experiment carried out for each strain. The animal experiment was independently repeated 3 times. The colony numbers in the culture samples from the oral cavities were counted. One asterisk represents $P < 0.05$ (*A. naeslundii* mixed with *S. gordonii* compared with single species at 150 min and 180 min).

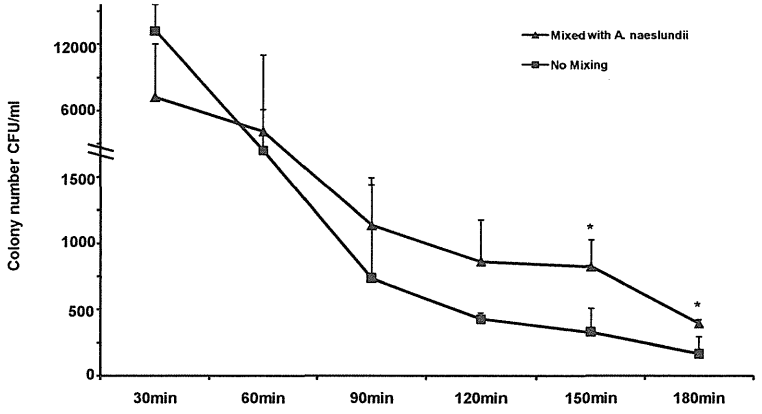

Fig. 3. *S. gordonii* mixed with *A. naeslundii* or single-species colonization on tooth surfaces. NOD/SCID.*e2f-1*$^{+/-}$ mice were used; for *S. gordonii* colonization, samples were poured on MS agar plate every 30 min after inoculation and this was continued till 180 min. Data are expressed as the mean ± SD of 3 independent assays. One mouse was used each time, and a total of 6 mice were used at 6 time points in 1 experiment carried out for each strain. The animal experiment was independently repeated 3 times. The colony numbers in the culture samples from the oral cavities were counted. One asterisk represents $P < 0.05$ (*S. gordonii* mixed with *A. naeslundii* compared with a single species at 150 min and 180 min).

ed-species inoculation (250.0 ± 80.2 and 220.0 ± 83.2 at 150 min and 180 min, respectively) was significantly higher than those with single-species inoculation (86.7 ± 23.1 and 73.3 ± 83.2), and the difference was observed at 150 min and 180 min.

***S. gordonii* colonization in mixed-species inoculation with *A. naeslundii* and *S. gordonii*:** The decrease in *S. gordonii* colony numbers with mixed-species inoculation was smaller than that with single-species inoculation. *S. gordonii* colonized better in single culture than in mixed inoculation with *A. naeslundii* at 30 min after inoculation (Fig. 3). From 90 min, the colony number of *S. gordonii* with mixed-species inoculation was higher than that with single-species inoculation. This was also observed at 150 min, and the colony number was significantly higher at 180 min.

Antibody staining of the samples in animal experiments: The polyclonal anti-rPAc mouse antibodies, which cross-reacted with the surface protein antigens of *S. gordonii*, but not of *A. naeslundii*, was used to differentiate *S. gordonii* from *A. naeslundii* where the *S. gordonii* fluoresced green using laser light (Figs. 4D and F); the *A. naeslundii* cells were dark (Fig. 4B). Photographs without the laser light showed single bacterial species and aggregation between *S. gordonii* and *A. naeslundii* (Figs. 4A, C, and E). Photographs of the in vivo samples reveal that *S. gordonii* numbers were fewer in the single-species samples than that in the mixed-species samples at the same time intervals (Figs. 5B and D). In the mixed-species in vivo samples, the number of *S. gordonii* was higher than that of single species, and many of them were aggregated with *A. naeslundii* (Figs. 5C and D).

DISCUSSION

The data reveal that the colony number of *A. naeslundii* at 90 min after inoculation is much higher in NOD/SCID.*e2f1*$^{-}$ mice than that in NOD.B10.D2 and NOD/SCID.*e2f1*$^{+/+}$ mice that were fed 1% sucrose

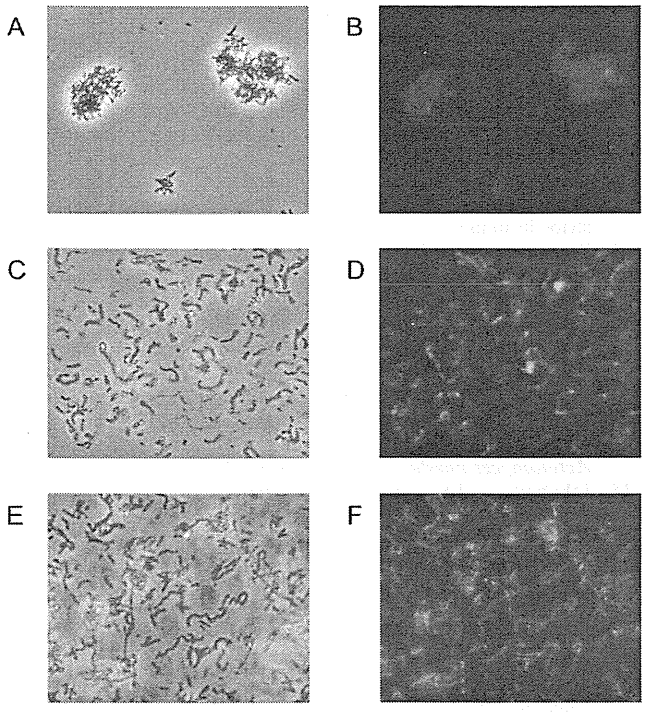

Fig. 4. Fluorescent antibody staining of *S. gordonii* in single and mixed cultures in vitro. Photographs with and without laser lights are seen. The culture was performed for 150 min, and *S. gordonii* was stained using polyclonal antibody and FITC-labeled secondary antibody. A and B: antibody staining of *A. naeslundii* (single inoculation) with normal and laser lights. C and D: fluorescent antibody staining of *S. gordonii* (single inoculation) with normal and laser lights. E and F: fluorescent antibody staining of mixed-species inoculation (*A. naeslundii* and *S. gordonii*) with normal and laser lights. The photographs represent the 3 independent experiments.

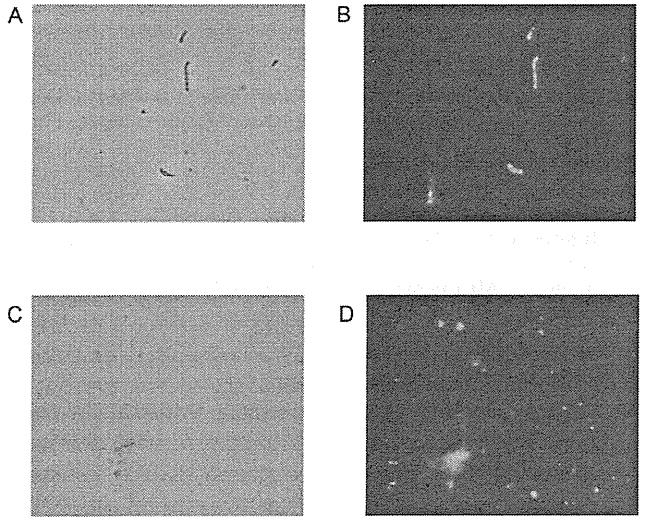

Fig. 5. Fluorescent antibody staining of *S. gordonii* in single and mixed culture in vivo. Photographs with and without laser lights are seen. NOD/SCID.*e2f-1*$^{+/-}$ mice were used; the samples were obtained from the tooth surfaces 150 min after inoculation. *S. gordonii* in the samples was stained using polyclonal antibody and FITC-labeled secondary antibody. A and B: antibody staining using *S. gordonii* (single inoculation) with normal and laser lights. C and D: antibody staining of mixed-species inoculation (*S. gordonii* and *A. naeslundii*) with normal and laser lights. The photographs represent the 3 independent experiments.

water before inoculation. NOD.B10.D2 mice have normal immunity and decreased saliva volume, which is higher than that in the NOD/SCID.*e2f1*$^{-/+}$ and *e2f1*$^{-/-}$ mice. We demonstrated that the NOD/SCID.*e2f1*$^{-}$ mice that have a decreased salive volume, lack IgA and IgG, and have decreased NK cells (17) are useful for studying *A. naeslundii* and *S. gordonii* colonization, which are the initial colonizers on human tooth surfaces (2-4,6,7). Further, saliva flow and immunity may play a central role in the initial colonization by these bacteria.

Long-term (24 h) feeding with 1% sucrose enhances the colonization of *A. naeslundii* in NOD/SCID. *e2f1*$^{+/-}$ mice, compared with short-term (1 h) feeding. Polysaccharides converted from sucrose by the original oral bacteria, such as streptococci in the mouse oral cavity, and the remaining sucrose were present in the oral cavity after feeding with 1% sucrose water for 24 h because the carbohydrate concentrations in mice fed 24 h was higher than that in mice fed for 1 h. Further, *A. naeslundii* degrades sucrose and polysaccharides such as fructan, giving free fructose and glucose (22,23); the presence of sucrose in the diet greatly increases the ability of *A. naeslundii* to produce fermentable products from sucrose through β-fructofuranosidase activity (22). The end products of sucrose degradation by *A. naeslundii* are volatile acids, especially acetic acid and lactic acid, and intracellular and extracellular polysaccharides (22), which are required for *A. naeslundii* to remain in the oral cavity and for other bacteria such as streptococci. Therefore, long-term 1% sucrose supply provides a more favorable condition for *A. naeslundii* and *S. gordonii* colonization on the tooth surface.

Dental plaque is a complex microbial ecosystem in which *A. naeslundii* and *S. gordonii* are early colonizers. The dynamic changes in the colonization of these bacteria are shown in an in vitro model, an artificial oral cavity environment that shows biofilm formation. However, the oral cavity is a complicated, multi-bacteria environment where saliva flow, tongue movement, and mastication are fundamental. In this study, we demonstrate the relationship of these bacteria in vivo and the dynamic changes show that colonization by both species is better from 150 min to 180 min when inoculated together than when inoculated separately (Figs. 2 and 3).

Direct observation of the 2 species was performed using photographs of the mixed-species inoculation and antibody staining of *S. gordonii*. Using in vitro samples, *S. gordonii* was identified using laser light and compared to photographs obtained using normal light (Figs. 4C and D). *A. naeslundii* did not fluoresce using laser light when compared to photographs with normal light (Figs. 4A and B). This revealed the fluorescent antibody staining was efficient in differentiating *S. gordonii* and *A. naeslundii*. The aggregation of *A. naeslundii* and *S. gordonii* can be clearly observed in Fig. 4F. Comparing the photographs of single species to 2 species, the number of *S. gordonii* observed was greater when using the mixed-species inoculation in the animal model (Figs. 5B and D). The photographs show that *A. naeslundii* and *S. gordonii* aggregated with each other at 150 min after inoculation (Fig. 5D). This aggregation is possibly because the colonization of the 2 species was higher than

that of a single species. Several factors may enhance the coexistence of these species. *A. naeslundii* has sialidase activity (24,25), which is not present in *S. gordonii* (24,26); this activity could potentially supply nutrients for *S. gordonii*. Further, *S. gordonii* produces several glycolytic and proteolytic enzymes lacking in *A. naeslundii* (24). Furthermore, the ability of *A. naeslundii* to bind surface receptors on *S. gordonii* contributes to its retention in biofilms under flowing saliva (26). Additionally, H_2O_2 production results in *S. gordonii* benefitting from *A. naeslundii*, as it is protected against oxidative stress (15). The aggregated cells in the mixd inoculation physically express more numbers of ligands to salivary pellicle receptors on the tooth surface than the non-aggregated cells in single inoculation. Therefore, the aggregation may be important for supporting initial colonizers on the tooth surface.

In summary, the NOD/SCID.*e2f1* mouse model is useful for studying the initial attachment of *A. naeslundii* and *S. gordonii*. Long-term sucrose supply enhances *A. naeslundii* colonization in NOD/SCID.*e2f1*$^-$ mice. To our knowledge, this study is the first to observe the dynamic changes in the initial attachment of 2 species using an in vivo model. The NOD/SCID.*e2f1* mouse model can be used in the future to elucidate the interaction between multiple species.

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Conflict of interest None to declare.

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E2f1-deficient NOD/SCID mice have dry mouth due to a change of acinar/duct structure and the down-regulation of AQP5 in the salivary gland

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Abstract Non-obese diabetic (NOD) mice have been used as a model for dry mouth. NOD mice lacking the gene encoding E2f1, a transcription factor, develop hyposalivation more rapidly progressively than control NOD mice. However, the model mice are associated with an underlying disease such as diabetes. We have now established E2f1-deficient NOD/severe combined immunodeficiency disease (NOD/SCID.E2f1$^{-/-}$) mice to avoid the development of diabetes (Matsui-Inohara et al., Exp Biol Med (Maywood) 234(12):1525–1536, 2009). In this study, we investigated the pathophysiological features of dry mouth using NOD/SCID.E2f1$^{-/-}$ mice. In NOD/SCID.E2f1$^{-/-}$ mice, the volume of secreted saliva stimulated with pilocarpine is about one third that of control NOD/SCID mice. In behavioral analysis, NOD/SCID.E2f1$^{-/-}$ mice drank plenty of water when they ate dry food, and the frequency and time of water intake were almost double compared with control NOD/SCID mice. Histological analysis of submandibular glands with hematoxylin–eosin stain revealed that NOD/SCID.E2f1$^{-/-}$ mice have more ducts than NOD/SCID mice. In western blot analysis, the expression of aquaporin 5 (AQP5), a marker of acinar cells, in parotid and in submandibular glands of NOD/SCID.E2f1$^{-/-}$ mice was lower than in NOD/SCID mice. Immunohistochemical analysis of parotid and submandibular acini revealed that the localization of AQP5 in NOD/SCID.E2f1$^{-/-}$ mice differs from that in NOD/SCID mice; AQP5 was leaky and diffusively localized from the apical membrane to the cytosol in NOD/SCID.E2f1$^{-/-}$ mice. The ubiquitination of AQP5 was detected in submandibular glands of NOD/SCID.E2f1$^{-/-}$ mice. These findings suggest that the change of acinar/duct structure and the down-regulation of AQP5 in the salivary gland cause the pathogenesis of hyposalivation in NOD/SCID.E2f1$^{-/-}$ mice.

Keywords Salivary glands · AQP5 protein · Down-regulation · Dry mouth · Ubiquitin · Disease model

Abbreviations

AQP5	Aquaporin 5
NOD	Non-obese diabetic
SCID	Severe combined immunodeficiency disease
SS	Sjögren's syndrome
BSA	Bovine serum albumin
RT-PCR	Reverse transcription/polymerase chain reaction
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
B2M	β2 microglobulin
HPRT	Hypoxanthine phosphoribosyl transferase

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SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
IP	Immunoprecipitation
WB	Western blot analysis
Ub-AQP5	Ubiquitinated-AQP5

Introduction

Saliva is essential for the preservation of oral health because of its many physiological functions, such as food digestion, antimicrobial actions, remineralization of teeth, and buffering of pH [44]. Salivary dysfunction results in impaired food and beverage intake as well as host defense and communication, all of which ultimately have an adverse influence on a person's quality of life. Sjögren's syndrome (SS) [10], the use of drugs with anticholinergic effects [41], and radiotherapy for head and neck cancers [14] are the most common causes of dry mouth. However, the pathophysiological features of dry mouth are not yet clear. Therefore, establishing dry mouth models in animals is important.

Non-obese diabetic (NOD) mice develop an anti-exocrine gland pathology similar to human SS [24]. NOD mice lacking the gene encoding E2f1, a transcription factor, further develop SS-like hyposalivation more rapidly progressively than do NOD mice [39]. However, such dry mouth model mice are associated with the underlying disease, diabetes [28, 39]. Recently, we established E2f1-deficient NOD/severe combined immunodeficiency disease (NOD/SCID.E2f1$^{-/-}$) mice [26]. Normally, simultaneous stimulation with both isoproterenol and pilocarpine, β-adrenergic and muscarinic agonists, respectively, provokes a small volume of salivary secretion in NOD/SCID.E2f1$^{-/-}$ mice. Moreover, NOD/SCID.E2f1$^{-/-}$ mice avoid the development of diabetes, and therefore, NOD/SCID.E2f1$^{-/-}$ mice are considered to be a useful model for the study of dry mouth.

Members of the E2F transcription factor family (E2f1–E2f5) are important regulators of cell proliferation, differentiation, and apoptosis. The most characteristic member of the E2F family is E2f1 [13, 18, 43], which controls the initiation of DNA synthesis and the subsequent transition of cells from the G_0–G_1 to S phase of the cell cycle [22, 34]. In the mouse pancreas, insulin secretion is impaired by E2f1 deficiency via the decrease of K^+ channel inward rectifier 6.2 [3]. Therefore, E2f1 may regulate the transcription of ion channels and thus may be involved in secretory function.

Aquaporins (AQPs) are a family of small integral plasma membrane proteins that primarily transport water across the membrane. So far, 13 mammalian isoforms of AQPs (AQP0–12) have been identified, and each AQP exhibits a unique pattern of expression in epithelial tissues [1]. AQP5 is highly expressed in salivary and in lacrimal glands and plays an important role in the exocrine function of those glands [1]. SS shows an autoimmune exocrinopathy of salivary and lacrimal glands, which results in dry mouth and dry eyes [10]. In human SS patients, AQP5 protein is distributed diffusely in the cytoplasm of salivary glands [46] and in lacrimal glands [35] and may be involved in dry mouth and dry eyes. Therefore, evaluation of AQP5 protein has been considered to be useful to characterize exocrine function.

Polyposia is a clinical sign of dry mouth in humans [44]. Treatment of polyposia is a major approach to care for patients with dry mouth. Analyzing polyposia in experimental animals has been difficult because of the lack of suitable animal models. However, Nakamura et al. [32] reported that muscarinic acetylcholine receptor-knockout mice show a behavior similar to polyposia by analysis with a novel method: the frequency of water intake preceded by eating was measured using video recording under noninvasive conditions. Using this useful approach, we conclusively demonstrate that NOD/SCID.E2f1$^{-/-}$ mice show the behavior of dry mouth under physiological conditions. Furthermore, we demonstrate the involvement of AQP5 in the hyposalivation of NOD/SCID.E2f1$^{-/-}$ mice.

Materials and methods

Materials

Bovine serum albumin (BSA) and the protease inhibitor cocktail were purchased from Roche (Basel, Switzerland). Anti-rabbit and anti-mouse IgG horseradish peroxidase-linked antibodies were purchased from Beckman Coulter (Fullerton, CA, USA). Mouse anti-ZO-1 antibody, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-mouse IgG, and ProLong Gold antifade reagent with DAPI were purchased from Molecular Probes (Eugene, OR, USA). ECL western blotting detection reagents and the immunoprecipitation starter pack were purchased from GE (Piscataway, NJ, USA). Pilocarpine and formalin were purchased from Wako (Osaka, Japan). Rabbit anti-AQP5 antibody was purchased from Millipore (Temecula, CA, USA). Mouse anti-actin antibody was purchased from Abcam (Cambridge, MA, USA). Rabbit anti-ubiquitin antibody was purchased from Cell Signaling (Beverly, MA, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Block Ace was purchased from Yukijirushi-Nyugyo (Sapporo, Japan). OCT compound was purchased from Sakura Finetechnica (Tokyo, Japan). Paper plugs were purchased from Morita (Osaka, Japan). Isoflurane was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). SYBR *Premix Ex Taq* II was purchased from TAKARA BIO (Shiga, Japan).

Animals

The experimental design was approved in accordance with the guidelines established by the Animal Office at the National Institute of Infectious Diseases (approval numbers: 209124, 210111, and 211125). The generation of NOD/SCID.E2f1$^{-/-}$ and NOD/SCID mice has been previously described [26]. NOD/SCID mice were used as a control because the salivary flow of NOD/SCID mice is similar to BALB/c mice [38] and prediabetic NOD mice [26, 38].

Measurement of saliva secretion

Male NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (16–20 weeks of age) were anesthetized by 1 % isoflurane inhalation in air after which saliva secretion was modulated by intraperitoneal injection of pilocarpine (0.05 mg/100 g body weight). The secreted saliva was then absorbed onto paper plugs inserted into the oral cavity. The saliva-saturated plugs were weighed and corrected for the original weight of the paper plug. The volume of secreted saliva was calculated as the increase in weight of the paper plug.

Assessment of prandial water drinking

The behavior of polyposia was analyzed according to the method previously reported by Nakamura et al. [32]. Briefly, mice were fasted overnight with free access to water. In the morning, each mouse was fed with about 3 g of dry pellet food (type CMF; Oriental Yeast Co., Tokyo, Japan) or wet paste food, prepared by mixing the powder form of pellet food (type CMF) with 1.5 times its weight of water, and eating behavior was recorded for 1 h using a video camera. The number of approaches to the water nozzle preceded by eating and the time of drinking were measured.

Preparation of lysates of parotid and submandibular glands

Mice were sacrificed by CO_2 asphyxiation, and their parotid and submandibular glands were removed. These glands were homogenized in 10 mM HEPES buffer (pH 7.2) containing 0.3 M sucrose, 2 mM EDTA, 0.2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail and then were centrifuged at 750×*g* for 10 min to remove the nuclear fraction. The supernatant was collected as the lysate fraction.

Reverse transcription/polymerase chain reaction

Total RNA was isolated from parotid and submandibular glands using TRIzol, isopropyl alcohol, and 75 % ethanol. Primers were synthesized as described previously [33]. Specific primers used were M_1R (sense: AGAAGAGGCTGCCACAGGTA, antisense: CAGACCCCACCTGGACTTTA), M_2R (sense: GAATGGGGATGAAAAGCAGA, antisense: GCAGGGT GCACAGAAGGTAT), M_3R (sense: CACAGCC AAGACCTCTGACA, antisense: ATGATGTT GTAGGGGGTCCA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: TCCACCACCCTGTTGCTGTA, antisense: ACCACAGTCCATGCCATCAC). Reverse transcription/polymerase chain reaction (RT-PCR) was performed with 1 μg total RNA in the One-Step RT-PCR kit master mix. Amplification reactions were performed at 94 °C for denaturation, 56 °C for annealing, and 72 °C for extension with 30 cycles in a TAKARA Thermal Cycler Dice mini (TAKARA). PCR products were separated by 2 % agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV illumination.

Real-time RT-PCR

Real-time PCR analysis was performed using the primers shown in Table S1. Real-time RT-PCR was performed on TAKARA Thermal Cycler Dice Real Time System *II* using the SYBR *Premix Ex Taq* II. The PCR reaction consisted of 12.5 μl of SYBR *Premix Ex Taq* II, 400 nM of forward and reverse primers, and 2.0 μl of template cDNA in a total volume of 25 μl. Cycling was performed using the Thermal Cycler Dice Real Time System Software 4.02 according to the manufacturer's instructions: 30 s at 95 °C, followed by 40 rounds of 5 s at 95 °C and 30 s at 60 °C. To verify that the used primer pair produced only a single product, a dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 60 to 95 °C. Relative RNA equivalents for each sample were obtained by normalizing to GAPDH, β-actin, β2 microglobulin (B2M), or hypoxanthine guanine phosphoribosyl transferase (HPRT) levels. Each sample was run in duplicate to determine sample reproducibility.

Immunoprecipitation

For IP, submandibular lysates containing 1 mg of protein were incubated overnight at 4 °C with 0.8 μg/ml of rabbit anti-AQP5 or anti-ubiquitin antibodies using the immunoprecipitation starter pack.

Western blot analysis

Protein concentrations in lysate fractions were determined by the method of Bradford [5]. Proteins were resolved by SDS-PAGE and were then transferred to nitrocellulose or polyvinylidene difluoride membranes (12.5 V, overnight). The membranes were blocked at room temperature for 50 min in Block Ace and then were probed for 120 min with the primary antibodies, rabbit anti-AQP5 (diluted

1:500), rabbit anti-ubiquitin (diluted 1:500), or mouse anti-actin (1:1,000). The blots were washed three times with 10 % Block Ace containing 0.05 % Tween 20 and were then probed for 90 min with the appropriate secondary antibody, anti-rabbit or anti-mouse IgG (diluted 1:10,000). Immunoreactivity was determined by the use of ECL western blotting detection reagents. Intensities of immunoreactivity were measured using a GS-800 densitometer (Bio-Rad, Hercules, CA, USA) and were analyzed using Quantity One Software version 4.6.1 (Bio-Rad).

Histological examination by hematoxylin and eosin staining

The parotid and submandibular glands removed as described above were fixed in neutral phosphate buffered 3.7 % formalin for 24 h. Following paraffin embedding, tissue sections were cut at 1 μm, deparaffinized, and stained with hematoxylin and eosin (HE). Three randomly selected areas of HE-stained glands (objective lens ×40, Olympus BX51) were visualized with 2D image analysis software WinROOF (Mitani, Japan). Images were prepared with Photoshop CS3 (Adobe, San Jose, CA, USA), and the areas concerning total gland and duct cells were blacked out visually. Pixel numbers of the black painted areas were counted with ImageJ version 1.33u (NIH, Bethesda, MD, USA), and duct cell areas were subtracted from total gland areas (total area) to calculate the areas of acinar cells (acinar area). Finally, the ratio of acinar cells to the total gland was calculated and depicted graphically.

Immunohistochemistry

The glands removed as described above were cut into equivalently sized sections, immediately embedded in OCT compound, and rapidly frozen in liquid nitrogen. Frozen sections were cut at a 7-μm thickness and mounted on APS-coated slides. Sections were dried at room temperature for 3 h and were then post-fixed in 100 % methanol at room temperature for 1 min and washed with PBS. Tissue sections were blocked with BSA/goat IgG in PBS. Sections were probed with the primary antibodies, rabbit anti-AQP5 (diluted 1:100) and mouse anti-ZO-1 (diluted 1:200) overnight at 4 °C. After three washes, the sections were probed for 2 h with the appropriate secondary antibodies, Alexa 488-labeled donkey anti-rabbit IgG (diluted 1:200) and Alexa 568-labeled donkey anti-mouse IgG (diluted 1:200) at room temperature. After three washes, to stain nuclei and to encapsulate the specimens, sections were embedded with Pro Long Gold antifade reagent with DAPI. The stained specimens were observed using an LSM5 Exciter (Carl Zeiss, Germany) confocal microscope and analyzed using LSM Image Browser (Carl Zeiss).

Statistical analysis

Statistical differences were determined by Student's *t* test. *P* values below 0.05 are regarded as statistically significant and are indicated by asterisks.

Results

Saliva secretion

First, we examined the effect of the cholinergic agonist pilocarpine on saliva secretion in NOD/SCID.E2f1$^{-/-}$ mice. Figure 1a, b shows the flow rates of saliva in each 1 min period and the cumulative amounts of saliva secreted in 10 min, respectively, following pilocarpine injection. The flow rates of saliva secretion in NOD/

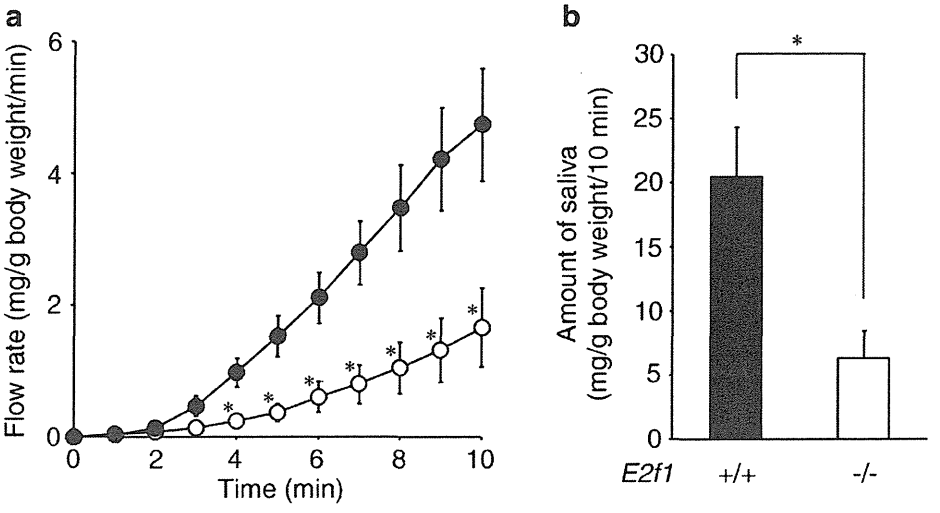

Fig. 1 Pilocarpine-induced saliva secretion in NOD/SCID.E2f1$^{-/-}$ mice. NOD/SCID.E2f1$^{-/-}$ mice (*open circles*) and NOD/SCID mice (*solid circles*) were stimulated by pilocarpine at 0 min. The volume of secreted saliva is indicated by the flow rate every 1 min (**a**). The total amount of saliva secreted in 10 min is indicated (**b**). +/+, NOD/SCID mice; −/−, NOD/SCID.E2f1$^{-/-}$ mice. Values are means ± SE from four independent experiments. *$P<0.05$, compared with NOD/SCID mice at each period (**a**) and total amount (**b**)

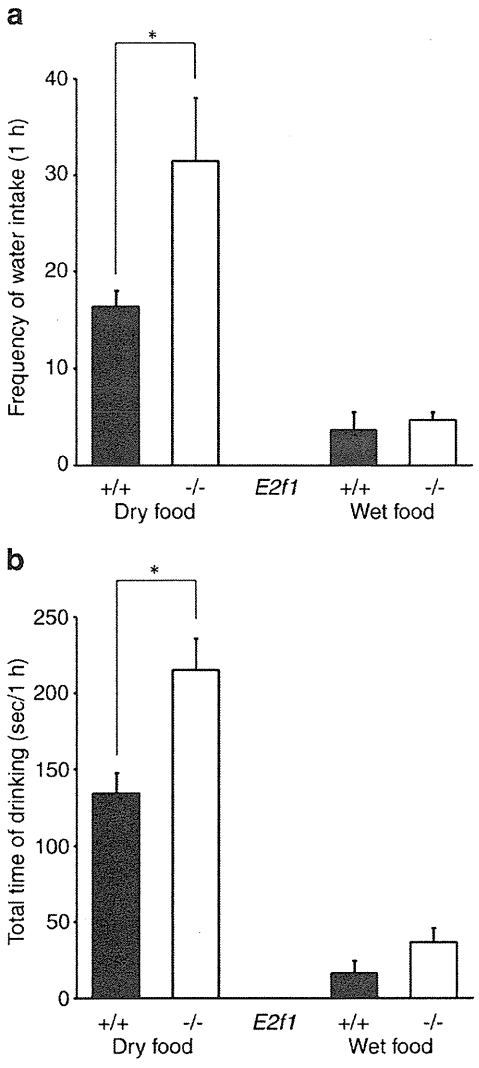

Fig. 2 Behavior of water drinking by NOD/SCID.E2f1$^{-/-}$ mice. The mice were fasted overnight, and in the morning, they were fed with dry pelleted food or with wet paste food. The frequency of water intake (**a**) and total time of drinking (**b**) are indicated. Values are means ± SE from eight independent experiments. +/+, NOD/SCID mice; −/−, NOD/SCID.E2f1$^{-/-}$ mice. *P<0.05

SCID.E2f1$^{-/-}$ mice were clearly decreased at 4–10 min after treatment (n=4; *P<0.05, compared with NOD/SCID mice at each period). The cumulative amounts of saliva secreted over 10 min were quite different between NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice, 20.5±3.9 mg (g body weight)$^{-1}$ (n=4) and 6.3±2.2 mg (g body weight)$^{-1}$, respectively (n=4; *P<0.05, compared with NOD/SCID mice). In addition, body weight and gland weight were not significantly different between NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (data not shown). These results strongly support previous studies [26, 42] and indicate that NOD/SCID.E2f1$^{-/-}$ mice secrete lower amounts of saliva following cholinergic stimulation.

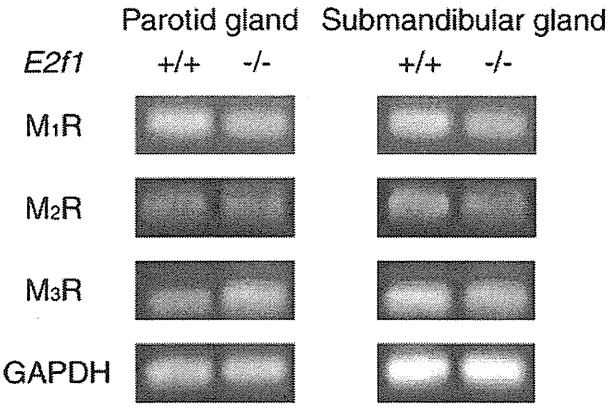

Fig. 3 mRNA levels of muscarinic receptors in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Muscarinic acetylcholine receptor subtypes M_1R, M_2R, M_3R and the housekeeping gene GAPDH were detected by RT-PCR (1 μg RNA) in parotid and in submandibular glands. +/+, NOD/SCID mice; −/−, NOD/SCID.E2f1$^{-/-}$ mice

Prandial drinking

In order to examine saliva secretion in NOD/SCID.E2f1$^{-/-}$ mice without exogenous agonist stimulation, we next studied the eating behavior of NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice. The results are shown in Fig. 2 and in Electronic supplementary materials (Fig. S1 and Movie S1). During eating of dry pelleted food, the control NOD/SCID mice rarely approached the water bottle nozzle (16.4±1.6 approaches in 60 min; n=7). In contrast, the eating behavior of NOD/SCID.E2f1$^{-/-}$ mice was frequently interrupted by drinking, resulting in a significantly greater number of approaches to the water bottle nozzle (31.4±6.6 approaches in 60 min; n=7). Such behavioral features are similar to those of salivarectomized rats [9]. On the other hand, when wet paste food was eaten instead of the dry pelleted food, no significant difference (n=3) in the frequency of prandial drinking was observed between NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice, 3.7±1.9 and 4.7±0.8 approaches in 60 min, respectively (Fig. 2a). As shown in Fig. 2b, the tendency of total time in touching the water nozzle was similar to the frequency of prandial drinking. In addition, the amount of water intake was not significantly different between NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (data not shown). These observations strongly suggest that polyposia in NOD/SCID.E2f1$^{-/-}$ mice is caused by hyposalivation.

Muscarinic receptor expression

We further investigated the characteristics of salivary glands in NOD/SCID.E2f1$^{-/-}$ mice. To elucidate whether the decrease of pilocarpine-induced saliva secretion was caused by receptor expression, we examined mRNA levels of muscarinic receptor subtypes M_1–M_3 in the parotid and submandibular

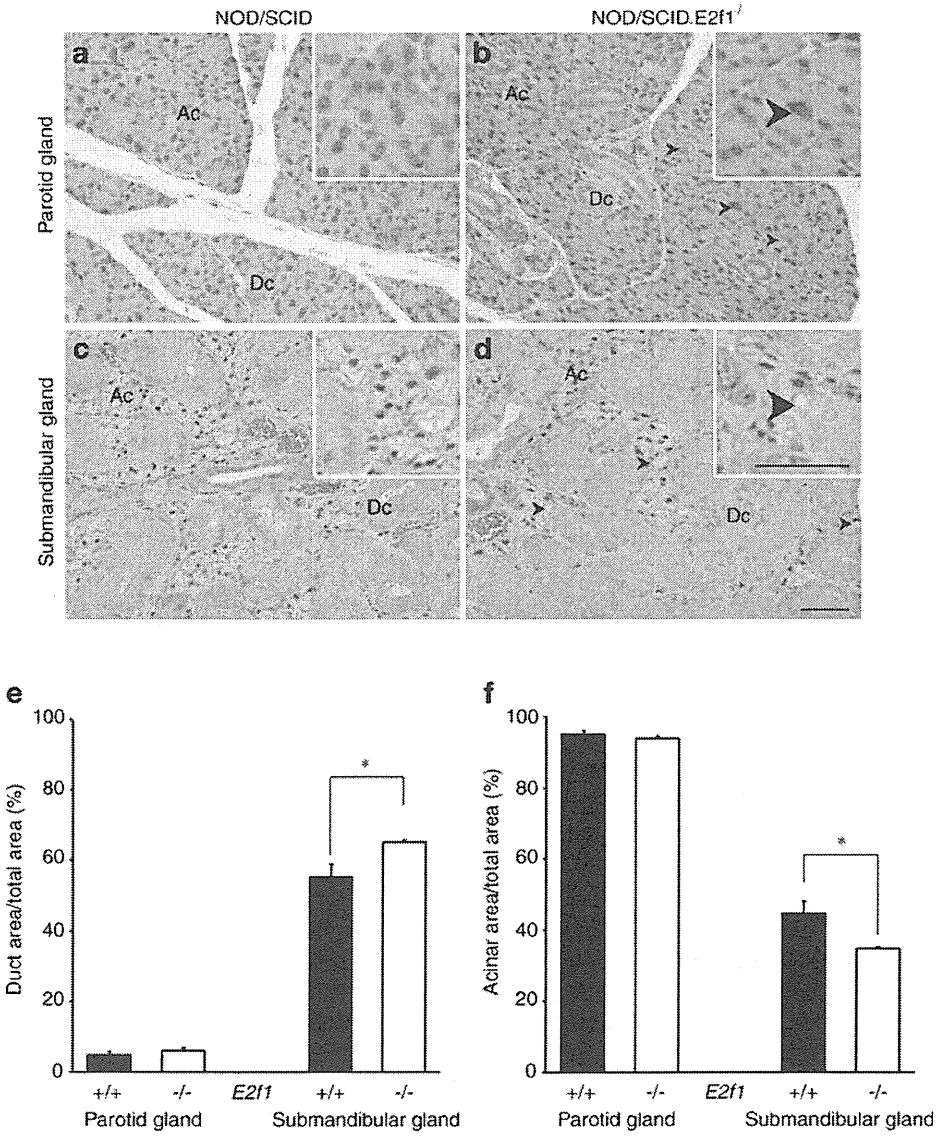

Fig. 4 Histological change of acinar/duct structures in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Histological sections of parotid (**a**, **b**) and submandibular (**c**, **d**) glands of NOD/SCID mice (**a**, **c**) and NOD/SCID.E2f1$^{-/-}$ mice (**b**, **d**) were stained by hematoxylin and eosin. The ratios of duct area/total area (**e**) and acinar area/total area (**f**) are represented from photographs. *Arrows* indicate abnormal features such as nuclear morphology (**b**) or vacuoles (**d**) in acinar cells. *Ac* acinar cells, *Dc* duct cells. *Scale bar*, 20 μm. +/+, NOD/SCID mice; −/−, NOD/SCID.E2f1$^{-/-}$ mice. Values are means ± SE from five independent experiments. *P<0.05

glands by RT-PCR. Levels of GAPDH were used for internal reference. The mRNA levels of M_1R, M_2R, and M_3R in the salivary glands were not different between the NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (Fig. 3). In real-time RT-PCR, mRNA expression levels of muscarinic receptors relative to β-actin, B2M, HPRT (data not shown), or GAPDH (Fig. S2) were no quantitative alteration in the submandibular gland. These results suggest that the decrease of pilocarpine-induced saliva secretion was not caused by changes in muscarinic receptor expression in the salivary glands.

Histological analysis

Matsui-Inohara et al. [26] reported that NOD/SCID.E2f1$^{-/-}$ mice have a tendency for increased numbers of duct cells. In order to confirm the increase of duct cells induced by E2f1 deficiency, we next performed a histological examination of the parotid and submandibular glands (Fig. 4a–d). Interestingly, the nuclear size was larger in parotid gland cells in NOD/SCID.E2f1$^{-/-}$ mice compared with NOD/SCID mice (arrowheads, Fig. 4b). Further, vacuoles were detected in submandibular gland cells in NOD/SCID.E2f1$^{-/-}$ mice (arrowheads, Fig. 4d). As Fig. 4e shows, the duct areas of submandibular glands in NOD/SCID.E2f1$^{-/-}$ mice (65.3 ±0.6 %, n=5) were larger than in NOD/SCID mice (55.4±3.5 %, n=5) (*P<0.05). In contrast, as Fig. 4f shows, the acini areas in NOD/SCID.E2f1$^{-/-}$ mice (34.7 ±0.6 %, n=5) were smaller than in NOD/SCID mice (44.6±3.5 %, n=5) (*P<0.05). These observations indicate that duct cells were increased while at the same time the acinar cells were decreased, suggesting that

histological changes such as the decrease of acinar cells may cause the decrease of saliva secretion in NOD/SCID.E2f1$^{-/-}$ mice.

AQP5 expression

To further evaluate the decrease in the number of acinar cells, we examined the expression of AQP5 protein, a marker of acinar cells, in the parotid and submandibular glands by western blotting. The expression of actin (Fig. 5a) was used for internal reference. The expression levels of AQP5 in both the parotid and submandibular glands of NOD/SCID.E2f1$^{-/-}$ mice were significantly lower than in the NOD/SCID mice (Fig. 5a). Expression levels of another water channel, AQP6, were not changed in those mice (data not shown). Figure 5b shows the relative intensity of AQP5 expression in the parotid and submandibular glands of NOD/SCID and of NOD/SCID.E2f1$^{-/-}$ mice. These results indicate that the expression of AQP5 protein in NOD/SCID.E2f1$^{-/-}$ mice is markedly decreased compared with the NOD/SCID mice.

AQP5 localization

AQP5 is localized in the apical membranes of salivary acinar cells and is believed to be involved in fluid secretion [27, 29]. We examined the localization of AQP5 in the parotid and submandibular glands of NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (Fig. 6). AQP5 was localized in the apical membranes of acinar cells in salivary glands, but not in duct cells. Interestingly, the localization of AQP5 in NOD/SCID.E2f1$^{-/-}$ mice was different from that in NOD/SCID mice. In contrast, ZO-1, a tight junction protein, was not different. In NOD/SCID.E2f1$^{-/-}$ mice, AQP5 was diffused and leaked from ZO-1 (arrowheads, Fig. 6d, f, j, and l), indicating that AQP5 may not localize at the apical membrane but rather in the cytosol near the apical membrane. In NOD/SCID mice, AQP5 was localized in the space framed by ZO-1 (arrows, Fig. 6a, c, g, and i). These observations indicate that apical membrane-localized AQP5 in NOD/SCID.E2f1$^{-/-}$ mice is less than in NOD/SCID mice, suggesting that the change of AQP5 localization may cause the decrease in saliva secretion in NOD/SCID.E2f1$^{-/-}$ mice.

AQP5 ubiquitination

To elucidate whether the change of AQP5 expression and localization results from AQP5 degradation, we next examined the ubiquitination of AQP5 in submandibular glands of NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (Fig. 7). The band of ubiquitinated-AQP5 (Ub-AQP5) was detected in NOD/SCID.E2f1$^{-/-}$ mice, but in contrast, this band was not detected in NOD/SCID mice. The bands detected at about 50 kDa are considered to be rabbit IgG, because another rabbit antibody was detected at the same position (data not shown). These results indicate that the ubiquitination of AQP5 protein occurs in NOD/SCID.E2f1$^{-/-}$ mice.

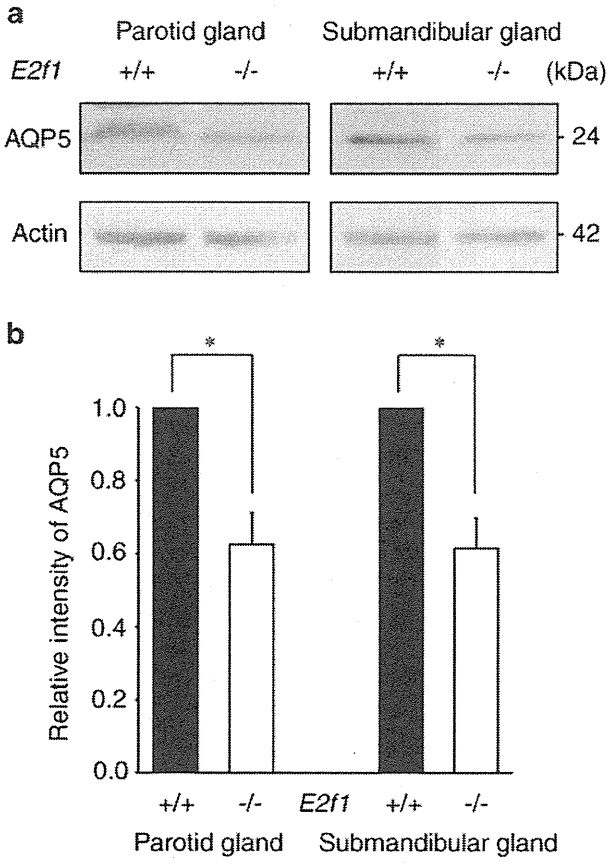

Fig. 5 AQP5 protein expression in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Parotid and submandibular glands were analyzed by western blotting (30 μg protein). AQP5 was detected using an anti-AQP5 antibody. Actin used as the internal reference (**a**). The intensities of the immunoreactive bands were quantified and normalized to that of the NOD/SCID mice samples. Immunoblot images are representative of data from four independent experiments, and values in the histograms are means ± SE of all four experiments (**b**). +/+, NOD/SCID mice; −/−, NOD/SCID.E2f1$^{-/-}$ mice. *$P<0.05$

Discussion

In the present study, we demonstrated that (1) NOD/SCID.E2f1$^{-/-}$ mice have the behavior of dry mouth, (2) duct cells increase and acinar cells decrease in the salivary glands of NOD/SCID.E2f1$^{-/-}$ mice, and (3) AQP5 protein is diffusely localized in the apical membrane of salivary acini in NOD/SCID.E2f1$^{-/-}$ mice.

Saliva secretion is controlled by the autonomic nervous system. Activation of the parasympathetic nerve leads to the secretion of salivary fluid, whereas the sympathetic nerve

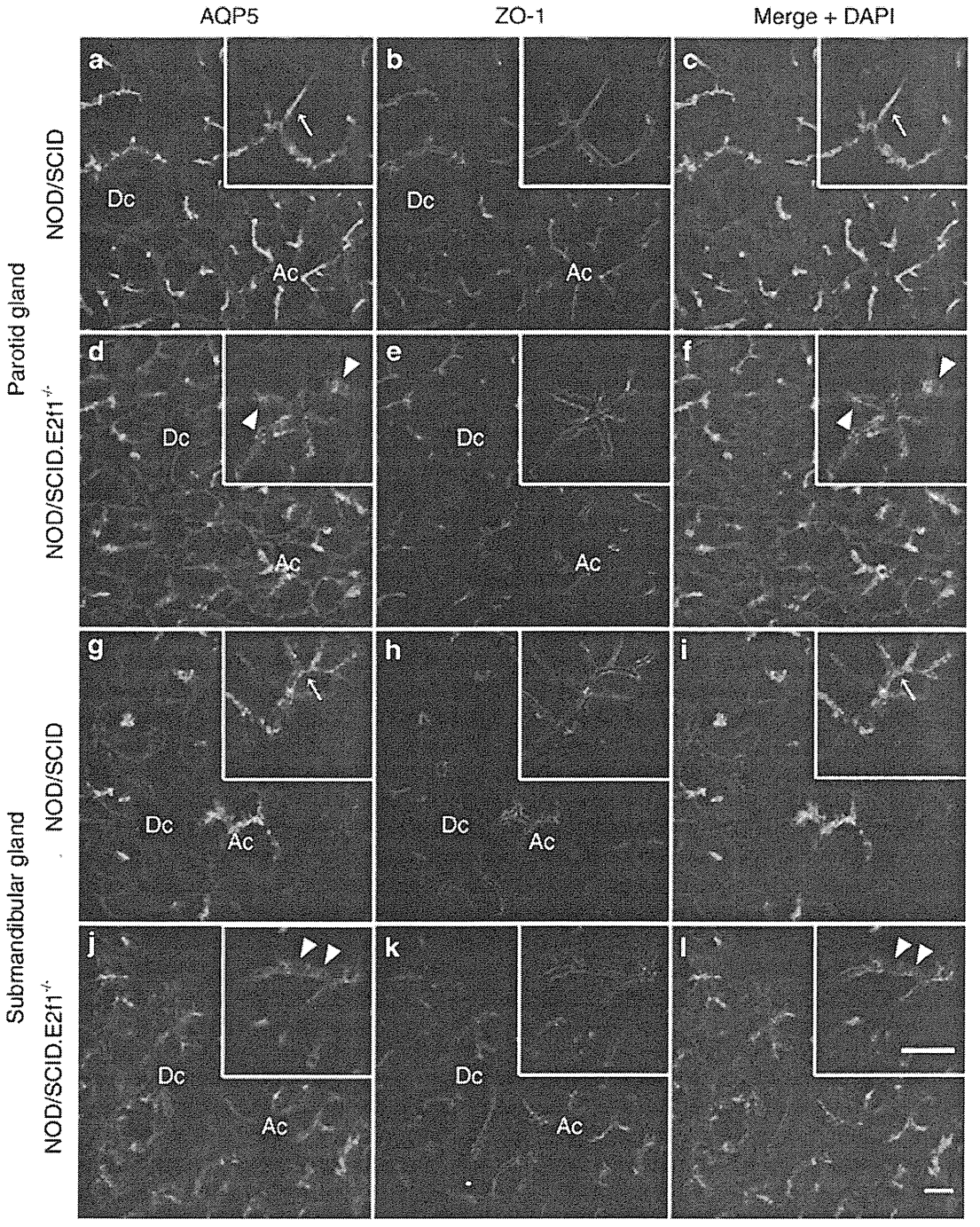

Fig. 6 AQP5 localization in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Cryosections of parotid (**a–f**) and submandibular (**g–l**) glands of NOD/SCID mice (**a–c**, **g–i**) and NOD/SCID.E2f1$^{-/-}$ mice (**d–f**, **j–l**) are immunostained by anti-AQP5 and anti-ZO-1 antibodies after which fluorescence was observed by confocal microscopy. Green fluorescence of Alexa488 shows the localization of AQP5. Red fluorescence of Alexa568 shows the localization of ZO-1. Nuclei were stained with DAPI as *blue*. *Arrows* indicate the normal localization of AQP5. *Arrowheads* indicate the diffuse localization of AQP5. *Ac* acinar cells, *Dc* duct cells. *Scale bar*, 10 μm

activation leads to secretion of salivary protein [37]. Cholinergic agonist stimulation mimics the activation of the parasympathetic nerve. In NOD/SCID mice, E2f1 deficiency led to a 30.7 % decrease in pilocarpine-induced saliva secretion (Fig. 1). In C57BL/6 mice, E2f1 deficiency causes a 44.5 % decrease in saliva secretion provoked by simultaneous stimulation with isoproterenol and pilocarpine [28]. In NOD mice, E2f1 deficiency caused a 61.3 % decrease in

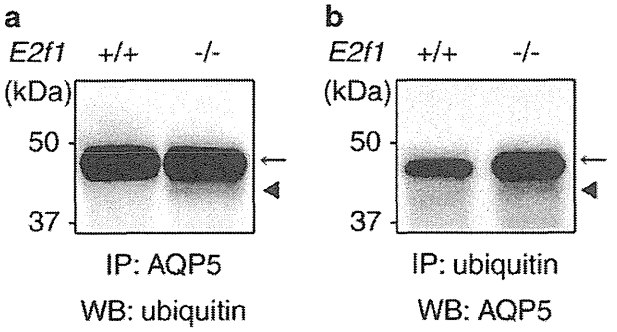

Fig. 7 AQP5 ubiquitination in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Submandibular gland lysates were immunoprecipitated with the anti-AQP5 (**a**) or anti-ubiquitin (**b**) antibodies, and then western blot analysis was performed (10 µl sample). Ub-AQP5 was detected using the anti-ubiquitin (**a**) or anti-AQP5 (**b**) antibodies. *Arrows* indicate the rabbit IgG. *Arrowheads* indicate the Ub-AQP5. *IP* immunoprecipitation, *WB* western blot analysis; +/+, NOD/SCID mice; −/−, NOD/SCID.E2f1$^{-/-}$ mice

saliva secretion induced by the stimulation with isoproterenol and pilocarpine [39]. In NOD/SCID mice, E2f1 deficiency results in a 63.0 % decrease in saliva secretion stimulated with isoproterenol and pilocarpine simultaneously [26]. These observations suggest that hyposalivation occurs in NOD/SCID.E2f1$^{-/-}$ mice. To characterize hyposalivation in physiological conditions, we further recorded prandial water drinking by NOD/SCID.E2f1$^{-/-}$ mice who had fasted overnight. When the NOD/SCID.E2f1$^{-/-}$ mice were fed dry food, the frequency and time of water intake were almost double compared with NOD/SCID mice (Fig. 2 and Electronic supplementary materials). On the other hand, there were no differences in water intake by either type of mouse when fed wet food. It has been reported that changes in drinking behavior, represented by an increase in water intake, are characteristic of animals with severe hyposalivation and are thought to be a form of compensatory behavior for insufficient saliva secretion elicited by dry food [4, 9, 12, 32]. Therefore, NOD/SCID.E2f1$^{-/-}$ mice have the behavior of hyposalivation in physiological conditions.

Salivary glands are mainly composed of two epithelial cell types, acinar and duct cells: acinar cells secrete the salivary fluid as well as most of the salivary proteins, and duct cells secrete some protein and modify the ionic composition of the saliva as they convey it to the mouth [48]. We also demonstrated the effect of E2f1 deficiency on the acinar/duct structure in NOD/SCID and NOD/SCID.E2f1$^{-/-}$ mice (Fig. 4). The E2f1 deficiency affects the ratio of acinar area/duct area in submandibular glands, but not in parotid glands. In parotid glands of NOD/SCID mice, acinar cells represent a high proportion of the gland. Thus, parotid glands contain few duct cells, meaning that an increase of the duct area cannot be detected by the histology of HE staining. Furthermore, the protein levels of AQP5, a marker of acinar cells, were decreased both in parotid and in submandibular glands of NOD/SCID.E2f1$^{-/-}$ mice (Fig. 5). Therefore, the number of duct cells increases and that of the acinar cells decreases in NOD/SCID.E2f1$^{-/-}$ mice compared with NOD/SCID mice. We previously reported that E2f1 deficiency significantly decreases levels of fibroblast growth factor receptor 2IIIb (FGFR-2b) in submandibular duct cells of NOD/SCID mice [26]. The transcription of FGFR-2b is activated by E2f1 [47]. The interaction between FGF-10, a member of the FGF family, and FGFR-2b is required for the development of mouse submandibular glands [16, 30, 36]. Further, FGF-10 heterozygous mice are viable and fertile but display hypoplasia of the submandibular and the lacrimal glands [8, 17]. Therefore, the disappearance of FGFR-2b induced by E2f1 deficiency suppresses FGF-10 signaling, inhibiting the maturation of duct cells. As a result, immature duct cells increase and acinar cells have no space to mature, causing the volume of saliva production to be decreased. Taken together, NOD/SCID.E2f1$^{-/-}$ mice have less volume of saliva because of the decrease in acinar cells, although further studies to characterize that mechanism are needed.

AQP5 is thought to play a fundamental role in water transport during the formation of saliva, tears, and other exocrine secretions [20]. In AQP5 knockout mice, saliva is significantly hypertonic and viscous, and the volume is smaller, directly indicating that AQP5 has an essential role in the normal secretion of saliva [25]. Interestingly, we found that AQP5 is diffusely localized near the apical membrane of parotid and submandibular acinar cells of NOD/SCID.E2f1$^{-/-}$ mice (Fig. 6). This localization of AQP5 in NOD/SCID.E2f1$^{-/-}$ mice was different from that in NOD/SCID mice. This diffuse localization occurs without functionality of the muscarinic receptors because in vivo muscarinic stimulation did not result in any significant translocation of AQP5 in rat parotid gland [11]. In the rats with an AQP5 point mutation, the mutant AQP5 failed to localize to the apical membrane in submandibular glands [19] and parotid glands [40]. The mutation has been considered to result in less efficient membrane trafficking and increase in lysosomal degradation of AQP5, which leads to lower expression of AQP5 in apical membranes of acinar cells [17] and consequently causes the mutant rats to have less saliva [31]. Therefore, it is conceivable that the diffuse localization of AQP5 causes to reduce fluid secretion in NOD/SCID.E2f1$^{-/-}$ mice. It has been reported that PACE4, a member of the subtilisin-like proprotein convertase family, is involved in the transcriptional activation of AQP5 during rat salivary acinar differentiation [2] and the transcription of PACE4 is up-regulated by E2f1 [49]. Therefore, AQP5 appears to be mutated by E2f1 deficiency via the down-regulation of PACE4 in NOD/SCID mice.

We also demonstrated that AQP5 ubiquitination occurs in NOD/SCID.E2f1$^{-/-}$ mice but not in NOD/SCID mice (Fig. 7). Protein ubiquitination usually occurs in sequence: addition of the first ubiquitin, mono-ubiquitination (molecular weight 7.5–8 kDa). After mono-ubiquitination, the attached ubiquitin can attach other ubiquitins (poly-ubiquitination) [6]. Ub-AQP5 in NOD/SCID.E2f1$^{-/-}$ mice was detected at about 47 kDa, which is about 24 kDa larger than non-Ub-AQP5. The increment in molecular weight is consistent with the size of three ubiquitins suggesting that AQP5 is poly-ubiquitinated in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Ubiquitin is best known for targeting protein destruction by proteasomes [6]. Therefore, we hypothesize that AQP5 is diffusively localized from the apical membrane causing AQP5 dysfunction, and subsequently, AQP5 is degraded via ubiquitination, causing the AQP5 protein level to be decreased in NOD/SCID.E2f1$^{-/-}$ mice. When AQP1 [23] or AQP4 [7] are ubiquitinated, their protein levels decrease in BALB/c fibroblasts or rat optic nerves, respectively. Those reports strongly support the present study, although further studies to characterize the mechanism involved are needed.

Because the salivary flow in NOD/SCID mice is similar to control mice [26, 38], we used NOD/SCID mice used as the control. However, the effects of the NOD gene background and the SCID mutation have not been clear. NOD mice have been reported to have lymphocyte infiltrates in their parotid and submandibular glands compared with BALB/c mice [15]. In contrast, there is a report that lymphocytic infiltrates of NOD mice were observed in submandibular glands, but not in parotid glands [45]. The SCID mutation has been reported to be involved in duct development [38]. In contrast, Matsui-Inohara et al. reported that the SCID mutation has no effect on duct development [26]. It has been reported that AQP5 in NOD mice is increased in the basolateral membranes of parotid and submandibular acinar cells compared with BALB/c mice [21]. On the other hand, it has been reported that AQP5 expression is increased in apical membranes of parotid acinar cells and in basolateral membranes of submandibular acinar cells in NOD mice compared with BALB/c mice [45]. Further study is needed to examine the effect of the NOD gene background and the SCID mutation on salivary gland function.

In conclusion, AQP5 shows a diffuse localization, ubiquitination, and decreased expression in NOD/SCID.E2f1$^{-/-}$ mice salivary glands. Additionally, the decrease of acinar cells and the increase in duct cells are observed. It is conceivable that these events trigger hyposalivation of NOD/SCID.E2f1$^{-/-}$ mice, although further study with central action regarding hyposalivation is needed.

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