

Localization of Serine Racemase and Its Role in the Skin

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D-Serine is an endogenous coagonist of the N-methyl-D-aspartate (NMDA)-type glutamate receptor in the central nervous system and its synthesis is catalyzed by serine racemase (SR). Recently, the NMDA receptor has been found to be expressed in keratinocytes (KCs) of the skin and involved in the regulation of KC growth and differentiation. However, the localization and role of SR in the skin remain unknown. Here, using SR-knockout (SR-KO) mice as the control, we demonstrated the localization of the SR protein in the granular and cornified layer of the epidermis of wild-type (WT) mice and its appearance in confluent WT KCs. We also demonstrated the existence of a mechanism for conversion of L-serine to D-serine in epidermal KCs. Furthermore, we found increased expression levels of genes involved in the differentiation of epidermal KCs in adult SR-KO mice, and alterations in the barrier function and ultrastructure of the epidermis in postnatal day 5 SR-KO mice. Our findings suggest that SR in the skin epidermis is involved in the differentiation of epidermal KCs and the formation of the skin barrier.

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INTRODUCTION

The N-methyl-D-aspartate (NMDA)-type glutamate receptor is one of the three major subtypes of ionotropic glutamate receptors and has a prominent role in the central nervous system (Nakanishi, 1992; Bliss and Collingridge, 1993; Ozawa *et al.*, 1998). Recent studies have demonstrated that in addition to being expressed in central nervous system, the NMDA

receptor is also expressed in keratinocytes (KCs) in the human skin and is involved in the regulation of KC growth and differentiation and in the maintenance of cutaneous barrier homeostasis (Fujiwara *et al.*, 2003; Nahm *et al.*, 2004).

The NMDA receptor is a heteromeric complex consisting of a GluN1 subunit and at least one of the four types of the GluN2 subunit (Kutsuwada *et al.*, 1992). D-Serine, a D-amino acid abundant in the mammalian brain, is an endogenous ligand of the glycine site of the NMDA receptor (Hashimoto *et al.*, 1992). The activation of NMDA receptors requires, besides the binding of glutamate to the GluN2 subunit, the binding of glycine or D-serine to the glycine site on the GluN1 subunit (Dingledine *et al.*, 1999). D-Serine synthesis is catalyzed by serine racemase (SR), an enzyme that is highly abundant in the brain and directly converts L-serine to D-serine (Wolosker *et al.*, 1999). D-Serine is efficacious in potentiating the activity of NMDA receptors (Fadda *et al.*, 1988; Matsui *et al.*, 1995), and its reduction was demonstrated to greatly decrease NMDA receptor activity (Mothet *et al.*, 2000). We previously demonstrated that NMDA receptor-mediated excitotoxicity can be attenuated in SR knockout (SR-KO) mice (Inoue *et al.*, 2008). Although the expression and roles of the NMDA receptor in the skin have been demonstrated, it is still unclear whether SR is localized in the skin epidermis and whether a mechanism of conversion of L-serine to D-serine by SR exists in KCs.

In this study, using SR-KO mice as the control, we localized SR expression by immunohistochemistry and confirmed the localization of this protein in the epidermis of wild-type (WT) mice and cultured WT KCs. We further investigated the

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⁷In accordance with Shiseido's company policy on limitation of animal experiments, these authors performed only *in vitro* assays, such as measurement of amino acids and quantitative real-time PCR analysis.

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Abbreviations: K10, keratin 10; KC, keratinocyte; P5, postnatal day 5; PBS, phosphate-buffered saline; NMDA, N-methyl-D-aspartate; SC, stratum corneum; SG, stratum granulosum; SR, serine racemase; SR-KO, serine racemase-knockout; TEWL, transepidermal water loss; TGase 3, transglutaminase 3; WT, wild type

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conversion of L-serine to D-serine in cultured WT and SR-KO KCs, the expression of proteins involved in KC differentiation, the ultrastructure of the epidermis, and the function of the epidermal barrier.

RESULTS

SR protein expression in epidermis and cultured KCs

We first examined the SR protein expression in the epidermis of the skin by western blot analysis. The SR protein signal was detected in the epidermis of WT mice, but not in the epidermis of SR-KO mice (Figure 1a). The expression level of SR protein in the epidermis was ~100 times lower than that in the brain (data not shown). We next examined the localization of the SR protein in the skin by immunofluorescence staining. As shown in Figure 1b, SR immunopositivity was detected in the granular and cornified layers of the skin in WT mice but not in those of SR-KO mice. To determine SR localization in the epidermal subcompartments, the differentiation markers involucrin and keratin 10 (K10), the former normally present in the granular and cornified layers (Tharakan *et al.*, 2010) and the latter in the spinous and granular layers (Fuchs *et al.*, 1992), were selected for their double immunofluorescence staining with SR. The double staining showed that some immunopositivity signals of SR overlapped with those of involucrin (Figure 1c) but rarely with those of K10 (Figure 1d), suggesting that SR is present mainly in the cornified layer and partially in the granular layer.

We further examined SR protein expression in the cultured KCs derived from the skin of WT or SR-KO mice. In agreement

with the finding of localization of SR in the granular and cornified layers of the skin in WT mice, SR protein expression was detected in confluent cultured WT KCs, but not in growth-phase KCs of this genotype (Figure 2a). In contrast, no SR was detected in either the growth-phase or confluent SR-KO KCs (Figure 2a). Consistent with the findings of immunocytochemical analysis, western blot analysis revealed the absence of SR in the lysates from growth-phase KCs derived from either WT or SR-KO mice. SR immunopositivity signals were detected in the lysates from confluent WT KCs, but not in the lysates from confluent SR-KO KCs (Figure 2b).

Synthesis of D-serine in cultured KCs derived from skin

The ability of SR to convert L-serine to D-serine was analyzed using confluent KCs derived from the skin of WT or SR-KO mice. Under our culture conditions without addition of L-serine, the concentration of intracellular D-serine was significantly higher in WT KCs than in SR-KO KCs (Figure 3a, left). There was no significant difference in intracellular L-serine concentration between WT and SR-KO KCs (Figure 3a, middle). The addition of 10 mM L-serine to the culture medium resulted in larger increases in intracellular D-serine and L-serine concentrations in WT than in SR-KO KCs (Figure 3b, left and middle). The concentration ratio of D-serine to total serine was significantly higher in WT KCs than in SR-KO KCs under both culture conditions—that is, with and without the addition of L-serine (Figure 3a and b, right).

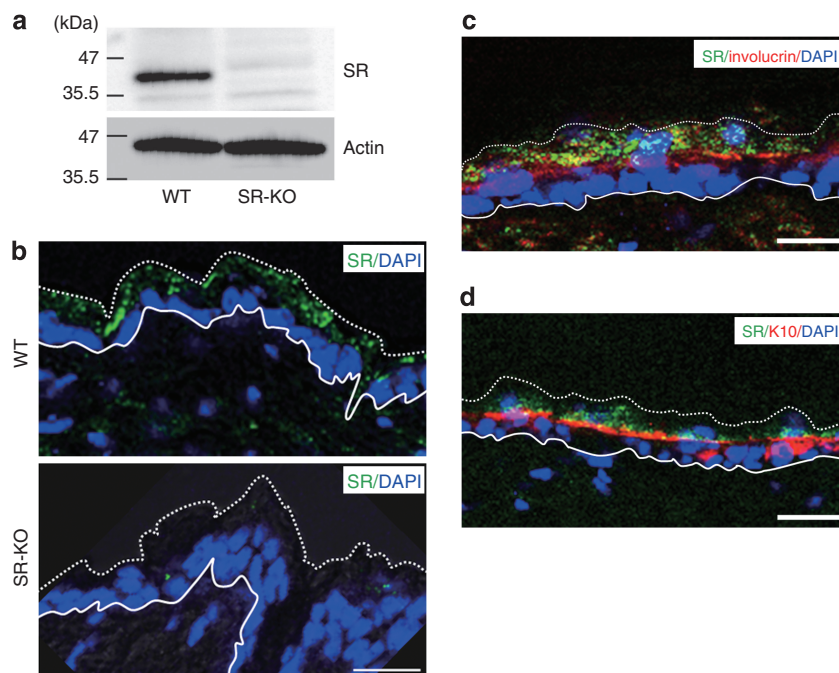


Figure 1. Expression of serine racemase (SR) in mouse skin. (a) Western blot analysis of epidermal proteins in wild type (WT) and SR-knockout (SR-KO) mice using anti-SR and anti-actin antibodies. (b) Immunohistochemical staining of skin from WT and SR-KO mice with anti-SR antibody (green). SR immunopositivity was detected in the granular and cornified layers of the skin from WT mice but not from SR-KO mice. (c, d) Double immunofluorescence staining of skin from WT mice using anti-SR (green) and anti-involucrin (c, red) or anti-K10 antibodies (d, red). K10, keratin 10. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). The dotted lines indicate the skin surface. The lines indicate the border between the epidermis and the dermis. Scale bars = 20 μm.

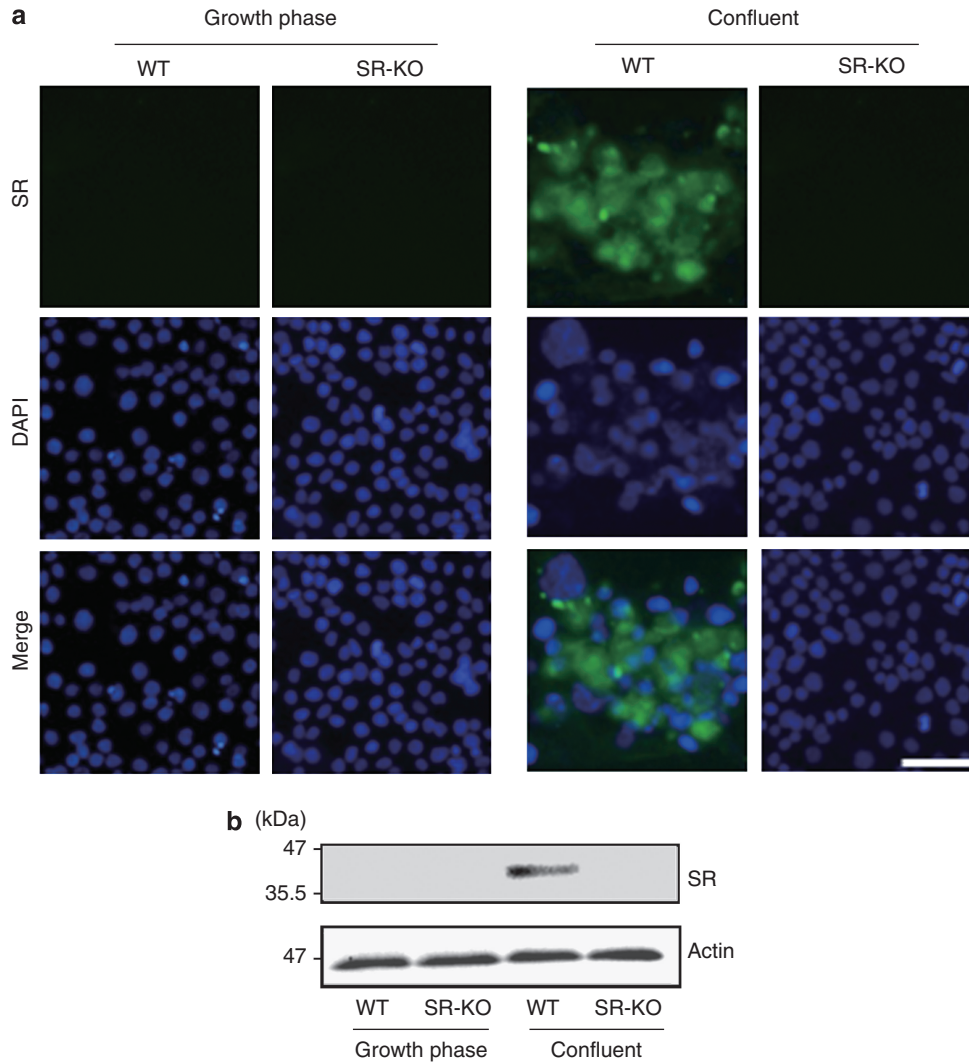


Figure 2. Expression of serine racemase (SR) in skin-derived cultured keratinocytes (KCs). (a) Immunofluorescence staining of SR (green) in growth-phase (left columns) and confluent KCs (right columns) derived from skin of wild type (WT) and SR-knockout (SR-KO) mice. SR immunopositivity was only detected in confluent WT KCs. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 100 μm. (b) Western blot analysis of proteins in the lysates from growth-phase and confluent KCs using anti-SR and anti-actin antibodies. The positions of protein size markers are indicated on the left side. The SR protein band of ~38 kDa was only detected in the lysates from confluent WT KCs.

Expression levels of mRNAs and proteins involved in differentiation of epidermal KCs

On the basis of the localization of SR in the cornified and granular layer of the epidermis, we examined by quantitative real-time PCR the mRNA expression levels of filaggrin, involucrin, and loricrin in the epidermis of WT and SR-KO mice that are involved in the differentiation of epidermal KCs (Steinert and Marekov, 1995). The mRNA expression level of transglutaminase 3 (TGase 3), an enzyme involved in the formation of the epidermal barrier (Nemes and Steinert, 1999; Hitomi, 2005), was also examined. The mRNA expression levels of involucrin and TGase 3 in the epidermis of SR-KO mice were significantly higher than those in the epidermis of WT mice (Figure 4). The expression levels of these protein markers and K10 were further examined by immunohistochemistry. Among the examined proteins, the involucrin

(Figure 5a and b) and K10 (Figure 5a and c) proteins showed significantly higher immunopositivity signal intensities in SR-KO mice than in WT mice. There were no marked differences in the immunopositivity signal intensities of filaggrin and loricrin between WT and SR-KO mice (data not shown).

Barrier function of skin

On the basis of the above changes observed in the SR-KO epidermis, we determined whether deletion of SR results in alteration in the barrier function of the skin. Because we detected SR expression in the skin of WT mice on postnatal day 5 (P5) (data not shown), skin permeability, transepidermal water loss (TEWL), and recovery of disrupted skin barrier were examined in newborn and P5 WT and SR-KO mice. The skin of either WT or SR-KO newborn mice was not permeable to toluidine blue (Supplementary Figure S1 online). However, the

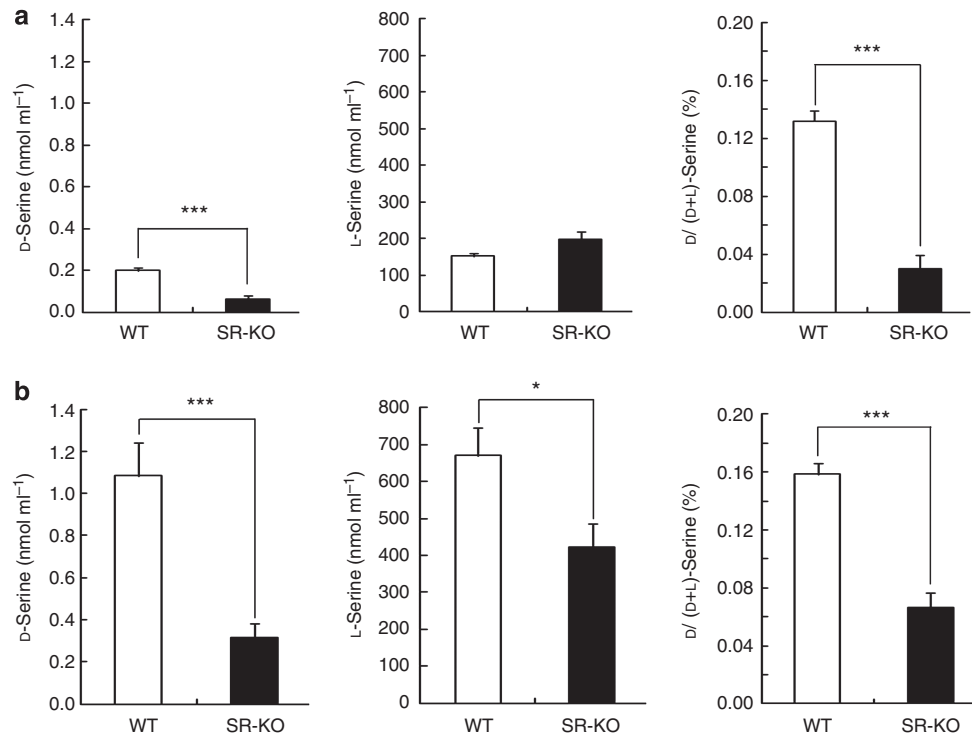


Figure 3. Concentrations of intracellular D-serine and L-serine in wild type (WT) and serine racemase-knockout (SR-KO) keratinocytes (KCs) cultured in medium with and without the addition of L-serine. (a) Concentrations of intracellular D-serine and L-serine in extracts of KCs cultured in medium without addition of L-serine. (b) Concentrations of intracellular D-serine and L-serine in extracts of KCs cultured in medium with addition of 10 mM L-serine. Data are presented as mean \pm SEM ($n=7$). * $P<0.05$; *** $P<0.001$; two-tailed Student's t -test.

level of TEWL in SR-KO mice was significantly higher than that in WT mice (Figure 6a). In the assay of barrier recovery, SR-KO mice exhibited significantly lower recovery rates at 4 and 6 hours after tape stripping than did WT mice (Figure 6b).

We also assessed the healing rate of wounds produced in the dorsal skin of adult WT and SR-KO mice, and found no marked difference in the time needed for complete healing of wounds between the two genotypes (Supplementary Figure S2 online).

Ultrastructural analysis of epidermis

The SR-KO mice did not show any gross histological abnormality in the skin examined with hematoxylin and eosin staining (data not shown). The dorsal skin obtained from P5 mice was further examined by electron microscopy. Although no marked change was observed in the formation of lamellar bodies, the exocytosis of lamellar granules, and the formation of lamellar bilayers in the epidermis of SR-KO mice (Supplementary Figure S3 online), the number of stratum corneum (SC) layers in this genotype of mice was significantly decreased when compared with WT mice (Figure 6c–e). Moreover, in the transition zone of the stratum granulosum (SG) of the SR-KO epidermis, the keratohyalin granules were markedly enlarged (Figure 6f and g).

DISCUSSION

The epidermis of the skin functions as a barrier against the environment through the uppermost layer of terminally

differentiated, denucleated KCs, namely, the cornified layer (Nemes and Steinert, 1999; Candi *et al.*, 2005), that forms the end point of epidermal differentiation and barrier formation. In this study, we found the localization of SR protein expression in the granular and cornified layers of the skin in WT mice, changes in the expression levels of markers of differentiation (involucrin, TGase 3, and K10) in SR-KO mice, and the alteration of the barrier function in SR-KO mice. These findings imply that SR is involved in the terminal differentiation of KCs and the formation of the epidermal barrier. Confluency is a stage representing the terminal differentiation of cultured WT KCs (Botta *et al.*, 2012). In our *in vitro* assay, SR immunopositivity was identified only in confluent WT KCs but not in growth-phase KCs that further suggests an association between SR expression and the terminal differentiation of KCs.

In view of the SR immunopositivity, SR seems predominantly expressed in the cornified layer, and with terminal differentiation many functional proteins in epidermal KCs are degraded, including the NMDA receptor. The question arising here is whether the racemization catalyzing the conversion of L-serine to D-serine exists in the epidermis. Although we were unable to directly examine SR activity in the cornified layer, we speculate about the function of SR in the epidermis on the basis of the following reasons. First, from the determined molecular weight of the SR protein (~ 38 KDa) from the skin, the protein is expected to be of full-length size and not degraded. Second, the overlapping of SR and involucrin expressions indicates that SR may at least be partially

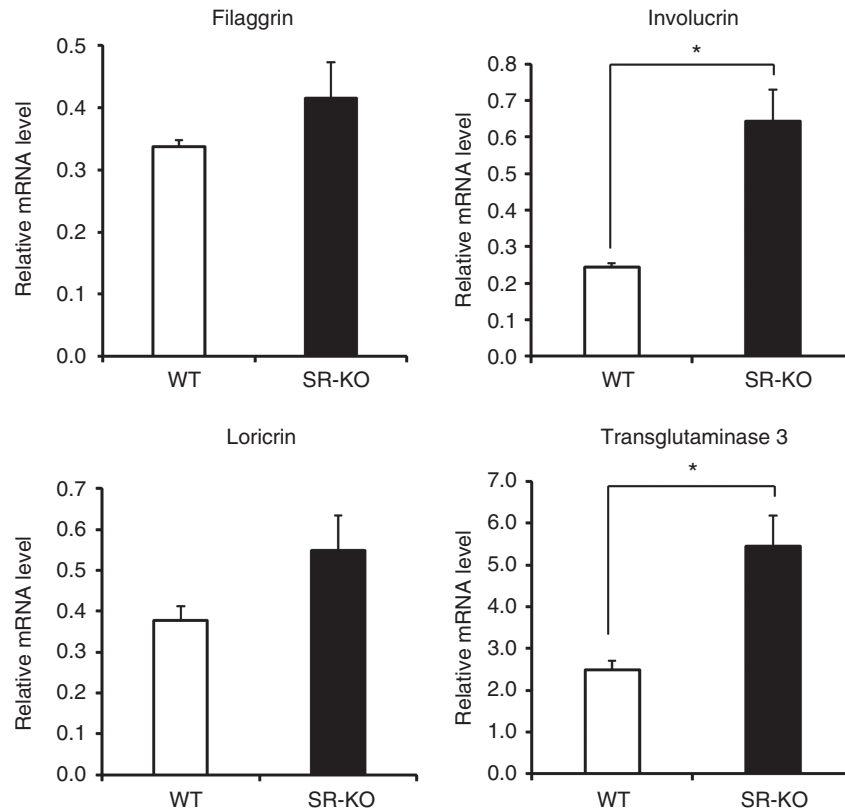


Figure 4. The mRNA expression levels of filaggrin, involucrin, loricrin, and transglutaminase 3 (TGase 3) in the epidermis as determined by quantitative real-time PCR analysis. The relative mRNA expression levels of filaggrin, involucrin, loricrin, and TGase 3 in the epidermis of wild type (WT) and serine racemase-knockout (SR-KO) mice were normalized against the level of glyceraldehyde-3-phosphate dehydrogenase mRNA. The mRNA expression levels of involucrin and TGase 3 in the epidermis of SR-KO mice were significantly higher than those of WT mice. Data are presented as mean \pm SEM ($n=3$). * $P<0.05$; two-tailed Student's t -test.

expressed in the granular layer. Third, the increased involucrin, TGase 3, and K10 expression levels in SR-KO epidermis indicate the association of SR function with the differentiation of KCs in the granular layer. Finally, our *in vitro* assay demonstrated the existence of a mechanism for the conversion of L-serine to D-serine through racemization by SR in the epidermal KCs. These findings suggest that SR and D-serine are required for KC differentiation and the maintenance of the physiological function of the skin. One study demonstrated that an enzyme isolated from frog skin secretions catalyzes the isomerization of L-amino acids in peptides to the D-type (Jilek *et al.*, 2005) that further suggests the importance of D-amino acids and racemization in skin functions.

Besides converting L-serine to D-serine, SR has the activity operating in reverse racemase mode, converting D-serine to L-serine (Foltyn *et al.*, 2005). The condensation reaction between L-serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase, is the first step in the *de novo* biosynthesis of ceramides (Holleran *et al.*, 1990; Hanada, 2003; Breiden and Sandhoff, 2013). As serine palmitoyltransferase strictly uses L-serine as its amino acid substrate (Hanada *et al.*, 2000), it is possible that SR in SG may have a role in the synthesis of ceramides by catalyzing the mutual

conversion of L-serine and D-serine to maintain an appropriate level of L-serine.

The increased level of TEWL and the significantly reduced rates of barrier recovery in P5 SR-KO mice reveal an alteration in the barrier function of the SR-KO skin. Formation of the skin barrier requires not only the formation of the SC lipid-enriched extracellular matrix, but also the corneocyte formation (Hohl, 1990; Nemes and Steinert, 1999). During the final stages of epidermal differentiation, outer SG cells transform into anucleate corneocytes, with highly resilient cornified envelopes. The significant decrease in the number of SC layers observed in SR-KO mice is assumed to result from the impairment in this transformational process that consequently exerts an influence on the barrier function of the epidermis or its recovery after acute disruption by tape stripping. The influx of calcium ions into KCs through the NMDA receptor has been shown to have an important role in KC differentiation. In one pharmacological study, blockade of keratinocytic NMDA receptors with MK-801 suppressed the expression of differentiation markers such as K10 and filaggrin (Fischer *et al.*, 2004a, b). Furthermore, parakeratotic cornification was demonstrated to be associated with the reduced level of NMDAR1(GluN1) expression (Fischer *et al.*, 2004b). Taken

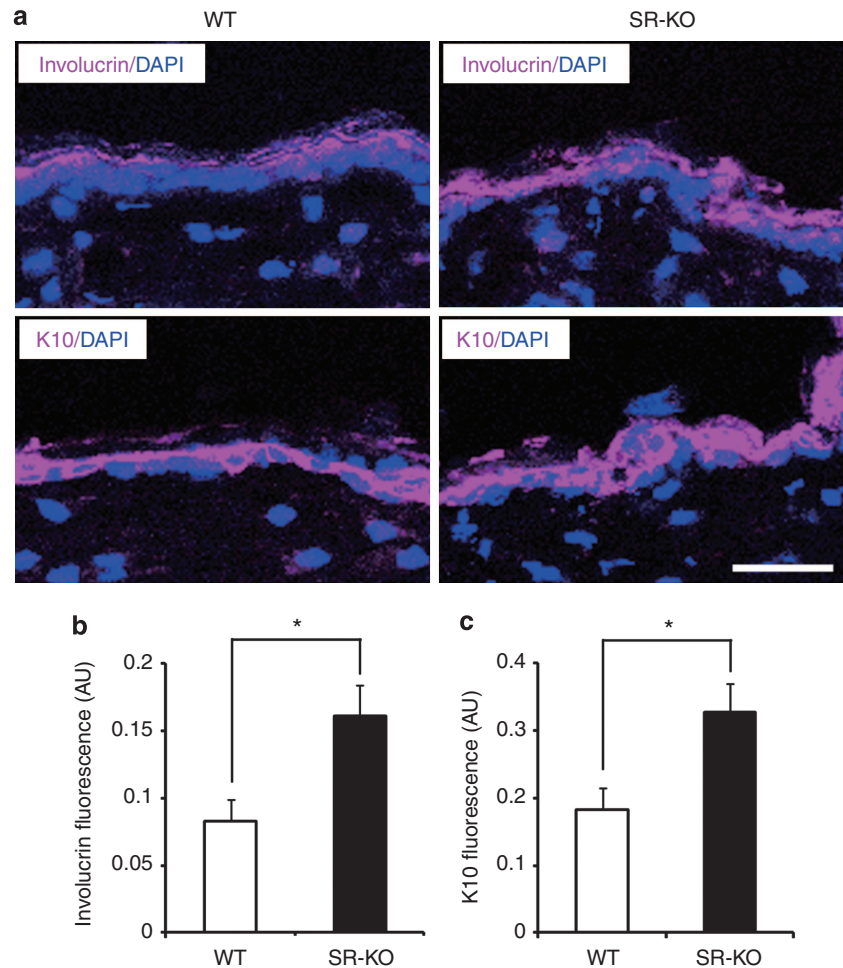


Figure 5. Expression of involucrin and keratin 10 (K10) in the epidermis of wild type (WT) and serine racemase-knockout (SR-KO) mice.

(a) Immunofluorescence staining of skin from WT and SR-KO mice with anti-involucrin (magenta) and anti-K10 antibodies (magenta). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 20 μ m. (b, c) Graphs showing the immunopositivity signal intensities of involucrin (b) and K10 (c) in the epidermis of WT and SR-KO mice. The expression levels of involucrin and K10 proteins were significantly higher in SR-KO mice than in WT mice. Data are presented as mean \pm SEM ($n=5$). AU, arbitrary units. * $P<0.05$; two-tailed Student's t -test.

together, the negative influence on NMDA receptor function resulting from the deficiency of D-serine in SR-KO mice may affect KC cornification.

Accordingly, an enlargement of keratohyaline granules was observed in the transition zone of the SG in the epidermis of the P5 SR-KO mice. Although there is no evidence showing a direct association between keratohyaline granules and barrier function of the skin, it is likely that the abnormally enlarged keratohyalin granules in the SG of SR-KO mice may indicate the effect of SR-KO on KC differentiation and may affect the production of filaggrin (Dale *et al.*, 1978) that is important for skin barrier (Candi *et al.*, 2005).

It is worth mentioning that our data on the recovery of barrier function are inconsistent with one previous report (Fuziwara *et al.*, 2003) in which the recovery of skin barrier after tape stripping in hairless mice was delayed by the topical application of NMDA receptor agonists, presumably through an NMDA receptor-mediated mechanism of accelerating

calcium influx into KCs and consequently perturbing the secretion of lamellar bodies, and such delay was erased by NMDA receptor antagonists. This inconsistency is probably attributed to the following reasons: (1) differences in the pharmacological and genetic approaches; (2) the different types of mice at different ages that were used for analysis; and (3) the developmental deletion of SR that affects the KC differentiation and leads to a significant decrease in the number of SC layers as observed in P5 mice that may overcome the influence resulting from an increase or a decrease in calcium influx into KCs on the secretory system of lamellar granules.

There is also another inconsistent finding in SR-KO mice: the lack of diffusion of toluidine blue into the skin and the increased TEWL. This inconsistency is probably attributed to the methodological validity. Dye diffusion is appropriate for the measurement of a large magnitude of barrier disruption, whereas TEWL is sensitive for the measurement of subtle

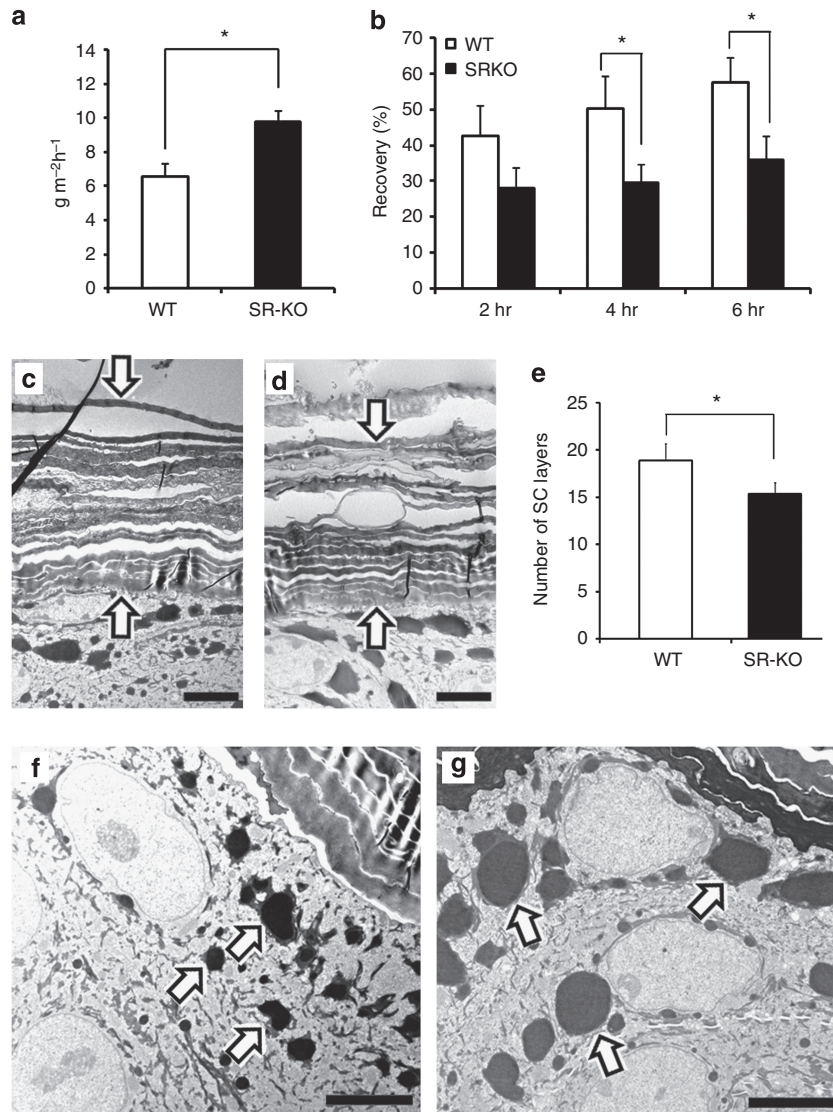


Figure 6. Barrier function and ultrastructural analysis of skin. (a) Transepidermal water loss (TEWL) in the dorsal skin of postnatal day 5 (P5) wild type (WT) and serine racemase–knockout (SR-KO) mice. (b) Barrier recovery of P5 mice 2, 4, and 6 hours after tape stripping. (c–g) Electron microscopy of P5 mice epidermis. Stratum corneum (SC) of (c) WT and (d) SR-KO mice. Arrows indicate the outermost and innermost layers of SC. (e) Bar graph showing the number of SC layers in WT and SR-KO mice. (f, g) Keratohyalin granules (arrows) in the transition zone of the stratum granulosum of the (f) WT and (g) SR-KO mice. Scale bars = 5 μm. Data are presented as mean ± SEM. **P* < 0.05; two-tailed Student’s *t*-test.

changes in barrier function (Indra and Leid, 2011). The effect of the deletion of SR on the skin barrier function observed in P5 mice needs to be examined in adult mice.

MATERIALS AND METHODS

Animals

Animal care and experimental protocols were carried out basically in accordance with the “Guidelines for the Care and Use of Laboratory Animals, DHEW, publication no. (NIH) 80-23, revised 1996” and approved by the Experimental Animal Committee of the University of Toyama (Authorization No. 2010-MED-61). The SR-KO mice with 100% C57BL/6 genetic background were generated as previously reported (Miya *et al.*, 2008). The WT and SR-KO mice were used for analyses in a genotype-blind manner.

Antibodies

The pET-His expression system (Novagen, Birmingham, UK) was used to produce a His-tagged fusion protein containing the full length of mouse SR amino acids. The glutathione *S*-transferase (GST) fusion protein expression system (GE Healthcare, Buckinghamshire, UK) was used to produce a GST-tagged fusion protein containing amino acid residue nos. 150–190 of the mouse SR (SR_{150–190}). Polyclonal antibodies against His-tagged SR were produced in rabbits and guinea pigs and were further purified using an antigen-affinity column coupled with the GST fusion protein SR_{150–190}. Antibodies against SR were used at a concentration of 0.5 mg ml⁻¹ for western blot analysis, immunohistochemistry, and immunocytochemistry. A rabbit polyclonal anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to

loricrin, involucrin, filaggrin, and K10 were purchased from Covance (San Diego, CA).

Western blot analysis

The skin epidermis from WT and SR-KO mice was homogenized in ice-cold mammalian protein extraction reagent (Pierce, Rockford, IL). Protein extracts (100 µg) were subjected to SDS-PAGE, and separated proteins were transferred onto polyvinylidene difluoride membranes. After blocking with a solution containing 5% skim milk in phosphate-buffered saline (PBS, pH 7.4), the membranes were incubated with a rabbit anti-SR or rabbit anti-actin (1:2,000) polyclonal antibody overnight at 4 °C, then with a horseradish peroxidase-conjugated secondary antibody for 1 hour. Protein bands were detected using an ECL chemiluminescence detection system (GE Healthcare).

Immunohistochemistry

Frozen skin tissues from WT and SR-KO mice were cut into 25-µm-thick sections using a freezing microtome and were mounted on slides. The cryosections of the skin were fixed in 0.1 M phosphate buffer (PB, pH 7.4) containing 4% (w/v) paraformaldehyde for 30 minutes, rinsed in PBS, and blocked with Protein Block Serum-Free (DakoCytomation, Carpinteria, CA) for 10 minutes at room temperature. The sections were then incubated with primary antibodies (guinea pig anti-SR, rabbit anti-SR, anti-involucrin, anti-filaggrin, anti-loricrin, or anti-K10 antibodies) diluted in PBS containing 1% BSA overnight at 4 °C. After washing in PBS, the sections were incubated with Alexa Fluor 488- and Alexa Fluor 594-conjugated species-specific secondary antibodies (Invitrogen, Carlsbad, CA) for 1 hour at room temperature. The sections were then washed in PBS, counterstained with 4',6-diamidino-2-phenylindole (Vector, Burlingame, CA), and coverslipped. Images were obtained using a confocal laser scanning microscope (Leica TCS-SP5, Leica Microsystems, Mannheim, Germany). For the quantitative analysis of immunopositivity signals, the obtained images were analyzed using the public domain Java image processing program ImageJ (National Institutes of Health, Bethesda, MD).

Culture of primary KCs

The excised skin samples from 1-day-old WT and SR-KO mice were floated on CnT-07 medium (CELLnTEC Advanced Cell Systems, Bern, Switzerland) supplemented with antibiotics (100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 0.25 µg ml⁻¹ amphotericin B) and 0.1% dispase II (Invitrogen), and incubated overnight at 4 °C. Epidermal sheets were then separated from the dermis with forceps and treated with TrySELECT (Invitrogen) for 30 minutes to isolate KCs. The cells were collected by centrifugation and then seeded in 3.5 cm culture dishes containing CnT-07 medium. They were then incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. Western blot analysis of KC proteins (100 µg) was performed as described above.

Immunocytochemistry

Confluent or growth-phase KCs that were cultured on glass-bottomed dishes were immersed in 4% paraformaldehyde for 30 minutes for fixation, incubated for 10 minutes in PBS containing 0.1% Triton X-100 for permeabilization, and blocked with PBS containing 3% BSA for 30 minutes. Thereafter, the cells were incubated with a guinea pig anti-SR antibody overnight at 4 °C, followed by incubation with donkey anti-guinea pig IgG conjugated with Alexa Fluor 488 for

1 hour at room temperature. The cells were rinsed in PBS after each treatment. Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole. Images were obtained using a fluorescence microscope.

Measurement of intracellular D-serine and L-serine in cultured KCs

Confluent KCs were further cultured in media supplemented with 10 mM L-serine for 48 hours. The concentration of intracellular D-serine and L-serine was analyzed by two-dimensional HPLC (Miyoshi *et al.*, 2011) as described in Supplementary Materials and Methods online.

Quantitative real-time PCR analysis

RNA was prepared from the epidermis of the back skin, then reverse transcribed and subjected to quantitative real-time PCR as described in Supplementary Materials and Methods online.

Cutaneous barrier function

TEWL was measured on the back skin of P5 mice using an evaporimeter (VapoMeter SWL2g; Delfin Technologies, Kuopio, Finland). In the assay of barrier recovery, epidermal barriers of P5 mice were disrupted by tape stripping until the TEWL reached 30–40 g m⁻² h⁻¹. In each animal, the percentage of recovery was calculated by the following formula: (TEWL immediately after barrier disruption – TEWL at indicated time point)/(TEWL immediately after barrier disruption – baseline TEWL) × 100%.

Electron microscopy

The back skin from P5 mice was minced into 1-mm-thick blocks and fixed immediately in 0.1 M cacodylate buffer (pH 7.4) containing 2% paraformaldehyde and 2% glutaraldehyde overnight at 4 °C. The samples were then washed with 0.1 M cacodylate buffer and followed by postfixation with 2% osmium tetroxide in 0.1 M cacodylate buffer at 4 °C for 3 hours. For observation of lamellar membrane and lamellar bodies, mouse skin was postfixed with 2.5% ruthenium tetroxide in 0.1 M cacodylate buffer. The blocks were ultrathin, sectioned at 70 nm, and were examined using a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

The number of SC layers was determined from osmium postfixed samples. Three points were selected at random and the number of SC layers was counted.

Statistical analyses

All values are presented as mean ± SEM. The statistical significance of difference between WT and SR-KO mice was determined by two-tailed Student's *t*-test. Values of *P* < 0.05 were considered statistically significant. For quantitative real-time-PCR data, *P*-values were corrected for type I errors using the Benjamini–Hochberg method.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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