Animal Models

Epidermal Hyperplasia and Appendage Abnormalities in Mice Lacking CD109

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CD109, a glycosylphosphatidylinositol-anchored glycoprotein, is highly expressed in several types of human cancer tissues, in particular, squamous cell carcinomas. In normal human tissues, human CD109 expression is limited to certain cell types including myoepithelial cells of the mammary, lacrimal, salivary, and bronchial glands and basal cells of the prostate and bronchial epithelium. Although CD109 has been reported to negatively regulate transforming growth factor- β signaling in keratinocytes *in vitro*, its physiologic role *in vivo* remains largely unknown. To investigate the function of CD109 *in vivo*, we generated CD109-deficient (*CD109*^{-/-}) mice. Although *CD109*^{-/-} mice were born normally, transient impairment of hair growth was observed. At histologic analysis, kinked hair shafts, ectatic hair follicles with an accumulation of sebum, and persistent hyperplasia of the epidermis and sebaceous glands were observed in *CD109*^{-/-} mice. Immunohistochemical analysis revealed thickening of the basal and suprabasal layers in the epidermis of *CD109*^{-/-} mice, which is where endogenous CD109 is expressed in wild-type mice. Although CD109 was reported to negatively regulate transforming growth factor- β signaling, no significant difference in levels of Smad2 phosphorylation was observed in the epidermis between wild-type and *CD109*^{-/-} mice. Instead, Stat3 phosphorylation levels were significantly elevated in the epidermis of *CD109*^{-/-} mice compared with wild-

type mice. These results suggest that CD109 regulates differentiation of keratinocytes via a signaling pathway involving Stat3. (Am J Pathol 2012; 181:1180–1189; <http://dx.doi.org/10.1016/j.ajpath.2012.06.021>)

CD109, a glycosylphosphatidylinositol-anchored cell surface glycoprotein, is a member of the α_2 -macroglobulin/C3, C4, C5 family of thioester-containing proteins.^{1–4} CD109 is a cell surface antigen expressed on a subset of fetal and adult CD34⁺ bone marrow mononuclear cells, phytohemagglutinin-activated T lymphoblasts, thrombin-activated platelets, leukemic megakaryoblasts, endothelial cells, and mesenchymal stem cell subsets.^{5–7} In addition, human CD109 is expressed in a limited number of cell types in normal tissues such as myoepithelial cells of the mammary, lacrimal, salivary, and bronchial glands and basal cells of the prostate and bronchial epithelium.^{8–12} High levels of CD109 expression have also been detected in various tumor cell lines and in tumor tissues including squamous cell carcinomas (SCCs) of the lung, esophagus, uterus, and oral cavity; malignant melanoma of the skin; and urothelial carcinoma of the urinary bladder.^{8–16} CD109 expression was significantly higher in well-differentiated SCCs of the oral cavity and in low-grade urothelial carcinomas of the urinary bladder than in moderately or poorly differentiated SCCs and in high-grade urothelial carcinomas, respectively.^{14,16} These findings suggest that CD109 expression is strictly controlled in normal tissues and is associated with tumor development.

Signals through the transforming growth factor (TGF)- β receptor system induce a wide range of biological responses including cell proliferation, differentiation, migration, and apop-

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tosis; tissue remodeling; immune response; and angiogenesis.^{17–19} Ligand-mediated assembly of TGF- β receptors I and II (TGFBR1 and TGFBR2, respectively) initiates an intracellular phosphorylation cascade; activated TGFBR2 transphosphorylates TGFBR1, which subsequently phosphorylates receptor-regulated Smads (R-Smads) such as Smad2/3, which in turn enables the R-Smads to bind a common mediator, Smad4. R-Smad/Smad4 complexes accumulate in the nucleus, where they act as transcription factors for target genes.^{20–22} TGF- β -mediated receptor activation also induces inhibitory Smads (I-Smads) such as Smad7, which compete with R-Smads in binding to activated TGFBR1, thereby negatively regulating signals.²³

CD109 functions as a negative regulator of TGF- β signaling in human keratinocytes; CD109, as a component of the TGF- β receptor system, inhibits activation of R-Smad, probably by direct modulation of receptor activity.^{24,25} Our recent study using cultured cells showed CD109 to be cleaved by furin, generating 180- and 25-kDa fragments; the 180-kDa fragment is partially secreted into the medium.²⁶ The negative effect of CD109 on TGF- β signaling requires this furin-mediated cleavage of CD109 because the resulting 180-kDa fragment is responsible for this negative effect.²⁶ CD109 associates with caveolin-1, a major component of caveolae, and promotes localization of TGFBRs into caveolar compartments to facilitate their degradation.²⁷ Although these findings show the importance of CD109 in the TGF- β signaling pathway *in vitro*, its physiologic functions *in vivo* have not yet been elucidated.

Recombinant CD109 regulates signal transducers and activators of transcription 3 (STAT3) activation in human keratinocytes.²⁸ Various cytokines and growth factors, such as IL-6, IL-20, IL-22, and epidermal growth factor, activate STAT3 in keratinocytes,^{29,30} whereas TGF- β suppresses STAT3 activation through IL-6 in epithelial cells.^{31,32} STAT3 is critical to such biological activities as cell proliferation, differentiation, migration, survival, and oncogenesis.^{29,30}

In the present study, we generated CD109-deficient mice (CD109^{-/-} mice) to investigate the physiologic roles of CD109 *in vivo*. The CD109^{-/-} mice showed transient impairment of hair growth, accompanied by kinked hair shafts, ectatic hair follicles with an accumulation of sebum, and persistent hyperplasia of the epidermis and sebaceous glands. These findings suggest that CD109 has a role in the normal development of skin.

Materials and Methods

Targeting Construct

Construction of the targeting vector started by modifying a pBlueScript II KS vector (Agilent Technologies, Inc., Santa Clara, CA) containing a LacZ reporter with a mouse nuclear localization signal upstream of a phosphoglycerine kinase (PGK) promoter driving a neomycin-resistant gene (*neo*) selection marker. The CD109 5' homology arm (1666 bp) was generated via PCR with PfuUltra High-Fidelity DNA polymerase (Agilent Technologies) using

Figure 1. Generation of mice lacking CD109. **A:** Schematic representation of the targeting vector. A portion of exon 1 and all of exon 2 were replaced by the *lacZ*-*PGK*-*neo* cassette, which was placed in-frame with the ATG start codon of CD109 (closed triangle). *DTa*, diphtheria toxin A gene; *neo*, neomycin-resistant gene. **B:** Mouse genotyping via Southern blot analysis. SpeI-digested genomic DNA isolated from the tails of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) CD109 mice were hybridized using the 5' probe shown in **A**. SpeI digestion yielded a 3.6-kb fragment for the wild-type allele and a 6.4-kb fragment for the mutant allele. **C:** Mouse genotyping using PCR. Primer positions are indicated by arrows (P1–P4) in **A**. PCR products amplified with P1 and P2 (wild-type allele) are 205 bp, and those with P3 and P4 (mutant allele) are 605 bp.

genomic DNA of the 129S6 mouse strain as a template and inserted into the EcoRV site of the vector. The CD109 3' homology arm (4893 bp) of intron 2 was inserted into the NotI site. This targeting vector (Figure 1A, middle) for the CD109 locus (Figure 1A, top) was designed to insert a *lacZ*-*PGK*-*neo* cassette 17 amino acids downstream from the methionine start, resulting in disruption of the remainder of the first coding exon and the entire second exon. Structure of the targeted CD109 allele is shown in Figure 1A (bottom). The final targeting vector was confirmed via DNA sequencing and restriction mapping.

Generation of CD109 Knockout/*lacZ* Knockin Mice

The targeting vector was linearized by digestion with KpnI, and introduced by electroporation into embryonic stem cells derived from 129S6 mice. After G418/diphtheria toxin A positive/negative selection, 10 embryonic stem cell clones with successful homologous recombination were isolated via Southern blot screening of SpeI-digested genomic DNA with a 5' probe (Figure 1A). One clone was injected into C57BL/6J blastocysts; chimeric mice were generated by PhoenixBio Co., Ltd. (Higashihiroshima, Japan). The genetic background of the mice used in the present study was C57BL6J/129S6. All mice were housed in polycarbonate cages containing hardwood chip bedding, at 25°C on a 12-hour light-dark cycle. All animal protocols were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (approval ID number 23121).

Table 1. List of Primary Antibodies

Antibodies to	Clone	Source	Application	Dilution	Pretreatment
CD109	C-9/mouse monoclonal	Santa Cruz Biotechnology	Immunoblotting	1:1000	NA
CD109	M-250/rabbit polyclonal	Santa Cruz Biotechnology	Immunoperoxidase	1:500	Autoclave
Smad7	H-79/rabbit polyclonal	Santa Cruz Biotechnology	Immunofluorescence	1:20	Water bath
β -Actin	AC-74/mouse monoclonal	Sigma-Aldrich	Immunoblotting	1:5000	NA
CD3	Rabbit polyclonal	Dako	Immunoperoxidase	1:500	Water bath
CD45R	RA3-6B2/rat monoclonal	eBioscience	Immunoperoxidase	1:3200	Water bath
Gr-1	RB6-8C5/rat monoclonal	eBioscience	Immunoperoxidase	1:100	Proteinase K, room temperature for 10 min
p63	4A4/mouse monoclonal	Sigma-Aldrich	Immunoperoxidase	1:100	Water bath
CK10	Rabbit polyclonal	Covance	Immunoperoxidase	1:1000	Water bath
CK14	AF64/rabbit polyclonal	Covance	Immunoperoxidase	1:1000	Water bath
Filaggrin	Rabbit polyclonal	Covance	Immunoperoxidase	1:500	Water bath
BrdU	B44/mouse monoclonal	BD Biosciences	Immunoperoxidase	1:100	2 nmol/L HCl, room temperature for 30 min
C-caspase-3	Rabbit polyclonal	Cell Signaling Technology	Immunoperoxidase	1:100	Water bath
pSmad2	Rabbit polyclonal	Cell Signaling Technology	Immunofluorescence	1:100	Water bath
(Ser465/467)			Immunoblotting	1:1000	NA
Smad2	Mouse monoclonal	Cell Signaling Technology	Immunoblotting	1:1000	NA
pStat3 (Tyr705)	Rabbit monoclonal	Cell Signaling Technology	Immunofluorescence	1:20	Water bath
			Immunoblotting	1:2000	NA
Stat3	Rabbit monoclonal	Cell Signaling Technology	Immunoblotting	1:2000	NA

NA, not applicable.

Genotyping of Mice After Germline Transmission

Genomic DNA from offspring was extracted from their tails. Genotyping of mice was performed via PCR, based on four primers: primer P1 (forward), 5'-GTCCGCGC-TTCTGGTGACAG-3'; primer P2 (reverse), 5'-GTGT-GACTGTTAGACAGTGCAG-3'; primer P3 (forward), 5'-CCATCGCCATCTGCTGCACG-3'; and primer P4 (reverse), 5'-ACGATCCTGAGACTTCCACAC-3' (Figure 1A). The PCR with rTaq polymerase (Takara Bio Inc., Ohtsu, Japan) was performed as follows: 96°C for 2 minutes; 32 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The expected PCR product sizes from wild-type and targeted alleles were 205 and 603 bp, respectively.

Tissue Preparation

After body weight measurement, mice were sacrificed under general anesthesia. Complete necropsies were performed, and resected organs were cut into 5-mm³ specimens and quickly frozen for protein extraction.

Antibodies

Primary antibodies used in the present study included anti-CD109 and anti-Smad7 monoclonal or polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti- β -actin and anti-p63 monoclonal antibodies (Sigma-Aldrich Corp., St. Louis, MO); anti-CD3 polyclonal antibody (Dako, Glostrup, Denmark), anti-CD45R and anti-Gr-1 monoclonal antibodies (eBioscience, Inc., San Diego, CA); anti-CK10, anti-CK14, and anti-filaggrin polyclonal antibodies (Covance, Inc., Princeton, NJ); anti-

BrdU (5'-bromo-2'-deoxyuridine) monoclonal antibody (BD Biosciences, San Jose, CA); and anti-cleaved caspase-3, anti-phospho-Smad2 (pSmad2), anti-Smad2, anti-phospho-Stat3 (pStat3), and anti-Stat3 monoclonal or polyclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA). Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody was purchased from Invitrogen Corp. (Carlsbad, CA), and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was purchased from Dako (Table 1).

Western Blot Analysis

Frozen mouse tissues were homogenized in SDS sample buffer (62.5 mmol/L Tris HCl [pH 6.8], 2% SDS, 25% glycerol, and 20 μ g/mL bromophenol blue) and sonicated until the tissues were no longer viscous. After measuring protein concentration using the DC Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA), the lysates were boiled at 100°C for 2 minutes in the presence of 2% β -mercaptoethanol. The lysates, containing 40 μ g proteins, were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 1 hour at room temperature in Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) with gentle agitation, and incubated with the primary antibody for 1 hour at room temperature. After the membranes were washed three times with Tris-buffered saline solution and Tween 20 buffer (20 mmol/L Tris HCl [pH 7.6], 137 mmol/L NaCl, and 0.1% Tween 20), they were incubated with secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. After the membranes were washed, the reaction was visualized using the ECL Detection Kit (GE Healthcare,

Buckinghamshire, UK) according to the manufacturer's instructions.

Histologic Analysis

The major organs were resected as described above. All tissues were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Sections 4 μm thick were prepared for H&E staining (which was performed using conventional methods) and immunohistochemistry (IHC). Epidermal thickness of the dorsal skin was measured from the basal lamina to the lower border of the stratum corneum using WinROOF software version 5.04 (Mitani Corp., Fukui, Japan). Lipid accumulation was visualized via Oil Red O (Sigma-Aldrich) staining on frozen sections of formalin-fixed skin tissues.

In Situ Hybridization

After anesthetization, the mice were perfused intravascularly with 4% (w/v) paraformaldehyde solution, and organs were resected and frozen in 2-methylbutane cooled in liquid nitrogen. Frozen sections 10 μm thick were prepared using a cryostat (Leica Microsystems GmbH, Wetzlar, Germany).

CD109-specific PCR products of ~400 bp with SP6 and T7 promoter fragments were generated using primers 5'-CCAAGCTATTTAGGTGACACTATAGAGAAGTGAACCTTCTCAGTGGC-3' and 5'-TGAATTGTAATACGACTCACTATAGGGGCACAAAGTACAGAAGGACGG-3'. The PCR products were gel purified and transcribed *in vitro* using SP6 or T7 RNA polymerase (Roche Applied Science, Penzberg, Germany) incorporating ^{33}P -UTP (PerkinElmer, Inc., Waltham, MA). SP6 RNA polymerase generated the sense probe as a control, and T7 RNA polymerase generated the antisense probe. The probes were subsequently DNase treated and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Slides were hybridized overnight at 60°C using 200 μL per slide hybridization solution consisting of 1×10^6 cpm radiolabeled probe and hybridization buffer (50% formamide, 10% dextran sulfate, 0.5 mol/L NaCl, $1 \times$ Denhardt's solution, 10 mmol/L Tris [pH 8.0], 1 mmol/L EDTA, 500 $\mu\text{g/mL}$ yeast transfer RNA, and 10 mmol/L dithiothreitol). After hybridization, slides were immersed in $2 \times$ standard saline citrate solution for 15 minutes at room temperature, and RNase buffer (20 $\mu\text{g/mL}$ RNase A, 0.5 mol/L NaCl, 10 mmol/L Tris [pH 8.0], and 1 mmol/L EDTA) for 30 minutes at 37°C. After intensive washing and dehydration, slides were dipped in twice-diluted Kodak Autoradiography Emulsion (type NTB; Eastman Kodak Co., Rochester, NY) and dried at room temperature for 30 minutes in a dark-room. Slides were then stored at 4°C for approximately 2 weeks in the dark, and were developed using Kodak D19 Developer and Fixer (Eastman Kodak).

Immunohistochemistry

Paraffin sections were prepared as described above. Slides were deparaffinized in xylene, and rehydrated in a graded series of ethanol. For antigen retrieval, they were

immersed in Target Retrieval Solution [pH 9.0] (Dako) and heated for 15 minutes at 121°C via autoclaving or incubated for 30 minutes at 100°C in a water bath. In the case of staining with anti-Gr-1 antibody, slides were pre-treated with proteinase K (100 $\mu\text{g/mL}$; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 minutes at room temperature for antigen retrieval. Nonspecific binding was blocked with 10% normal goat serum for 10 minutes at room temperature. Sections were incubated with primary antibodies for 1 hour at room temperature. Endogenous peroxidase was inhibited using 3% hydrogen peroxide in PBS for 15 minutes. The slides were incubated with the secondary antibody conjugated to horseradish peroxidase-labeled polymer (EnVision+; Dako) for 15 minutes at room temperature, except for the slides incubated with anti-CD45R or anti-Gr-1 antibodies, which were incubated with N-Histofine Simple Stain Mouse MAX PO (Rat) (Nichirei Biosciences, Inc., Tokyo, Japan), for 30 minutes at room temperature. Reaction products were visualized using diaminobenzidine (Dako); nuclear counterstaining was performed using hematoxylin.

BrdU Incorporation Analysis

Wild-type (CD109^{+/+}) and homozygous (CD109^{-/-}) mice were injected intraperitoneally with BrdU (Sigma-Aldrich), 5 mg per 100 g body weight, at postnatal day (P) 14 and P28. After 2 hours, the mice were sacrificed under general anesthesia, and dorsal skin was resected. The skin tissues were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Sections 4 μm thick were prepared on slides. The slides were deparaffinized, rehydrated, and immersed in 2 mol/L HCl for 30 minutes at room temperature for antigen retrieval. Immunohistochemical analysis using anti-BrdU antibody was performed as described above.

Immunofluorescence Staining

Deparaffinization, hydration, antigen retrieval, and blocking steps were performed as described above. The sections were incubated with primary antibody for 2 hours at room temperature. The slides were incubated with Alexa Fluor 488 (1:500)-labeled secondary antibody for 30 minutes at room temperature. The images were visualized under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Cell Culture

Both wild-type and CD109-deficient keratinocytes were isolated from neonatal (P0) dorsal skin using the CELLnTEC Advanced Cell Systems (CELLnTEC, Bern, Switzerland). They were maintained in fully defined, low-calcium (0.07 mmol/L) medium, CnT-07 (CELLnTEC) in an incubator at 37°C and 5% CO₂. They were then subcultured via trypsinization and plated on 3.5-cm dishes in CnT-07 medium. At 70% to 80% confluency, cells were starved for 6 hours in medium free of growth factor. They were washed once, treated with 0.1 nmol/L

TGF- β_1 (PeproTech, Inc., Rocky Hill, NJ) for 1 to 4 hours, and then lysed as described above.

In Vivo Wound Healing Assay

Male *CD109*^{+/+} and *CD109*^{-/-} sibling mice (approximately 22 to 28 g body weight and 8 to 12 weeks old) were anesthetized; their dorsal skin was shaved and swabbed with 70% ethanol before the procedure. For wounding, 4-mm punch biopsies (Kai Industries Co., Ltd., Seki, Japan) were performed on the shaved area. Wounds were separated by a minimum of 6 mm of uninjured skin. The diameter of the wound areas was measured at 0, 1, 3, 5, and 7 days after wounding, and wound closure was evaluated as a percentage of the initial wound size.

Statistical Analysis

The Student's *t*-test was used to analyze differences in epidermal thickness, wound healing, and IHC-positive ratios between *CD109*^{-/-} and *CD109*^{+/+} mice. *P* < 0.05 was considered significant.

Results

Generation of *CD109* Knockout/*lacZ* Knockin Mice

To inactivate the *CD109* gene, we engineered a targeting vector in which the 3' part of exon 1 and the entire exon 2 were replaced with a *lacZ*-PGK-*neo* cassette (Figure 1A). The *lacZ* sequence was placed in-frame with the start codon of *CD109*. Male chimeras were generated from one of the targeted embryonic stem cell clones, and germline transmission of the targeted locus was ascertained by mating to C57BL/6J females. The genotypes of wild-type (*CD109*^{+/+}), heterozygous (*CD109*^{+/-}), and homozygous (*CD109*^{-/-}) mice were determined using Southern blot analysis (Figure 1B) and PCR (Figure 1C). Both heterozygous and homozygous mice were born normally, and there was no significant difference in body weight or life span among wild-type, heterozygous, and homozygous mice.

Mouse *CD109* Is Expressed in Skin and Testes

To assess tissue distribution of *CD109*, whole lysates from various organs of *CD109*^{+/+} and *CD109*^{-/-} mice were prepared and subjected to Western blot analysis using anti-*CD109* antibody. Two bands with molecular masses of ~150 and ~180 kDa were detected in the lysates from skin and testes of *CD109*^{+/+} mice, but were undetectable in *CD109*^{-/-} mice, indicating that the two bands were *CD109*-specific (Figure 2A). The bands detected in cerebrum, cerebellum, and liver were nonspecific because they were detected in both *CD109*^{+/+} and *CD109*^{-/-} mice. *CD109* mRNA expression in the skin and testes was also examined via *in situ* hybridization. *CD109*-specific signal was detected in the epidermis and the seminiferous tubules of *CD109*^{+/+} mice but not in *CD109*^{-/-} mice (Figure 2B).

Figure 2. *CD109* is expressed in the skin and testes in mice. **A:** Western blot analysis for *CD109* expression in various tissues of adult *CD109*^{+/+} and *CD109*^{-/-} sibling mice. *CD109*-specific bands of ~150 and ~180 kDa were detected in the skin and testes of *CD109*^{+/+} mice (arrows), but were undetectable in *CD109*^{-/-} mice. Blots probed with anti- β -actin antibody are shown as a loading control. **B:** *In situ* hybridization for *CD109* mRNA expression in the skin and testes. ³²P-labeled antisense ribonucleotide probes for *CD109* were hybridized to frozen sections of the skin and testes of 6-week-old *CD109*^{+/+} and *CD109*^{-/-} mice. *CD109* mRNA was detected in the epidermis of the skin and the seminiferous tubules of the testes of *CD109*^{+/+} mice but not in *CD109*^{-/-} mice. Arrowheads indicate the boundary of the epidermis. Signals were captured by darkfield microscopy. Scale bars = 100 μ m. **C:** Immunohistochemical analysis of *CD109* expression in mice. *CD109* protein is expressed in the squamous epithelium of the skin and tongue and the seminiferous tubules of the testes of adult *CD109*^{+/+} mice but not in *CD109*^{-/-} mice. Images of dorsal skin, sole skin, and tongue are the same magnification. Scale bars = 100 μ m.

We subsequently performed IHC analysis of endogenous *CD109* protein expression in various mouse tissues. *CD109* protein was expressed in squamous epithelium of the skin and tongue, and seminiferous tubules of the testes in *CD109*^{+/+} mice but not *CD109*^{-/-} mice (Figure 2C). *CD109* expression was detected in the basal and suprabasal layers of the epidermis, including the infundibular portion of the hair follicle (Figure 2C). Tissue distribution of *CD109* was compatible with the results of X-gal staining for *LacZ* expression in *CD109*-deficient (*lacZ* knockin) mice (data not shown). Mouse *CD109* expression was not detected in either myoepithelial cells of the mammary and salivary glands or in bronchial basal cells.

CD109^{-/-} Mice Exhibit Transient Impairment of Hair Growth

Macroscopically, impairment of hair growth in *CD109*^{-/-} mice was first observed at P7, and persisted until P28 (Figure 3A). Hair of *CD109*^{-/-} mice was much sparser and less directed than hair of *CD109*^{+/+} mice. Hair growth then recovered, and no severe impairment was

Figure 3. $CD109^{-/-}$ mice develop skin abnormalities. **A:** Macroscopic images of hair growth impairment in a $CD109^{-/-}$ mouse. Images of the dorsal skin of a $CD109^{-/-}$ mouse and its $CD109^{+/+}$ sibling were obtained every 7 days (not all images are shown). Impairment of hair growth in the $CD109^{-/-}$ mouse was apparent from P7 to P28, but then hair growth recovered, and no severe impairment was observed after P35. **B:** Microscopic images of skin abnormalities in $CD109^{-/-}$ mice. H&E-stained sections of the dorsal skin were prepared from $CD109^{+/+}$ and $CD109^{-/-}$ mice at each age. Hair follicles of $CD109^{-/-}$ mice exhibited ectasia from P14 to P21 (arrowheads), and hair shafts were kinked at P14 (arrow). Hyperplasia of the epidermis and sebaceous glands was first observed at P7, and remained at P70. All images are the same magnification. Scale bar = 100 μ m. **C:** Oil Red O staining of the skin of $CD109^{+/+}$ and $CD109^{-/-}$ mice. Accumulation of sebum in the ectatic hair follicles was apparent in $CD109^{-/-}$ mice at P14 (arrowheads). Scale bar = 50 μ m. **D:** Comparison of epidermal thickness between $CD109^{+/+}$ and $CD109^{-/-}$ mice. H&E-stained sections of the dorsal skin were prepared from $CD109^{+/+}$ and $CD109^{-/-}$ mice at each age ($n = 3$), and epidermal thickness of interfollicular epidermis was measured as described in *Materials and Methods*. Values are given as mean $\pm$ SD. NS, not significant. * $P < 0.05$; ** $P < 0.01$.

observed after P35 (Figure 3A). However, hair direction remained irregular in $CD109^{-/-}$ mice after P35. All $CD109^{-/-}$ mice exhibited the same phenotype to varying degrees. $CD109^{-/-}$ mice, generated from $CD109^{+/+}$ mice backcrossed 10 times to the C57BL/6J strain, also showed the same phenotype, eliminating the influence of mouse genetic background of C57BL6J/129S6. No apparent abnormalities were observed in other organs, including testes in $CD109^{-/-}$ mice.

CD109 Deficiency Results in Epidermal Hyperplasia and Appendage Abnormalities

To examine histologic aberrations causing transient impairment of hair growth in $CD109^{-/-}$ mice, H&E-stained

skin specimens were prepared from P0 to P70 $CD109^{+/+}$ and $CD109^{-/-}$ mice (Figure 3B). There were no apparent differences in hair follicles between $CD109^{+/+}$ and $CD109^{-/-}$ mice at P0, P3, and P5 (Figure 3B; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>) or in inferior segments of hair follicles at P7 (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). At P14, some hairs of $CD109^{-/-}$ mice failed to penetrate the epidermis, and their shafts were kinky or zigzagged (Figure 3B), whereas all hairs of $CD109^{+/+}$ mice penetrated the epidermis. Many hair follicles of $CD109^{-/-}$ mice at P21 were ectatic; some did not have hair shafts (Figure 3B). However, the number of hair follicles did not significantly differ between $CD109^{+/+}$ and $CD109^{-/-}$ mice from P0 to P70 except at P21, when hair follicles of $CD109^{-/-}$ mice were difficult to count because of distortion of the hair follicles. Oil Red O staining of the skin of $CD109^{-/-}$ mice revealed accumulation of sebum in the ectatic hair follicles (Figure 3C). Inflammatory cell infiltration was observed in the dermis of $CD109^{-/-}$ mice, which was accompanied by the appearance of skin appendage abnormalities (Figure 3B; see also Supplemental Figure S2 at <http://ajp.amjpathol.org>). The infiltrating cells included $CD3^{+}$ cells (T lymphocytes), $Gr-1^{+}$ cells (neutrophils), and a small number of $CD45R^{+}$ cells (B lymphocytes) (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Hyperplasia of the epidermis and sebaceous glands became apparent at P7 in $CD109^{-/-}$ mice (Figure 3B; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>), and were sustained until P70. Epidermal thickness of the dorsal skin was quantified by measuring the length between the basal lamina and the lower border of the stratum corneum; differences between $CD109^{+/+}$ and $CD109^{-/-}$ mice (three mice per group) were statistically analyzed. Significant differences were observed between the two groups at all time points after P7 except for P35 and P42 (Figure 3D). Epidermal hyperplasia was also observed at infundibular portions of hair follicles in $CD109^{-/-}$ mice. Epidermal hyperplasia was noted in dorsal skin and in sole skin, which does not have hair (Figure 4B). These findings indicated that CD109 deficiency causes epidermal hyperplasia accompanied by ectatic hair follicles and impairment of normal hair growth.

Thickening of the Basal and Suprabasal Layers Is the Cause of Epidermal Hyperplasia

To further investigate the epidermal hyperplasia, the dorsal skin epidermis of $CD109^{+/+}$ and $CD109^{-/-}$ mice was analyzed at IHC using proliferation, differentiation, and apoptotic markers (Figure 4, A and C). Compared with epidermis in $CD109^{+/+}$ mice, the epidermis of $CD109^{-/-}$ mice exhibited apparent thickening of the basal and suprabasal layers, which were positive for the basal cell markers p63 and CK14.^{33,34} Thickness of the spinous and granular layers, which were positive for CK10 and filaggrin, respectively, were similar between $CD109^{+/+}$ and $CD109^{-/-}$ mice. We also examined the sole skin of adult $CD109^{+/+}$ and $CD109^{-/-}$ mice at P56. Epidermal hyperplasia and basal and suprabasal layer thickening

Figure 4. CD109 deficiency causes thickening of the epidermal basal and suprabasal layers. **A** and **B**: Immunohistochemical analysis of the epidermis of *CD109*^{+/+} and *CD109*^{-/-} mice. Dorsal (**A**) and sole (**B**) skin sections from *CD109*^{+/+} and *CD109*^{-/-} mice were immunostained using antibodies against p63, cytokeratin (CK) 14, CK10, and filaggrin. Thickening of the basal and suprabasal layers, which were positive for p63 and CK14, was observed in *CD109*^{-/-} mice compared with *CD109*^{+/+} mice. No apparent difference was observed in the spinous and granular layers, which were positive for CK10 and filaggrin, respectively, between *CD109*^{+/+} and *CD109*^{-/-} mice. All images in **A** and **B** are the same magnification. Scale bars = 100 μ m. **C**: The ratios of BrdU-positive cells or cleaved caspase-3-positive cells in epidermis of *CD109*^{+/+} and *CD109*^{-/-} mice. Whereas no significant difference was detected in cleaved caspase-3-positive ratio between *CD109*^{+/+} and *CD109*^{-/-} mice, the BrdU-positive ratio was significantly increased in the epidermis of *CD109*^{-/-} mice compared with *CD109*^{+/+} mice at P14 and P28 ($n = 3$). NS, not significant. * $P < 0.05$; ** $P < 0.01$.

were observed in the glabrous epidermis of *CD109*^{-/-} mice (Figure 4B), which suggested that the presence of hair follicles is not related to epidermal hyperplasia in CD109-deficient mice. Proliferation of the basal cells was assessed via BrdU incorporation, and apoptosis of keratinocytes was assessed by cleaved caspase-3 staining. Although no significant difference in the cleaved caspase-3-positive ratio was detected between the two mouse groups (Figure 4D), the BrdU-positive ratio was significantly increased in the epidermis of *CD109*^{-/-} mice compared with *CD109*^{+/+} mice (Figure 4C; see also Supplemental Figure S3 at <http://ajp.amjpathol.org>). These findings suggest that CD109 regulates proliferation and differentiation of keratinocytes.

Effect of CD109 Deficiency on TGF- β Signal Is Undetectable in Epidermis

CD109 is a component of the TGF- β receptor complex, and inhibits TGF- β /Smad signaling *in vitro*.^{25–27} To understand the mechanism underlying the epidermal hyperplasia and appendage abnormalities in CD109-deficient mice, we examined the influence of CD109 deficiency on TGF- β /Smad signaling using fluorescence IHC. Smad2

phosphorylation, which is reportedly increased by knock-down of CD109 expression,²⁶ was assessed in the epidermis by staining with anti-pSmad2 antibody. However, we found no significant difference in the number of pSmad2-positive cells in the epidermis between *CD109*^{+/+} and *CD109*^{-/-} mice (four mice per group) from P7 to P28 (Figure 5A; see also Supplemental Figure S4 at <http://ajp.amjpathol.org>). We also examined expression of the TGF- β -inducible protein Smad7 in the epidermis, which negatively regulates the strength and duration of TGF- β signaling.³⁵ No apparent difference in Smad7 staining was observed between *CD109*^{+/+} and *CD109*^{-/-} mice (data not shown).

Figure 5. TGF- β signal activation was undetectable, but Stat3 phosphorylation was enhanced in the epidermis of CD109-deficient mice. **A**: Fluorescence immunostaining of pSmad2 in the epidermis of *CD109*^{+/+} and *CD109*^{-/-} mice. Dorsal skin sections from *CD109*^{+/+} and *CD109*^{-/-} mice at P14 were immunostained using an antibody against pSmad2. A similar nuclear staining was observed in epidermis of both *CD109*^{+/+} and *CD109*^{-/-} mice. Nuclear counterstaining was performed using DAPI. All images are the same magnification. Scale bar = 100 μ m. **B**: Fluorescence immunostaining of pStat3 in the epidermis of *CD109*^{+/+} and *CD109*^{-/-} mice. Dorsal skin sections from *CD109*^{+/+} and *CD109*^{-/-} mice at P14 were immunostained using an antibody against pStat3. The level of pStat3 was elevated in the epidermis of *CD109*^{-/-} mice compared with *CD109*^{+/+} mice. Nuclear counterstaining was performed using DAPI. Scale bar = 100 μ m. **C**: Time course of pStat3 and pSmad2 after TGF- β ₁ (0.1 nmol/L) stimulation in *CD109*^{+/+} and *CD109*^{-/-} keratinocytes. Phosphorylation of Stat3 and Smad2 was determined using Western blot analysis. Levels of pSmad2 induced by TGF- β ₁ in *CD109*^{+/+} and *CD109*^{-/-} keratinocytes were almost the same at each time point, whereas levels of pStat3 in *CD109*^{-/-} keratinocytes were significantly elevated under growth factor-free conditions compared with those in *CD109*^{+/+} keratinocytes. Expression of total Stat3 and Smad2 is also shown. Expression of β -actin is shown as a loading control. **D**: *In vivo* wound healing assay in *CD109*^{+/+} and *CD109*^{-/-} mice. The assay was performed as described in Materials and Methods. No significant difference was observed between *CD109*^{+/+} and *CD109*^{-/-} mice.

Stat3 Phosphorylation Was Enhanced in the Epidermis of $CD109^{-/-}$ Mice

Recently, recombinant CD109 protein was reported to both down-regulate TGF- β signaling and up-regulate STAT3 phosphorylation in human N/TERT-1 keratinocytes *in vitro*.²⁸ In addition, STAT3 reportedly affects psoriasis-like epidermal hyperplasia.^{36,37} To examine the effect of CD109 deficiency on Stat3 activation *in vivo*, we assessed Stat3 phosphorylation in the epidermis by staining with anti-pStat3 antibody. We found that Stat3 phosphorylation levels were significantly elevated in the epidermis of $CD109^{-/-}$ mice compared with that of $CD109^{+/+}$ mice, on and after P7 (Figure 5B; see also Supplemental Figure S5 at <http://ajp.amjpathol.org>). We also isolated primary keratinocytes from neonatal (P0) dorsal skin and examined Stat3 phosphorylation and TGF- β /Smad signaling induced by TGF- β_1 in them. Primary keratinocytes were cultured for at least 7 days; Stat3 phosphorylation was assessed in the absence or presence of TGF- β_1 . The level of Stat3 phosphorylation in $CD109^{-/-}$ keratinocytes was high without TGF- β_1 stimulation compared with that in $CD109^{+/+}$ keratinocytes, although the levels of Stat3 phosphorylation were decreased by TGF- β_1 stimulation in both $CD109^{+/+}$ and $CD109^{-/-}$ keratinocytes (Figure 5C). However, the levels of Smad2 phosphorylation induced by TGF- β_1 were almost the same at each time point between $CD109^{+/+}$ and $CD109^{-/-}$ keratinocytes (Figure 5C).

In addition, we performed an *in vivo* wound healing assay to evaluate the effect of CD109 on wound healing, which is regulated not only by TGF- β signaling³⁸ but also by Stat3 phosphorylation.^{29,30,36} The dorsal skin of $CD109^{+/+}$ and $CD109^{-/-}$ mice was wounded via punch biopsy, and wound closure was measured as described in *Materials and Methods*. However, there was no significant difference in wound closure between $CD109^{+/+}$ and $CD109^{-/-}$ mice at each time point (Figure 5D).

Discussion

CD109 is a GPI-anchored cell surface protein that exhibits a confined expression to a limited number of cell types. Using Northern blot analysis, CD109 expression was detected only in human and mouse testes.⁸ Using IHC analyses, CD109 immunoreactivity was detected in human myoepithelial cells of the mammary, salivary, lacrimal, and bronchial glands, and basal cells of the prostate and bronchial epithelia.^{10,11} In the present study, we analyzed mouse CD109 expression using IHC, and detected CD109 in the epidermis of the skin, the squamous epithelium of the tongue, and the seminiferous tubules of the testes. However, we could not detect its expression in myoepithelial cells of the mammary and salivary glands or the basal cells of the bronchial epithelium, which indicates that the expression pattern of mouse CD109 is different from human CD109.

To investigate the function of CD109 *in vivo*, we generated $CD109^{-/-}$ mice, which exhibited skin abnormalities but no apparent abnormalities in other tissues. Skin ab-

normalities in $CD109^{-/-}$ mice included epithelial hyperplasia, primarily due to basal and suprabasal layer thickening, with increased basal cell proliferation, impairment of hair growth, ectatic hair follicles, and sebaceous gland hyperplasia. Among these abnormalities, epithelial hyperplasia could be the primary phenotype as a result of CD109 deficiency because the epidermal hyperplasia was observed not only in the dorsal skin but also in the sole skin, which does not have skin appendages. Impairment of hair growth and ectatic hair follicles may be secondary changes caused by narrowing of the infundibular portion of the hair follicles due to epithelial hyperplasia, because no developmental or differentiation abnormalities were observed at histologic analysis from P0 to P5, and hyperplasia of epidermis and sebaceous glands emerged at P7, before kinked hair shafts and ectatic hair follicles at P14. Inflammatory cell infiltration was also observed in the skin of $CD109^{-/-}$ mice. The infiltrating cells included T lymphocytes, neutrophils, and a small number of B lymphocytes. Although the precise mechanism of infiltration of these cells in the skin of $CD109^{-/-}$ mice remains elusive, it may be due to psoriasis-like skin alterations^{36,37} or to a reaction to accumulation of sebum. Alternatively, the inflammation might contribute to epidermal hyperplasia and hair follicle degeneration. Although CD109 is highly expressed in the seminiferous tubules of the testes in both humans and mice, $CD109^{-/-}$ mice did not exhibit any apparent abnormality in the testes; hence the function of CD109 in the testes remains unclear.

At IHC analyses of human cancer tissues, we have previously reported that CD109 is highly expressed in SCCs of the lung and oral cavity.^{10,14} In our study using SCCs of the oral cavity, CD109 was expressed in all well-differentiated SCCs, but in only 89% and 64% of moderately and poorly differentiated SCCs, respectively, which implies that CD109 expression is correlated with the differentiation stage of SCCs. In the present study, we found that CD109 deficiency results in thickening of the basal and suprabasal cell layers in the epidermis, with increased basal cell proliferation. These findings suggest that CD109 regulates proliferation and differentiation of keratinocytes *in vivo*, even in malignant tumors. Further investigation is necessary to clarify the role of CD109 in the development of cancer in humans.

CD109 is a negative regulator of TGF- β signaling in keratinocytes *in vitro*; overexpression of CD109 inhibits TGF- β signaling, whereas knockdown of CD109 up-regulates its signaling.^{25–28} Because activation of TGF- β signaling suppresses cell proliferation of keratinocytes *in vitro*,¹⁷ we evaluated the status of Smad2, one of the R-Smads, and Smad7, one of the I-Smads,^{20–23} in $CD109^{-/-}$ mice. Cutaneous wound healing is reportedly delayed in transgenic mice that overexpress Smad2 in the epidermis,³⁹ and hyperplasia of the epidermis and sebaceous glands is induced in transgenic mice that overexpress Smad7 in the epidermis.^{35,40} The skin phenotype of Smad7 transgenic mice was similar to that of $CD109^{-/-}$ mice observed in the present study. Epidermal hyperplasia in Smad7 transgenic mice may be due to suppression of R-Smad (eg, Smad2) phosphorylation and a reduction in TGFBR1 and TGFBR11 protein levels,

resulting in blockage of TGF- β -induced growth arrest in keratinocytes.⁴⁰ However, CD109 deficiency influenced neither levels of Smad2 phosphorylation and Smad7 expression nor wound-healing ability in the epidermis of *CD109*^{-/-} mice. These findings suggest either that long-lasting effects of CD109 deficiency on the TGF- β signaling pathway may be masked by feedback signals or other compensatory mechanisms or that CD109 does not modulate TGF- β /Smad2 signaling in mouse epidermis but is associated with a different signaling pathway.

CD109 can reportedly regulate STAT3 activation in human keratinocytes.²⁸ In addition, up-regulation of STAT3 is associated with psoriasis,^{36,37} which is a common disease characterized histologically by epidermal hyperplasia, altered epidermal differentiation, and local accumulation of acute and chronic inflammatory cells.^{30,36,41} We therefore evaluated the level of Stat3 phosphorylation in *CD109*^{-/-} mice. Immunofluorescence analysis showed that Stat3 phosphorylation was elevated in the epidermis of *CD109*^{-/-} mice compared with *CD109*^{+/+} mice. Enhanced Stat3 phosphorylation was also observed in primary keratinocytes isolated from *CD109*^{-/-} mice under cytokine- and growth factor-free conditions, although TGF- β ₁ stimulation markedly down-regulated Stat3 phosphorylation. Stat3 up-regulation is associated with increased proliferation and impaired differentiation of keratinocytes, and results in psoriasis-like skin alterations including hyperkeratosis and acanthosis with inflammatory infiltrates *in vivo*.^{30,36,37,41} This phenotype is similar to the skin abnormalities observed in *CD109*^{-/-} mice. Thus, our findings suggest that CD109 regulates Stat3 activation, which could be associated with epidermal hyperplasia in *CD109*^{-/-} mice. Further analyses of cytokine or growth factor signaling pathways will provide insight into the precise mechanism of skin abnormalities developed in *CD109*^{-/-} mice.

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