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Keratinocytes-specific SOX2 overexpression suppressed pressure ulcer formation following cutaneous I/R injury via enhancement of amphiregulin production

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Abstract

Ischemia-reperfusion (I/R) injury is a key player in the pathogeneses of pressure ulcer formation. Our previous work demonstrated that inducing transcription factor sex-determining region Y-box 2 (SOX2) promotes cutaneous wound healing via epidermal growth factor receptor (EGFR) signaling pathway enhancement. However, its protective effect on cutaneous I/R injury was not well characterized. We aimed to assess the role of SOX2 in cutaneous I/R injury and the tissue-protective effect of SOX2 induction in keratinocytes in cutaneous I/R injury. SOX2 was transiently

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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expressed in keratinocytes following cutaneous I/R injury. Ulcer formation was significantly suppressed in keratinocytes-specific SOX2-overexpressing mice. SOX2 in skin keratinocytes significantly suppressed the infiltrating inflammatory cells, apoptotic cells, vascular damage, and hypoxic areas in cutaneous I/R injury. Oxidative stress-induced mRNA levels of inflammatory cytokines expression were suppressed, and antioxidant stress factors and amphiregulin (AREG) was elevated by SOX2 induction in skin keratinocytes. Recombinant (r) AREG administration suppressed pressure ulcer development following cutaneous I/R injury in mice and suppressed oxidative stress-induced reactive oxygen species production and apoptosis *in vitro*. These findings support that SOX2 in keratinocytes might regulate cutaneous I/R injury via AREG production, resulting in oxidative stress suppression. rAREG can be a potential therapeutic agent for cutaneous I/R injury.

INTRODUCTION

The increased risk of pressure ulcers with aging is becoming an international problem. A pressure ulcer is a common skin disease in the elderly that sometimes progresses to intractable ulcers, requiring a significant investment of human and medical resources for treatment. Nonblanchable erythema and purpura develop in the early stages of pressure ulcers, followed by epidermal cell necrosis and a chronic stage transition. Ischemia-reperfusion injury refers to cellular damage caused by blood reperfusion to ischemic tissue. Cutaneous ischemia-reperfusion injury causes a cascade of harmful events, such as vascular injury, thrombosis, inflammatory cell infiltration, inflammatory cytokine production, cell apoptosis, and tissue necrosis. The oxidative stress and subsequent inflammation caused by ischemia-reperfusion injury is an important factor in pressure ulcer development (Inoue et al., 2020; Peirce et al., 2000; Salcido et al., 1994; Stadler et al., 2004; Uchiyama et al., 2015a; Uchiyama et al., 2015b). Recently, we identified sex-determining region Y-box 2 (SOX2) as one of the responsible genes for the high potential for oral mucosa wound healing (Iglesias-Bartolome et al., 2018), and reported that SOX2 induction in skin keratinocytes promotes cutaneous wound healing through a mechanism dependent on EGFR signaling activation by increased EGF ligand expression (Uchiyama et al., 2019).

Nuclear factor erythroid 2-related factor 2 (Nrf2) has been known as the master regulator of antioxidant genes (Pizzimenti et al., 2021). In homeostatic conditions, Nrf2 is anchored by Keap1 and undergoes rapid proteasome-dependent degradation. With the inhibition of Keap1, Nrf2 is translocated to the nucleus and binds to the antioxidant response element under oxidative stress, resulting in increased antioxidant enzyme heme oxygenase 1 (HO-1) or NQO1 production (Li et al., 2014). Previously, the enhanced Nrf2 activation and oxidative stress in cutaneous I/R injury and its potential as a therapeutic target were revealed (Sekiguchi et al., 2018). Amphiregulin (AREG) is one of the epidermal growth factors involved in promoting keratinocyte proliferation, and its expression is induced when keratinocytes are damaged (Cook et al., 1991; Kennedy-Crispin et al., 2012). Additionally, oxidative stress on keratinocytes increases AREG expression (Miyazaki et al., 1996). A positive feedback signaling loop between Nrf2 and AREG acts protectively against mechanical stress (Reiss et al., 2014). AREG has a relatively low affinity for the receptor compared with other EGFR ligands; thus, it is less likely to cause receptor internalization

and continuous downstream MAPK/ERK signaling pathway activation (Zaiss et al., 2015). Therefore, AREG and its targeting of Nrf2 might have therapeutic potential for several diseases.

Based on previous studies and our results, we hypothesize that SOX2 has protective effects against tissue damage caused by cutaneous I/R injury. However, the possible role of SOX2 in the skin during I/R injury was unclear. Hence, this study aimed to examine the changes in SOX2 expression in the skin during cutaneous I/R injury and the possible protective effect of SOX2 induction in skin keratinocytes on ulcer development following cutaneous I/R injury and its cellular and molecular mechanisms focusing on SOX2 inducible AREG, and the effect of rAREG treatment as a potential therapeutic agent for cutaneous I/R injury.

Results

SOX2 expression during cutaneous I/R *in vivo*

First, we investigated the SOX2 expression during cutaneous I/R injury in wild type (WT) mice *in vivo*. SOX2 mRNA levels increased in the skin during ischemia by 3.82 fold compared with that before I/R, and highly increased at 4 h following reperfusion by 65.4 fold compared with that before I/R, and then gradually decreased to the basal levels at 24 h following reperfusion (Figure 1a). Immunofluorescence staining was performed to assess the amount and distribution of SOX2 during cutaneous I/R injury in WT mice. SOX2 expression was observed in hair follicles (−12 h, stable state) but not in the epidermis. SOX2 expression was transiently elevated in keratinocytes and hair follicles at 4 and 12 h following reperfusion (Figure 1b). *In vitro*, H_2O_2-induced oxidative stress significantly increased SOX2 expression in a time-dependent manner in mouse primary keratinocytes (Figure 1c).

Keratinocytes-specific SOX2 expression protected the development of ulcers following cutaneous I/R injury

We compared the wound area using a single-cycle cutaneous I/R injury mouse model in K14CreERTM/LSL-SOX2 (transgenic [TG]) mice treated for 5 consecutive days with an intraperitoneal injection of tamoxifen (Tam) dissolved in sunflower oil as SOX2 overexpressing (SOX2) mice or sunflower oil as a control (Ctrl) [Figure 2a] and confirmed SOX2 expression (Supplementary Figure S1a, b). Results showed that SOX2 in keratinocytes significantly inhibited cutaneous ulcer formation following cutaneous I/R (Figure 2b, c). The wound size of SOX2 mice at 5 days following reperfusion was 56.3% of Ctrl mice and was significantly smaller than that of Ctrl mice from 1 to 9 days following reperfusion. However, the size of the ulcers in the SOX2 and Ctrl groups was similar on day 12. To assess the effect of Tam dissolved in sunflower oil and sunflower oil alone on wound healing following cutaneous I/R injury, the size of the wound area was evaluated in WT mice treated with Tam dissolved in sunflower oil, sunflower oil, and phosphate-buffered saline (PBS) as a negative control (Supplementary Figure S2a). No significant difference was found in the size of wounds between Tam- and PBS-treated mice. Conversely, the sunflower oil group showed significantly less ulcerative area than the Tam group (Supplementary Figure S2b). Histopathological examination revealed the deep ulcer,

prominent dermal edema, and hypodermal inflammatory cell infiltration in the I/R skin site on days 1, 3, and 5 in Ctrl mice. In contrast, ulceration in the I/R skin was not seen on days 1 and 3 in SOX2 mice (Figure 2d). Then, we examined the effect of keratinocyte-specific SOX2 overexpression on infiltrating inflammatory cells into the cutaneous I/R site (Supplementary Figure S2c). Immunohistochemical studies revealed that the numbers of infiltrating myeloperoxidase $(MPO)^+$ neutrophils in SOX2 mice were significantly fewer compared with those of Ctrl mice on days 1 and 3 following reperfusion (Figure 2e), and the numbers of $CD68^+$ macrophages in SOX2 mice were significantly fewer on day 1 following reperfusion (Figure 2f).

Keratinocytes-specific SOX2 overexpression protected tissue damage via enhancing EGF ligands expression and Nrf2 activation in the cutaneous I/R site

We previously demonstrated that cutaneous I/R injury induces a reduced number of vessels and increased hypoxic area and apoptotic cells in the I/R skin site (Sekiguchi et al., 2018; Yamazaki et al., 2020). Therefore, we investigated the effect of keratinocytes-specific SOX2 overexpression on vascular loss and apoptosis around the cutaneous I/R injury area using skin tissue obtained from the I/R site 1 day following reperfusion and surrounding normal area (stable state). Results showed a significantly reduced number of $CD31^+$ endothelial cells in the I/R area compared with stable state in Ctrl mice, and a significantly larger number of $CD31^+$ endothelial cells in the I/R area in SOX2 mice compared with those in Ctrl mice (Figure 3a). The hypoxic area in the skin tissue was analyzed using an antibody against pimonidazole, which is a marker of hypoxia. The enhanced hypoxic area in the I/R site in Ctrl mice was significantly reduced in SOX2 mice (Figure 3b). The elevated numbers of TUNEL and DAPI double-positive cells in the epidermis and dermis in the I/R skin in Ctrl mice were significantly less elevated in SOX2 mice (Figure 3c). We next investigated the effect of keratinocytes-specific SOX2 overexpression on the mRNA levels of proinflammatory cytokines, an antioxidant enzyme, and EGFR ligands in the I/R area and surrounding normal area (stable state) by real-time polymerase chain reaction (PCR). Elevated mRNA levels of proinflammatory cytokines, including inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α by cutaneous I/R injury in Ctrl mice were slightly decreased in SOX2 mice. In contrast, mRNA expression of HO-1 and EGFR ligands, including AREG and heparin-binding EGF-like growth factor (HBEGF), following I/R injury in Ctrl mice was significantly enhanced in SOX2 mice (Figure 3d). We examined the antioxidant transcription factor Nrf activation, which is promoted by its uncoupling from Keap1 during oxidative stress, by utilizing OKD48 (B6-ARE-NRF2/LUC) mice. OKD48 mice and TG mice were crossed, OKD48/TG mice were generated, and both expressed genotypes were confirmed to evaluate the effect of keratinocytes-specific SOX2 overexpression on Nrf2 activation (OKD48 signal) (Supplementary Figure S3a, b). Murine skin was harvested from cutaneous I/R sites and surrounding normal skin area (stable state) immediately following reperfusion (0 h), and OKD48 signals were visualized by luciferin. Results showed the OKD48 signal was significantly enhanced in the I/R skin area in OKD48/SOX2 mice compared with that in the I/R skin area in OKD48/Ctrl mice (Figure 3e).

SOX2 expressing keratinocytes enhanced antioxidative Nrf2 activation and attenuated apoptosis via enhancement of AREG expression *in vitro*

Next, we examined the keratinocytes-specific SOX2 overexpression on oxidative stress-induced antioxidant signaling *in vitro*. OKD48/TG mouse-derived primary skin keratinocytes were cultured, and OKD48 signals were examined with or without H_2O_2 (0.5 mM) stimulation and treatment of 4OH-Tam dissolved in dimethyl sulfoxide (DMSO) (SOX2 overexpressing keratinocytes) or DMSO as the vehicle (control: Ctrl). Results showed a significantly increased OKD48 signal by H_2O_2 stimulation in Ctrl primary keratinocytes, which was significantly enhanced in SOX2 primary keratinocytes (Figure 4a). We investigated the effect of SOX2 overexpression in primary keratinocytes on the mRNA expression of proinflammatory cytokines, antioxidant enzymes, and EGFR ligands. Quantitative PCR analysis revealed that H_2O_2 stimulation increased the mRNA levels of iNOS and TNF-α expression in Ctrl primary keratinocytes and suppressed those expressions in SOX2 overexpressing cells. In contrast, H_2O_2 stimulation induced the mRNA levels of HO-1, AREG, and HBEGF in Ctrl primary keratinocytes, which were significantly enhanced in SOX2 overexpressing cells (Figure 4b). The increase in protein levels of HO-1 and AREG expression in SOX2 overexpressing keratinocytes under H_2O_2 stimulation was revealed by immunoblotting assay (Figure 4c). Next, we examined whether SOX2 overexpression affects the H_2O_2-induced apoptosis in keratinocytes *in vitro*. TUNEL staining revealed a significantly reduced number of apoptotic cells in SOX2 primary keratinocytes compared with the Ctrl keratinocytes after H_2O_2 stimulation (Figure 4d). Furthermore, siAREG treatment significantly reduced the preventing effects of SOX2 on H_2O_2-induced apoptosis in keratinocytes (Supplementary Figure S4, Figure 4e). We also confirmed that SOX2 induction significantly enhanced the H_2O_2-induced AREG expression but not HBEGF in HaCaT cells (Figure 4f).

rAREG protected ulcer formation following cutaneous I/R injury via suppressing ROS production

We previously showed that SOX2 directly regulates EGFR ligand expression (Uchiyama et al., 2019). Interestingly, AREG has been reported to inhibit oxidative stress (Papaiahgari et al., 2007). Then, we analyzed the expression and distribution of AREG in cutaneous I/R and surrounding normal areas (stable state) in Ctrl and SOX2 mice by immunostaining. AREG-positive area in stable skin was found in keratinocytes and dermis in SOX2 mice. The AREG-positive area was not observed in the I/R skin in Ctrl mice, whereas it was observed in the dermis in SOX2 mice (Figure 5a). However, the administration of Tam did not enhance AREG expression in the stable state and I/R skin in WT mice (Supplementary Figure S5a). Furthermore, the molecular network of AREG under SOX2-expressing skin was examined using a published RNA-seq data set generated from skin samples of Ctrl and SOX2 mice (Uchiyama et al., 2019). Ingenuity® Pathway Analysis (Regulator effects analytic) identified several genes downstream of AREG, determining the activation of tumor cell line proliferation, DNA endogenous promoter, and invasive tumor activation and inhibition of organismal death (Supplementary Figure S5b), suggesting a role for AREG in regulating cell proliferation or survival in the skin. We next compared the size of cutaneous ulcers following I/R injury in WT mice treated with subcutaneous injection of recombinant mouse AREG (rAREG) or PBS as control around the I/R site to assess

the effect of rAREG on cutaneous ulcer I/R injury. Results showed that the size of pressure ulcers was significantly reduced on day 1 and partially suppressed from days 2 to 5 following reperfusion in rAREG-treated mice compared with those in control mice (Figure 5b). Next, we examined the effect of rAREG treatment on oxidative stress in mouse primary keratinocytes *in vitro*. The OKD48 signal in primary keratinocytes from OKD48 mice was quantified with or without H_2O_2 and treatment with rAREG or PBS as a control. The rAREG treatment significantly suppressed the OKD48 signal in primary keratinocytes (Figure 5c). Moreover, we examined the influence of rAREG treatment on oxidative stress-inducing apoptosis in mouse fibroblasts. TUNEL staining shows that the elevated number of apoptotic cells after H_2O_2 stimulation was significantly less elevated in rAREG-treated fibroblasts (Figure 5d). Finally, we investigated the effect of rAREG treatment on oxidative stress that affects fibroblasts and vascular endothelial cells with reactive oxygen species (ROS) detection assay using NIH3T cells and human umbilical vein endothelial cells (HUVECs) *in vitro*. Results showed that rAREG treatment suppressed H_2O_2-induced intracellular ROS accumulation in both NIH3T3 cells and HUVECs in a concentration-dependent manner (Figure 5e).

DISCUSSION

The current study investigated the protective effect of keratinocytes overexpression of the transcription factor SOX2 on the development of pressure ulcers and its possible mechanisms using a mouse model of cutaneous I/R injury.

SOX2 is essential for maintaining the stemness and self-renewal of stem cells; furthermore, it is well-known as one of the key transcription factors that can together regenerate an induced pluripotent stem cell (Takahashi and Yamanaka, 2006). Recent studies revealed that the quiescent and self-renewing stem cells maintain low ROS levels and reside in a hypoxic environment (Emmerson et al., 2018; Lee et al., 2018). SOX2 induction might provide stem cell phonotypes to keratinocytes, enhancing the resistance to oxidative stress and tissue regeneration. We previously reported the effect of SOX2 expression in keratinocytes to promote healing in the skin (Uchiyama et al., 2019); however, this study demonstrated that SOX2 expression in keratinocytes had a protective function against tissue damage caused by cutaneous I/R injury, and the possible mechanism of action focused on oxidative stress.

First, it was revealed that the mRNA and protein levels of SOX2 expression were transiently enhanced following I/R injury in WT mice. Additionally, it was demonstrated that the H_2O_2-induced oxidative stress increased mRNA SOX2 expression in keratinocytes. These results suggested that cutaneous I/R injury-induced oxidative stress might temporarily induce SOX2 expression in epidermal keratinocytes. The results of the cutaneous I/R injury experiment with control and keratinocyte-specific SOX2 expressing mice demonstrated that cutaneous SOX2 expression partially protected ulcer formation after cutaneous I/R injury. Furthermore, the results of fewer neutrophils and macrophages, numerous $CD31^+$ vessels, smaller hypoxic area, and reduced number of apoptotic cells revealed by immunostaining using skin in cutaneous I/R site supported that SOX2 in keratinocytes might prevent ulcer development following I/R injury via suppressing inflammation and vascular damages.

The disappearance of the difference in ulcer area between the SOX2 and Ctrl groups in the second half of the observation period could be due to: a decreased number of local SOX2-positive keratinocytes because of ulcer formation or a possible protective effect of sunflower oil as the vehicle on cutaneous I/R injury. A recent study revealed the tissue-protective effect of sunflower oil in an I/R animal model (La Russa et al., 2021). The results showed fewer ulcerative areas in the sunflower oil-treated group compared with the PBS-treated group (Supplementary Figure S2b); thus, indicating the accelerated wound healing effect of sunflower oil, which was canceled by Tam. However, the mechanism underlying the counteraction of Tam in accelerating cutaneous wound healing is unclear. Further investigation using a different experimental model during the wound healing process will be required.

The results of the effect of SOX2 on gene expression associated with proinflammatory cytokines and antioxidant genes and Nrf2 activation using the OKD48 model suggest that SOX2 in keratinocytes suppresses oxidative stress-induced inflammation and apoptosis via Nrf2-antioxidant response enhancement in cutaneous I/R injury. OKD48 consists of an oxidative stress-inducible promoter, luciferase, and an Nrf2 fragment that contributes to stress-dependent stabilization, and its activation was correlated with HO-1 expression (Oikawa et al., 2012; Sekiguchi et al., 2019; Suzuki et al., 2015). Our results showing enhanced OKD48 signaling in the SOX2 expression group may indicate that SOX2 enhances oxidative stress in keratinocytes; however, this might be countered by the tissue-protective effects of SOX2. Wang et al. (2016) demonstrated that dipeptidyl peptidase–4 inhibitors induce prolonged Nrf2 activation, resulting in upregulated metastasis-associated protein expression and metastasis promotion in xenograft mouse models (Wang et al., 2016). Recent studies have shown that anwulignan, a monomer extracted from Schisandrae Sphenantherae Fructus, alleviates tissue damage by enhancing Nrf2/ARE signaling (Liu et al., 2021; Liu et al., 2022). These results suggest that Nrf2 activation is also enhanced by other factors besides oxidative stress. Based on our results and these findings, we propose a model in which SOX2 enhances the Nrf2-ARE binding complex, resulting in the enhancement of antioxidative gene expression. Furthermore, a recent study reported that forced Keap1 downregulation causes Nrf2 activation, resulting in increased SOX2 expression in diabetic bone marrow-derived multipotent stromal cells (Rabbani et al., 2019). Moreover, several studies reported that Nrf2 regulates SOX2 expression in glioma stem cells and squamous malignancies (Härkönen et al., 2023; Zhu et al., 2013). Altogether, these results indicate that Nrf2 activation by oxidative stress might be a key factor that induces SOX2 and AREG expression in keratinocytes following cutaneous I/R injury.

The results of the siAREG experiments using keratinocytes indicate that AREG might be a key player, acting as a tissue-protective molecule against oxidative stress. Furthermore, our result revealing partially suppressed ulcer formation in I/R injury by rAREG administration and a strong effect of rAREG in counteracting H_2O_2-inducing oxidative stress following antioxidant response *in vitro* suggested the importance of AREG, thereby inducing the protective effect of cutaneous I/R injury. Moreover, Ingenuity® Pathway Analysis using upregulated genes in SOX2-expressing skin in TG mice compared with control TG mice (Uchiyama et al., 2019) revealed AREG regulating cell proliferation and cell death

inhibition through several downstream genes, including MYBL2, BIRC5, and FOXM1 (Bongiovanni et al., 2011; Maruyama et al., 2014).

This study has several limitations. First, previous studies have reported the use of three I/R cycles in a mouse model (Saito et al., 2008; Stadler et al., 2004). Repeated I/R cycles increase tissue damage; additionally, they could alter the outcomes. Second, the difference in the wound healing process between humans and mice should be considered before translation to humans if this cutaneous I/R mouse model shows a time course similar to that of human bedsores. Third, the effect of the solution to dissolve Tam on ulcer development at the I/R skin site should be excluded to investigate the precise effect of SOX2 on cutaneous I/R injury using a different solution.

Finally, we propose a model for the role of SOX2 in keratinocytes to regulate cutaneous I/R injury. The oxidative stress induced by cutaneous I/R stimulation activates Nrf2-ARE signaling, which in turn induces SOX2 upregulation and AREG. SOX2 induction in keratinocytes might induce the Nrf2-SOX2-AREG network in the stable state and enhance its antioxidant and anti-apoptosis function under oxidative stress (Figure 5f). Altogether, our findings revealed new potential strategies for the use of AREG as a therapeutic agent for pressure ulcer prevention.

MATERIALS AND METHODS

The detailed protocols and statistical analysis are described in Supplementary Materials and Methods online.

Mice

All experiments were approved by the Gunma University Animal Care and Experimentation Committee (#20–024) and carried out in accordance with the approved guidelines. C57BL/6 WT mice were purchased from the SLC (Shizuoka, Japan). K14CreERTM/LSL-SOX2 (Transgenic: TG) mice were generated as previously described (Lu et al., 2010). OKD48 mice were kindly provided from Dr. T. Iwawaki (Department of Life Science, Kanazawa Medical University, Ishikawa, Japan) (Oikawa et al., 2012).

Single I/R cycle and analysis *in vivo*

The single cutaneous I/R model that has been previously reported was used (Sekiguchi et al., 2018; Uchiyama et al., 2015b). The dorsal skin was gently pulled up and trapped between two round ferrite magnetic plates for 12 hours, and then plates were removed. We defined 12 h ischemia and canceling of ischemia as single I/R in our study, and we performed single I/R cycle. The time point of removal of magnets plates (reperfusion, 0 h) was defined as starting observation time (Day 0). The size of skin ulcer was measured on photographs using Image J (version2.3.0; NIH, Bethesda, Maryland), as reported previously (Uchiyama et al., 2015a).

Histological examination and immunofluorescence staining

Murine skins were fixed by formalin and embedded in paraffin or were excised, fixed in 4% paraformaldehyde in phosphate buffered saline for 30 minutes for frozen section.

Immunohistochemical staining of paraffin and frozen sections and analyses were performed as previously described (Uchiyama et al., 2017).

Cell culture and treatments

Primary keratinocytes were isolated from TG mice as previously described (Lichti et al., 2008). Mouse embryonic fibroblast cells (NIH3T3) and HUVECs were purchased from ATCC (Manassas, VA).

Statistical analysis

P values were calculated using Student's t-test (two-sided) or by one-way analysis of variance followed by Tukey-Kramer test. Data analysis was done with R, version 3.6.3 (The R Foundation for Statistical Computing: https://www.r-project.org/). Error bars represent standard errors of the mean, and numbers of experiments (n) are indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

No datasets were generated or analyzed during this study.

Abbreviations:

I/R	ischemia-Reperfusion
SOX2	sex determining region Y-box2
EGFR	epidermal growth factor receptor
AREG	amphiregulin
Nrf2	nuclear factor erythroid 2-related factor 2
HO-1	heme oxygenase 1
WT	wild type
TG mouse	transgenic mouse
Tam	tamoxifen
PBS	phosphate-buffered saline
DMSO	dimethyl sulfoxide

HBEGF	heparin binding-epidermal growth factor-like growth factor
MPO	myeloperoxidase
TNF	tumor necrosis factor
OKD48	keap1-dependent oxidative stress detector No-48
ROS	reactive oxygen species
HUVECs	human umbilical vein endothelial cells

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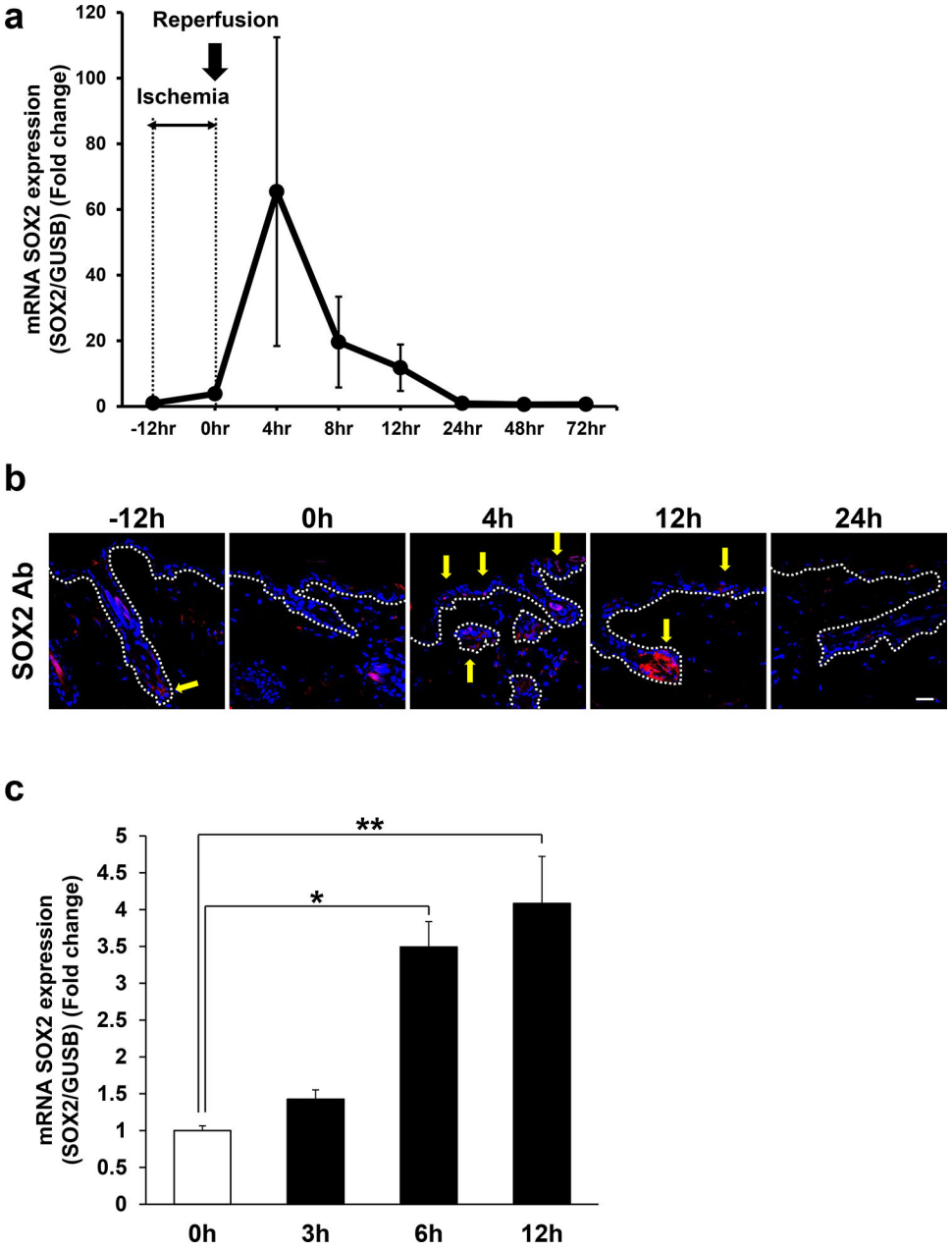

Figure 1. Examination of SOX2 expression level changes in wild type mice following I/R stimulation in the skin.

(a) The quantitative evaluation by qPCR assay showing the mRNA expression of SOX2; n = 4. **(b)** Representative immunofluorescence images of SOX2; Scale bar = 100 μm. Arrows indicate SOX2-expressing keratinocytes in epidermis and hair follicles (yellow); n = 3, Scale bar = 20 μm. **(c)** Quantification of SOX2 mRNA levels in mouse primary keratinocyte quantitative RT-PCR. Cells are treated with H_2O_2 for the indicated times. The amount of SOX2 expression in nontreated cells is assigned a value of 1; n = 3. All data are expressed as mean ± standard error of the mean. **(a, c)** A one-way analysis of variance followed by the Tukey–Kramer test is used. $^{**}p < 0.01$, $^{*}p < 0.05$.

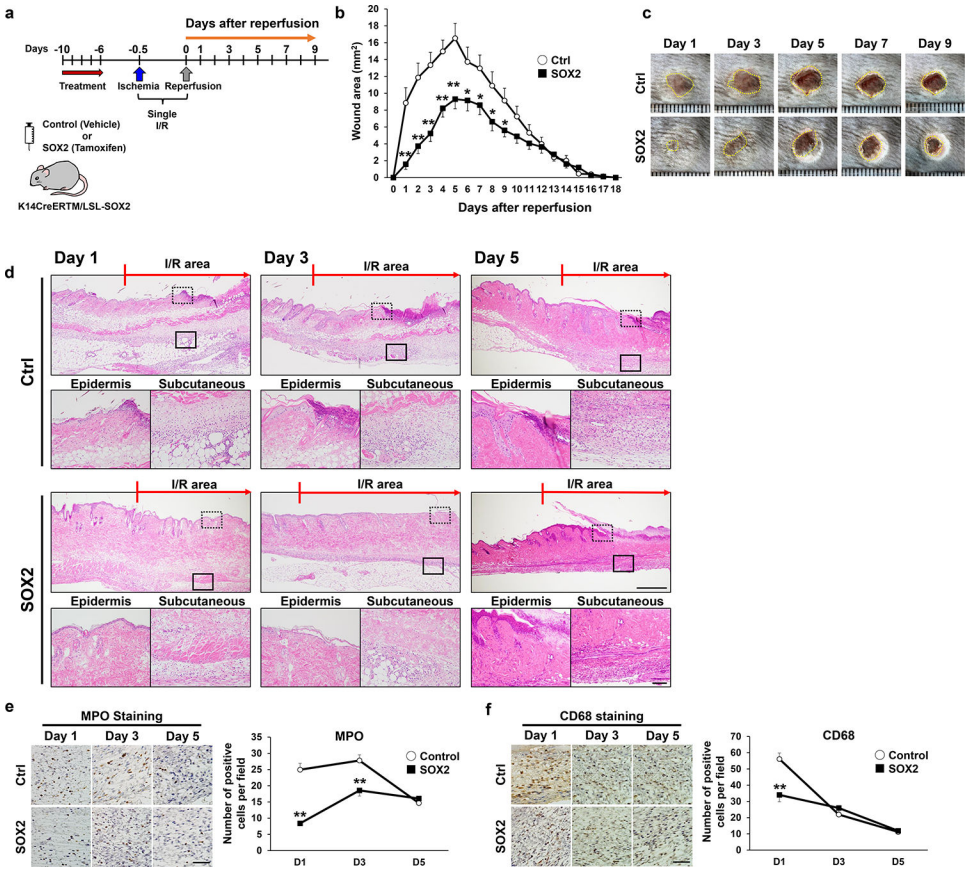

Figure 2. SOX2-expressing skin keratinocytes suppressed the formation of ulcers and infiltration of inflammatory cells following cutaneous I/R injury.

(a) Time course in *in vivo* experiments using K14CreERTM/LSL-SOX2 (TG) mice. Tamoxifen and vehicle were administered daily by intraperitoneal injection starting 5 days before the initiation of ischemic stimulation by magnet pinching. **(b)** The size of the wound area following I/R injury in control (Ctrl) or SOX2-overexpressing (SOX2) mice; n = 18 wounds per group. **(c)** The wound photographs are taken following cutaneous I/R in Ctrl or SOX2 mice at 1, 3, 5, 7, and 9 days following reperfusion. The dotted line indicates the wounded area. **(d)** Representative hematoxylin–eosin stained images of mouse skin tissue at 1, 3, and 5 days following I/R injury. The red arrows indicate the I/R areas. The black dotted square shows the epidermis (lower left) and the black line square shows the subcutaneous area (lower right); Scale bar = 500 μm at low magnification (upper panels), and 100 μm at high magnification (lower panels); n = 5. **(e–f)** Immunohistochemical images showing infiltrating inflammatory cells stained for (e) MPO^+ neutrophils, and **(f)** $CD68^+$ macrophages on days 1, 3, and 5. The right panels show quantitative evaluation by counting the number of positive cells in six random fields of view; n = 3, Scale bar = 50 μm. All data are expressed as mean ± standard error of the mean. **(b)** Student's t-test (two-sided) and **(e, f)** one-way analysis of variance followed by Tukey–Kramer test are used. $^{**}p < 0.01$, $^{*}p < 0.05$.

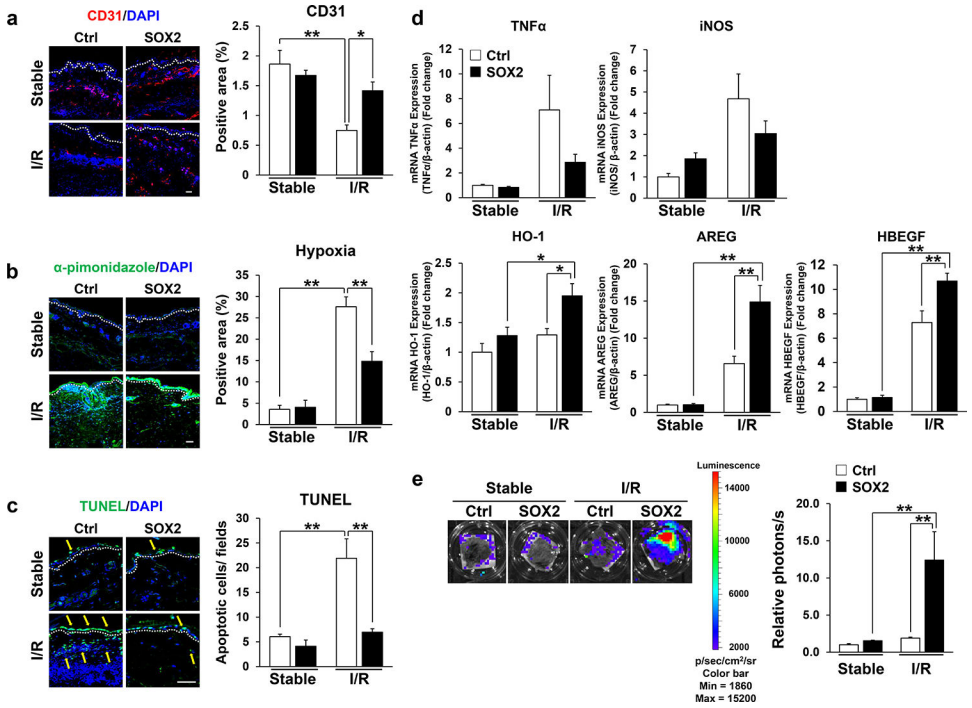

Figure 3. SOX2-expressing skin keratinocytes suppressed the reduction of vascularity and inflammation in the I/R area following cutaneous I/R.

(a–c) Representative immunofluorescence images of the I/R area and surrounding normal area (stable state) from Ctrl or SOX2 mice on day 1 showing **(a)** vascular area ($CD31^+$ endothelial cells), **(b)** hypoxic area (α-pimonidazole$^+$), and **(c)** apoptotic cells (TUNEL staining). Scale bar = 50 μm. The right panels show quantitative evaluation performed by **(a, b)** measuring positive area or **(c)** counting number of positive cells in the epidermis and dermis in six random fields of view; **(a, b)** n = 4, **(c)** n = 3. **(d)** Quantification of mRNA expression levels in the I/R area on day 1 using skin tissue from Ctrl or SOX2 mice by qPCR; n = 4–8. **(e)** Images visualizing the OKD48 signal with or without I/R injury in OKD48/Ctrl and OKD48/SOX2 mice; n = 4. The right panel shows OKD48 signal quantification. The value of 1 was assigned in a stable state skin in OKD48/Ctrl mice. All data are expressed as mean ± standard error of the mean. **(a-e)** A one-way analysis of variance followed by the Tukey–Kramer test is used. $^{**}p < 0.01$, $^{*}p < 0.05$.

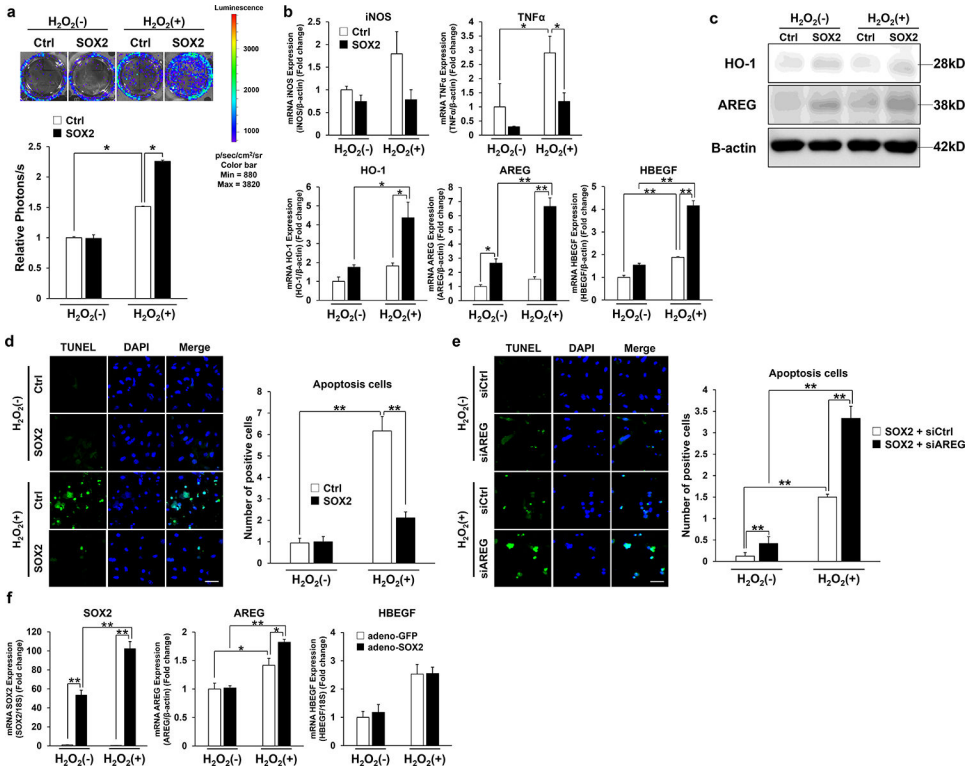

Figure 4. SOX2-expressing skin keratinocytes suppressed oxidative stress-induced inflammation and apoptosis *in vitro*.

(a) Images visualizing the OKD48 signal with or without H_2O_2-induced oxidative stress in Ctrl or SOX2-expressing primary keratinocytes from OKD48/TG mice. The lower panel showing the OKD48 signal quantification. Values are determined by relative luminescence signals in n = 3 per group. **(b)** qPCR assay showing mRNA expression levels at 3 h after H_2O_2 stimulation (0.5 mM) in Ctrl or SOX2-expressing primary keratinocytes from OKD48-LUC/TG mice; n = 3. **(c)** Representative immunoblotting images showing expression of each protein in mouse keratinocytes; n = 3. **(d)** Representative images and quantification of TUNEL staining at 6 h after H_2O_2 stimulation (0.5 mM) in Ctrl or SOX2-expressing primary keratinocytes from TG mice. Quantification is performed by counting positive cells in n = 3 per group. Scale bar = 50 μm; **(e)** Representative images and quantification of TUNEL staining at 6 h after H_2O_2 stimulation (0.5 mM) in Ctrl or SOX2-expressing primary keratinocytes from TG mice after siRNA treatment. Quantification is performed by counting positive cells in n = 4 per group; Scale bar = 50 μm. **(f)** qPCR assay showing mRNA expression levels at 3 h after H_2O_2 stimulation (0.5 mM) in HaCaT cells treated with adeno-GFP or adeno-SOX2; n = 3 per group. **(a, b, and d–f)** A one-way analysis of variance followed by the Tukey–Kramer test is used. $^{**}p < 0.01$, $^{*}p < 0.05$.

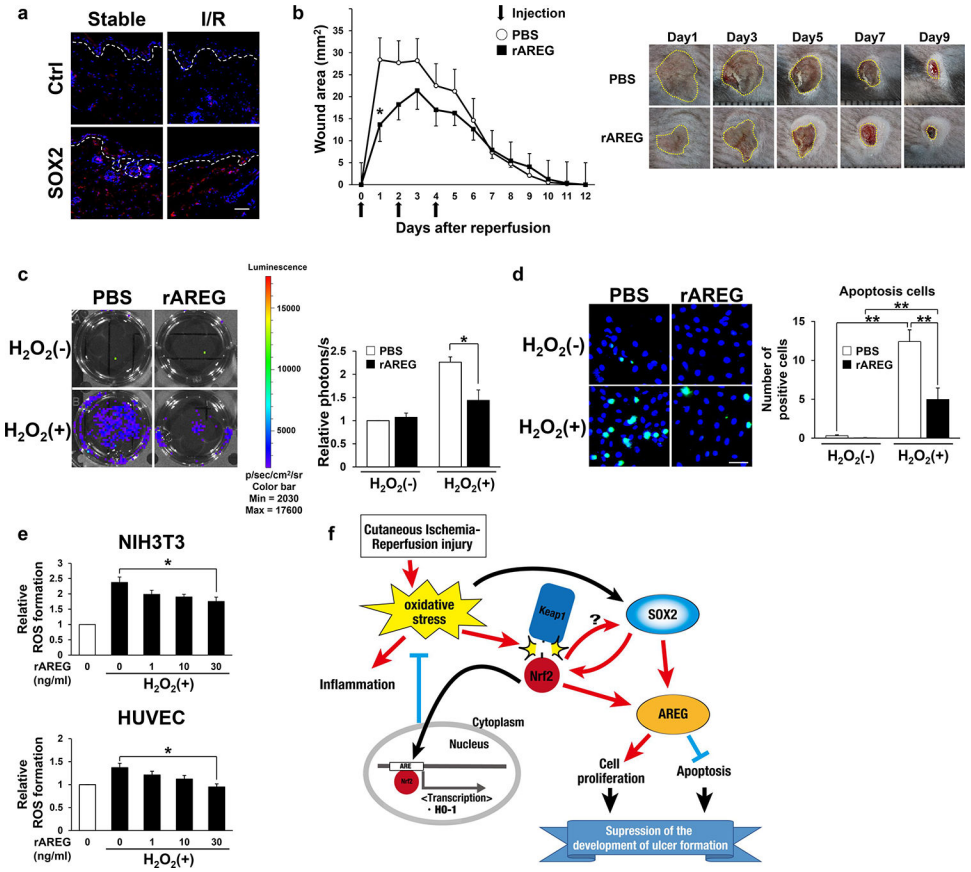

Figure 5. rAREG administration suppressed the development of I/R injury-induced skin ulcers *in vivo* and oxidative stress-induced ROS production *in vitro.*

(a) Representative immunofluorescence image showing the change of AREG expression and localization in I/R injury skin site in Ctrl or SOX2 mice; n = 3. Scale bar = 50 μm. **(b)** The size of the wound area following I/R injury in WT mice treated with subcutaneous injection of rAREG or PBS as a control. The right panel reveals the photographs of the wound following cutaneous I/R in PBS or rAREG-treated WT mice at 1, 3, 5, 7, and 9 days following reperfusion; n = 8 for day 0 to day 2, and n = 5 for day 3 to day 12, **(c)** Images visualizing the OKD48 signal with or without H_2O_2-induced oxidative stress in primary keratinocytes from OKD48-LUC mice treated with PBS or rAREG. The right panel shows OKD48 signal quantification. The values are determined by relative luminescence signals in n = 3 per group. **(d)** Representative images and quantification of TUNEL staining at 6 h after PBS or H_2O_2 stimulation (0.5 mM) in mouse fibroblasts (NIH3T3) cells treated with or without rAREG. The values are determined by counting positive cells in n = 5 per group; Scale bar = 50 μm. **(e)** Quantification of H_2O_2-induced intracellular ROS production in (top panel) NIH3T3 cells and (bottom panel) human vascular endothelial cells (HUVEC) treated with or without rAREG. ROS formation in cells without H_2O_2 treatment is assigned a value of 1; n = 4 in each group. All data are expressed as mean ± standard error of the mean. **(b)** Student's t-test (two-sided) and **(c-e)** one-way analysis of variance followed by

the Tukey–Kramer test is used. $^{**}p < 0.01$, $^{*}p < 0.05$. **(f)** Schematic model summarizing the mechanistic roles of SOX2 in keratinocytes regulating cutaneous I/R injury.