Loricrin and NRF2 Coordinate Cornification

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Cornification involves cytoskeletal cross-linkages in corneocytes (the brick) and the secretion of lipids/adhesion structures to the interstitial space (the mortar). Because the assembly of lipid envelopes precedes corneocyte maturation, loricrin is supposed to be dispensable for the protection against desiccation. Although the phenotypes of Lor knockout (LKO) mice are obscure, the antioxidative response on the KEAP1/NRF2 signaling pathway compensates for the structural defect in utero. In this study, we asked how the compensatory response is evoked after the defects are repaired. To this end, the postnatal phenotypes of LKO mice were analyzed with particular attention to the permeability barrier function primarily maintained by the mortar.

Ultrastructural analysis revealed substantially thinner cornified cell envelopes and increased numbers of lamellar granules in LKO mice. Superficial epidermal damages triggered the adaptive repairing responses that evoke the NRF2-dependent upregulation of genes associated with lamellar granule secretion in LKO mice. We also found that corneodesmosomes are less degraded in LKO mice. The observation suggests that loricrin and NRF2 are important effectors of cornification, in which proteins need to be secreted, cross-linked, and degraded in a coordinated manner.

INTRODUCTION

The ultimate barrier function of the epidermis resides in the stratum corneum (SC) that is formed above the tight junction, an evolutionarily ancient paracellular diffusion barrier. The SC is often structurally compared with the bricks and mortar, which correspond to corneocytes and interstitial lamellated lipid, respectively (Nemes and Steinert, 1999). Loricrin (LOR) forms cornified cell envelopes (CEs) (Mehrel et al., 1990), a heavy disulfide (—S—S—)/ε-(γ-glutamyl) lysine cross-linked macromolecular structure that is formed inside the cell periphery (Rice and Green, 1977). Despite its quantitative significance, the gene knockout study of the thiol-rich CE protein revealed that LOR is dispensable for the epidermal permeability barrier as assessed by transepidermal water loss (TEWL) levels or the passive diffusion of the Lucifer yellow dye (Koch et al., 2000). These observations suggest that LOR does not affect the inside-out or the outside-in permeability barrier function. Nonetheless, Koch et al. (2000) found that Lor knockout (LKO) mice exhibit a delay in the development of the epidermal barrier formation in utero. The relative abundance of thiol (—SH) to disulfide (—S—S—) in the stratum granulosum (SG) appears to be primarily responsible for this delay because abundant thiol-reactive electrophiles, such as sulforaphane, in the amniotic fluid rescues the phenotype (Huebner et al., 2012). Mechanistically, sulfur-rich CE protein depletion activates the KEAP1/NRF2 signaling pathway (Yamamoto et al., 2018). Keratinocyte (KC) antioxidants, such as small proline-rich proteins (i.e., SPRRs) (Huebner et al., 2012) or late cornified envelopes (Ishitsuka et al., 2016), are massively incorporated in LKO CEs. Accordingly, inhibiting NRF2 DNA binding activity through the transgenic introduction of the dominant-negative Nrf2mutant abrogates the adaptive response, leading to lethal desiccation in LKO mice (Huebner et al., 2012). Because wild-type (WT) dominant-negative Nrf2 mice are apparently healthy (Huebner et al., 2012), the observations suggest that LOR and the KEAP1/NRF2 system collectively promote cornification: cytoskeletal cross-linkage (the brick) and the paracellular secretion of lipids/adhesion structures (the mortar) (Eckhart et al., 2013). However, how the compensatory response is evoked and maintained in terrestrial conditions remains a matter of interest. To address this, we sought to further clarify the postnatal phenotypes in LKO mice with a particular focus on the SC permeability barrier function that primarily depends on the mortar.

RESULTS

LOR is mandatory for corneocyte maturation

According to the CE assembly model by Steinert et al. (Kalnin et al., 2001), LOR is a major CE reinforcement component (Mehrel et al., 1990) that organizes the cross-linkages of cytoskeletal proteins, such as suprabasal
keratin (K) 1/K10 or FLG (Steinert and Marekov, 1995). A recent mass spectrometry–based study (Rice et al., 2016) and the photosensitive phenotype in LKO mice (Ishitsuka and Roop, 2018) support this view. Using transmission electron microscopy, CEs are observed as electron-dense deposits formed inside the cell periphery (Lavker and Matoltsy, 1970). Neonatal back skin from LKO mice on the FVB background was subjected to TEM, and SC was observed at the first (SC1) and second (SC2) layers. (a) Representative TEM images from P0 WT and LKO neonatal back skin on the FVB background. Arrows indicate the cornified CEs. Bar = 100 nm. The CE thickness was measured at the SC2 under TEM. The results presented are from fields at a magnification of 15,000; n = 8, ***, \( P < 0.001 \). (b) Representative TEM images from P0 WT, LKO, NKO, and DKO back skin on the BALB/c background. Arrows indicate the CEs. Bar = 100 nm. The CE thickness was measured at the SC2 under TEM. The results presented are from fields at a magnification of 15,000; n = 12; ***, \( P < 0.005 \) and ****, \( P < 0.001 \). CE, cornified envelope; DKO, Nrf2−/−Lor double knockout; LKO, Lor knockout; LOR, loricrin; NKO, Nrf2 knockout; P0, neonatal; SC, stratum corneum; TEM, transmission electron microscopy; WT, wild type.

**Figure 1. LOR is mandatory for corneocyte maturation.** Neonatal back skin was subjected to TEM, and SC was observed at the first (SC1) and second (SC2) layers. (a) Representative TEM images from P0 WT and LKO neonatal back skin on the FVB background. Arrows indicate the cornified CEs. Bar = 100 nm. The CE thickness was measured at the SC2 under TEM. The results presented are from fields at a magnification of 15,000; n = 8, ***, \( P < 0.001 \). (b) Representative TEM images from P0 WT, LKO, NKO, and DKO back skin on the BALB/c background. Arrows indicate the CEs. Bar = 100 nm. The CE thickness was measured at the SC2 under TEM. The results presented are from fields at a magnification of 15,000; n = 12; ***, \( P < 0.005 \) and ****, \( P < 0.001 \). CE, cornified envelope; DKO, Nrf2−/−Lor double knockout; LKO, Lor knockout; LOR, loricrin; NKO, Nrf2 knockout; P0, neonatal; SC, stratum corneum; TEM, transmission electron microscopy; WT, wild type.

SC paracellular barrier structure in LKO epidermis

Before LOR completes disulfide (–S–S–)-mediated cytoskeletal cross-linkages, the corneocyte outer coat, termed corneocyte lipid envelopes, is formed (Nemes and Steinert, 1999; Swartzendruber et al., 1987). Specifically, the ε-(γ-glutamyl) lysine isopeptide bond–mediated cross-linkage of the three scaffolding components envoplakin, periplakin, and involucrin is indispensable for corneocyte lipid envelopes formation (Sevilla et al., 2007). However, at this point of the CE assembly, corneocyte maturation is incomplete, and CEs remain immature/fragile (immature/fragile cornified envelope [CEf]) (Michel et al., 1988), considering the relative abundance of the earlier scaffolding components, as in the psoriatic epidermis (Ishida-Yamamoto et al., 1996) or LKO mice (Ishitsuka and Roop, 2018; Koch et al., 2000; Rice et al., 2016). Because the psoriatic epidermis, which harbors CEf (Ishida-Yamamoto et al., 1996; Michel et al., 1988), exhibits increased lamellar granule (LG) numbers (Ghadially et al., 1996), one could compare that LKO mice increase the LG storage. Indeed, we found an increase in cytoplasmic LG numbers in FVB neonatal LKO mice (Figure 2), whereas the areas of secreted LGs were significantly smaller in LKO mice (Figure 3). This change was accompanied by increased transcripts and proteins of ABCA12, which is essential for LG biogenesis and secretion (Zuo et al., 2008), in the neonatal mice on both backgrounds (Figure 4, data not shown). With respect to the effects of Nrf2 on LG biogenesis, NKO mice harbored significantly increased LG numbers compared with that for WT mice, whereas LG numbers were not significantly decreased in DKO mice compared with those in LKO mice (Figure 3b), suggesting that NRF2 may not affect LG biogenesis per se. However, it is noteworthy that the structure of SC lamellae remained undisturbed in LKO mice, unlike psoriatic epidermis (Ghadially et al., 1996). These observations suggest that LOR is nonessential for the SC permeability barrier function (Koch et al., 2000) primarily maintained by the biogenesis and secretion of LGs.

Desmosomal plaque proteins, such as desmoplakin, are incorporated into the CE through ε-(γ-glutamyl) lysine cross-linkage during the scaffolding phase (Nemes and Steinert, 1999), and the modified desmosome corneodesmosomes (CDs) replace the living layer–type adhesion structure (Ishida-Yamamoto et al., 2018, 2011). Therefore, LG-derived CDs preferentially bind to the immature CE (Allen et al., 2001; Serre et al., 1991) in advance of corneocyte maturation (Hohl et al., 1991; Mehrel et al., 1990) and shedding (Jonca et al., 2002; Serre et al., 1991; Simon et al., 2001, 1997). The deficiency of CD protein corneodesmosin (CDSN) (Leclerc et al., 2009; Matsumoto et al., 2008) as well as its premature proteolysis (Descargues et al., 2005) lead to lethal desmosomal defects in mice. The transcripts of Cdsn were significantly increased in LKO mice compared with those in WT or DKO mice (Figure 4). Accordingly, the numbers of undegraded CDs were significantly higher in LKO mice than in WT mice (Figures 5 and 6). Although the previous study revealed an increased incorporation of desmoglein 1 into the detergent/reductant-insoluble fraction (isolated CEs) in LKO epidermis (Rice et al., 2016), the transmission electron microscopy analysis did not reveal...
apparent numerical changes in desmosomes in the stratum spinosum (data not shown). Therefore, it is the enhanced LG secretion rather than the increased desmosomes that appears to be primarily responsible for the numerical changes of CDs. However, in contrast to the LG numbers, CDs remained undegraded in LKO and DKO mice compared with those in WT and NKO mice, respectively. The C-terminus of CDSN (Jonca et al., 2002) undergoes disulfide (–S–S–) cross-linkage to CEs (Hohl et al., 1991; Simon et al., 2001, 1997) concurrently with serine protease–mediated proteolytic cleavage on the N-terminus (Simon et al., 2001, 1997). Therefore, these observations suggest that LOR modifies the stability of CDSN during cornification. To address this, the protein extracts from heat-split neonatal epidermis, using SDS buffer that contained a reducing agent (β-mercaptoethanol), were subjected to immunoblotting using an anti-CDSN antibody recognizing the N-terminus. LKO/DKO mice exhibited stronger bands of undegraded CDSN (40–48 kDa) (Simon et al., 2001) than WT/NKO mice (Figure 6c). These results suggest that it is LOR, not the transcription factor NRF2, that promotes CD processing by enhancing disulfide (–S–S–) cross-linkage.

NRF2 promotes LG secretion

Neonatal mice undergo adaptive responses on commencing a terrestrial life (Huebner et al., 2012), and we found that neonatal LKO mice exhibited increased levels of Abca12 and Cdsn transcripts in an NRF2-dependent manner (Figure 4). Because tissue regeneration mimics organ development (Wood et al., 2002), the repairing responses after the abrasion of differentiated epidermal layers (Gregorio et al., 2010) could recapitulate the development in utero (Huebner et al., 2012; Ishitsuka et al., 2016). Adult BALB/c mice were subjected to a tape-stripping (TS) procedure, and the dependence of LKO mice on the transcription factor NRF2 was analyzed. When compared with DKO mice, LKO mice exhibited significantly increased levels of Abca12/Cdsn transcripts, in addition to NAD(P)H quinone dehydrogenase.
1 (Nqo1) (Figure 7). Thus, the activation of the KEAP1/NRF2 system in LKO mice may represent endogenous adaptive responses against minor epidermal damages or defects.

We have characterized the importance of the KEAP1/NRF2 signaling pathway in the recovery of epidermal barrier function after primary inflammatory assaults, such as psoriasiform tissue reaction (Ogawa et al., 2020a). The recovery rates of TEWL levels could correspond to the efficiency of LG secretion (Menon et al., 1992) rather than biogenesis itself (Ghadially et al., 1996). To address this, we quantified the areas of LGs secreted into the interstitial space in neonatal mice. In contrast to neonatal FVB mice (Figure 3a), we found that BALB/c neonatal mice exhibited a substantial increase in the secretory areas during cornification, that is, migration from SG2/SG1 (between the second and uppermost SG) to SG1/SC1 (between the SG1 and SC1) (Figure 8a). This increase was abrogated in NKO/DKO mice, compared with that in WT/LKO mice (Figure 8a and b), suggesting that NRF2 promotes LG secretion. Correspondingly, adult NKO/DKO mice exhibited significantly decreased recovery rates of TEWL following the controlled TS procedure (Kuo et al., 2013), although both adult WT and LKO mice showed comparable recovery rates (Figure 8c). An electrophile dimethyl fumarate (DMF) was orally administrated to LKO and DKO mice to exogenously activate the KEAP1/NRF2 signaling pathway (Yamamoto et al., 2018). As was observed in the imiquimod-induced psoriasiform tissue reaction (Ogawa et al., 2020a), DMF treatment resulted in a more prompt recovery than vehicle treatment (Figure 8d). DKO mice also responded to the treatment to some extent, presumably depending on the NRF2-independent pathway (Schulze-Topphoff et al., 2016), which was also observed in the imiquimod setting (Ogawa et al., 2020a). However, the recovery responses in DMF-treated DKO mice remained slow compared with that in LKO mice (Figure 8d).

**DISCUSSION**

**Cell-autonomous activation of NRF2 in LKO mice**

The LG-mediated transport of lipids protects terrestrial mammals from desiccation (Nemes and Steinert, 1999), and that of the adhesion molecule CDSN maintains corneocyte cohesion and desquamation (Jonca et al., 2002; Leclerc et al., 2009; Matsumoto et al., 2008). The KEAP1/NRF2 signaling pathway induces SPRR2s (Huebner et al., 2012) or LCEs (Ishitsuka et al., 2016) during epidermal tissue development to compensate for the loss of LOR. In accordance with previous reports, the thinner electron-dense deposits in LKO corneocytes underscore the primary function of LOR as a major CE protein by promoting disulfide (−S−S−)-mediated cytoskeletal cross-linkages on exposure to ambient air (Hohl et al., 1991; Mehrel et al., 1990). Therefore, the upregulated expression levels of Abca12 and Cdsn in LKO mice could be an NRF2-dependent, cell-autonomous recovery response reinforcing the SC permeability barrier (Huebner et al., 2012). Our results may offer an explanation to the very different consequences resulting from the activated KC reduction−oxidation signaling in the epidermis; constitutive activation leads to pathologic hyperkeratosis (Schäfer et al., 2014, 2012; Wakabayashi et al., 2003), whereas timely signaling in LKO mice results in the development of a very mild phenotype (Schäfer and Werner, 2015).

However, what is the cause of the NRF2-mediated adaptive responses in LKO epidermis? The lack of the
sulfur-rich structural protein would profoundly affect the epidermal sulfur metabolism in LKO SG, that is, the formation of disulfide (−S−S−) cross-linkages (Van Scott and Flesch, 1954). Therefore, it is most likely that the thiol-laden cytoskeleton-associated protein KEAP1 (Dinkova-Kostova et al., 2002; Kang et al., 2004) senses the cytoplasmic reduction–oxidation imbalance, and its conformational changes disrupt the phylogenetically ancient interaction with the effector protein NRF2 (Itoh et al., 1999; Katoh et al., 2005; Kobayashi et al., 2002) during the upward migration of KCs. As a consequence, antioxidative SPRRs (Vermeij and Backendorf, 2010; Vermeij et al., 2011) are massively induced, albeit LKO CE, Nrf2 knockout mice. The lysates were subjected to immunoblotting using the anti-CDSN antibody recognizing the N-terminus subjected to serine protease-mediated proteolysis. CD, corneodesmosome; CDSN, corneodesmin; DKO, Nrf2−Lor double knockout; LKO, Lor knockout; NKO, Nrf2 knockout; P0, neonatal; SC, stratum corneum; WT, wild type.

epidermis may be a tissue-intrinsic cue that directs recovery from the primary injury (Ishitsuka et al., 2020; Ogawa et al., 2020a), and the KEAP1/NRF2 signaling pathway is a major regulator of such adaptive tissue responses (Huebner et al., 2012; Ishitsuka et al., 2016). Although the oxidative amniotic milieu promotes disulfide (−S−S−) cross-linkages and assists the maturation of LKO CEs, the consequences of dominant-negative Nrf2 transgene introduction in LKO mice must be cautiously interpreted (Huebner et al., 2012) because the mutant Nrf2 primarily abrogates the cell-autonomous adaptive responses.

LOR and desquamation
Another important but rather unexpected finding was the retention of undegraded CDs in the LKO SC. The absence of CDSN (Leclerc et al., 2009; Matsumoto et al., 2008) or aberrantly activated serine protease activity lymphoepithelial
Kazal-type related inhibitor deficiency (Descargues et al., 2005) leads to premature SC detachment and severe desmosomal defects. LGs contain the serine protease kallikreins (Ishida-Yamamoto et al., 2005) that break down CDs. Although LGs secrete both kallikreins and lymphoepithelial Kazal-type related inhibitor (Ishida-Yamamoto et al., 2005), the results suggest that disulfide (–S–S–)–rich LOR directly interacts with CDSN and modifies its structure in the upper SC, as previously indicated (Mils et al., 1992; Serre et al., 1991; Simon et al., 2001, 1997). After secretion, the kallikrein-mediated proteolytic cleavage of CDSN begins from the N-terminus, which is responsible for its homophilic adhesive property (Jonca et al., 2002). By contrast, the C-terminus remains undegraded and covalently binds to CEs through disulfide (–S–S–) cross-linkages (Simon et al., 2001).

These observations highlight previously unappreciated functions of LOR because LOR may coordinate desquamation. Although we have not determined why delayed CD processing does not cause substantially thickened SC in LKO mice, as observed in the psoriatic (Allen et al., 2001; Simon et al., 2008) or palmoplantar (Mils et al., 1992; Serre et al., 1991) epidermis, these results suggest that LOR provides the structural basis of desquamation, which is hardwired into the epidermal differentiation program.

### Evolutional significance of LOR

Overall, LOR is dispensable for the protection against desiccation per se, unless it acquires nuclear-localizing signals and moves to the nucleus (Ishida-Yamamoto et al., 2000; Suga et al., 2000). The nuclear-localizing signal-harboring mutant LOR gene disturbs the SC permeability barrier, as assessed by the TEWL levels or the passive diffusion of the Lucifer yellow dye, irrespective of the presence or absence of WT LOR (Suga et al., 2000).

Consequently, what is LOR for? Similar to the salivary PRP, the alternative CE constituent SPRRs are structurally less organized (Williamson, 1994). The biochemical property of the PRP is advantageous in counteracting the harmful effects of xenobiotics, such as plant polyphenols (tannins) (Williamson, 1994). Furthermore, the high cysteine content of SPRRs allows the efficient quenching of ROS (Vermeij and Backendorf, 2010; Vermeij et al., 2011). By contrast, the primary protein structure of LOR favors intramolecular/intermolecular disulfide cross-linkages in vivo (Hohl et al., 1991; Mehrel et al., 1990). This biochemical peculiarity renders the purification of the native LOR protein in the exogenous gene expression system as significantly harder (Vermeij et al., 2011) as described in another cytoskeletal thiol-laden protein KEAP1 (Dinkova-Kostova et al., 2002).

The differentiation of cutaneous tissue highly depends on the gene expression program of the KCs, and tissue imprinting endows the isolated KCs with phenotypes peculiar to their tissue origin. Neither corneal KCs nor esophageal KCs are able to differentiate into the SC (Doran et al., 1980). However, epidermal KCs do not differentiate into fully matured corneocytes when kept in submerged cultures, and the in vitro CEs (Rice and Green, 1977) do not contain as much amount of LOR as the epithelial CEs in vivo (Steinert et al., 1998). Because the in vitro CEs resemble the CE (Michel et al., 1988; Steinert et al., 1998) because they are abundant with IVL (Simon and Green, 1984) or SPRRs (Steinert et al., 1998), LKO CEs may represent an aquatic
pattern of epidermal differentiation. Indeed, the phylogenetic analysis revealed that the K1/K10 pair, which partner with LOR through disulfide (S–S–S–) cross-linkages (Rice et al., 2016), is deleted from the keratin gene cluster in cetaceans (Ehrlich et al., 2019). Analogous to the psoriatic epidermis harboring the Ce (Ishida-Yamamoto et al., 1996; Michel et al., 1988), the dolphin epidermis expresses stress or hyperproliferation-associated K6/K17 (Ehrlich et al., 2019), which are also abundant in neonatal LKO CE (Rice et al., 2016; unpublished observations).

The decreased sulfur content in the LKO epidermis presumably makes the differentiating epidermal layers susceptible to NRF2 activation and may enhance the LG-mediated secretion of peptidases/peptidase inhibitors, such as secretory leukocyte peptidase inhibitor (Schäfer et al., 2012). The adaptive response becomes evident particularly when superficial epidermal damages are present, as observed in ichthyoses (Schäfer et al., 2012; Wakabayashi et al., 2003), psoriasis (Ogawa et al., 2020a), or atopic dermatitis (Ogawa et al., 2020b). In accordance with our previous works (Ishitsuka and Roop, 2020; Ishitsuka et al., 2020), we would like to conclude that LOR and NRF2 serve as important effectors of cornification, in which proteins need to be secreted, cross-linked, and degraded in a coordinated manner.

**MATERIALS AND METHODS**

**Mice**

All mice used in this study were maintained under specific pathogen-free conditions at the animal facility of the University of Tsukuba, and all procedures were approved by the University of Tsukuba Ethics Committee. LKO mice were maintained on the FVB background and backcrossed into the BALB/c background (Charles River Laboratories, Wilmington, MA) using DartMouseTM speed congenic service (a BALB/c content percentage >99% was confirmed). Separately, NKO mice on a BALB/c background were obtained from the Riken BioResource Research Center (Tsukuba, Japan) (Itoh et al., 1997) and crossed with the BALB/c LKO mice. WT mice were purchased from Charles River Laboratories (Yokohama, Japan). Age-matched mice (aged 8–12 weeks) were randomly selected from a pool for experiments housed in the same colony.

**Transmission electron microscopy**

The samples from neonatal back skin were fixed in half-strength Karnovsky fixative, followed by further fixation in 1% osmium tetroxide in distilled water. After en bloc staining with uranyl acetate, specimens were dehydrated in ethanol and embedded in Epon812 (Taab, Berkshire, United Kingdom). Ultrathin sections were stained with uranyl acetate and lead citrate.

To count CDs or LGs, montages were prepared using Adobe Photoshop, version 6.0 (Adobe Systems, San Jose, CA) at a magnification of 150,000, as described in a previous study (Igawa et al., 2011). We counted undegraded CD components in the lowermost SC layer (SC1) to the fifth SC layer (SC5) or cytoplasmic LGs in the SG3. All analyses were performed on at least four mice of each genetic background.

**TS-mediated abrasion of differentiated epidermal layers**

The back skin of adult mice was depilated (Epilat; Kracier, Tokyo, Japan), TS was performed using 20 strokes of a book tape (Scotch; Nichiban, Tokyo, Japan), as previously described (Kuo et al., 2013). Age-matched mice (aged 8 weeks) were purchased from Charles River Laboratories (Yokohama, Japan). The back skin was collected for histological/photographic analysis at the indicated time points. For TEWL measurement, TS was conducted using an adhesive tape (CELLOTAPE; Nichiban, Tokyo, Japan). TS was performed using 20 strokes of a book tape (Scotch; Nichiban, Tokyo, Japan), as previously described (Kuo et al., 2013). The back skin was carefully attached to the middle back skin, and SC was gently removed using four or five strokes. This typically resulted in spots of punctate hemorrhage and increased the TEWL to 100 ± 15 gm⁻²h⁻¹; afterward, we recorded the recovery response for up to 96 hours. The TEWL from the skin surface was measured at room temperature (22–25 °C) at 40–60% humidity with a VAPOSCAN AS-VT100RS system (ASCH Japan, Tokyo, Japan). Individual values were compiled as two consecutive measurements on the same site on at least two independent sites on the back. The recovery response was indicated as percentage recovery rates of TEWL levels to the
into 0.22-μm pore nitrocellulose membranes (10600001; GE Healthcare, Buckinghamshire, United Kingdom). The membranes were incubated overnight with the following primary antibodies: anti-CDSN (4.7 μg/ml, 25973-1-AP; Proteintech) and anti-tubulin (1 μg/ml, 017-25031; Fujifilm Wako Pure Chemical), followed by a 60-minute incubation with horseradish peroxidase–labeled secondary antibodies against rabbit or mouse IgG (0.04 μg/ml, sc-2004 or sc-2005; Santa Cruz Biotechnology). Antibody binding was visualized and enhanced with SuperSignal West Dura Extended Duration Substrate (34075; Thermo Fisher Scientific) and an image analysis system (LAS4000 Mini; Fujifilm Wako Pure Chemical).

**Statistical analysis**

Data were expressed as mean ± SEM. Comparisons were conducted using the Mann–Whitney U test (between two groups) or two-way ANOVA (time course/grouped experiments) using Prism 6 software (GraphPad Software, San Diego, CA). In all analyses, P < 0.05 was considered statistically significant.

**Data availability statement**

No datasets were generated or analyzed during this study.

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