PRDX4 Improved Aging-Related Delayed Wound Healing in Mice
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Aging-related delayed wound healing is an issue of concern worldwide. Oxidative stress is involved in wound healing. Antioxidative enzymes have various roles in this process. PRDX4, a member of the PRDX family, is upregulated after injury. To investigate the effects of PRDX4 on aging-related wound healing, we subjected C57BL/6J (wild-type), human Prdx4–transgenic (i.e., hPrdx4+/+/), Prdx4-knockout (i.e., hPrdx4−/−) mice of three age groups (young, adult, and aged) to skin wound formation. The overexpression of PRDX4 accelerated wound healing in adult and aged mice but not in young mice. Aged hPrdx4+/+ mice showed reduced oxidative stress and inflammation, lower numbers of neutrophils, increased macrophage infiltration, increased angiogenesis, and increased GF levels. The granulation tissue of adult and aged hPrdx4+/+ mice was richer in fibroblasts than that in the matched wild-type mice. PRDX4 deficiency was associated with mortality in adult and aged mice. In vitro, the overexpression of PRDX4 promoted the proliferation and migration of fibroblasts derived from adult or aged mice and made fibroblasts more resistant to the cytotoxicity of hydrogen peroxide. PRDX4 is essential for wound healing and can improve the healing process from multiple aspects, suggesting that it may be very beneficial to wound treatment, especially for the elderly.


INTRODUCTION
With the progress of clinical medicine, the elderly population is gradually increasing, and society is aging (Kanasi et al., 2016). More and more attention has been paid to the care of the elderly. Owing to limitations in mobility, aged people, especially those who stay in bed for long periods of time, are more susceptible to skin injury caused by pressure and shear force (Pittman, 2007). Aging is becoming an increasingly important aspect in the study of wound healing. In comparison with the wounds in young people, skin wounds in the elderly show significantly delayed healing, which is associated with serious complications, mortality, and high treatment costs (Hardman and Ashcroft, 2008; Keylock et al., 2008). It is therefore of great significance to understand the causes and mechanisms of delayed wound healing in the elderly and to identify suitable treatments.

Wound healing is an intricate biological process that consists of three overlapping but distinct phases: the inflammatory, proliferative, and remodeling phases. In the inflammatory phase, large numbers of inflammatory cells are recruited under the guidance of chemokines to fight infection and cleanse the wound (Rodrigues et al., 2019). Various cytokines are released by these cells, some of which (e.g., IL-1β and TNFα) can affect the progression of the inflammatory phase (Eming et al., 2014). The proliferative phase is characterized by the replacement of blood clots with fleshy granulation tissue formed by activated fibroblasts (Sgonc and Gruber, 2013). From the beginning of the proliferative phase, activated fibroblasts migrate into the wound bed and proliferate in response to GFs, such as FGF2, TGFβ1, and PDGF, serving as a scaffold for other cells and components (Bainbridge, 2013; Sgonc and Gruber, 2013). In addition to fibroblasts, macrophages are another important element in granulation tissue and a key player during the inflammatory to the proliferative phase (Kim and Nair, 2019). The absence of macrophages in this period will reduce granulation tissue formation, resulting in delayed wound healing, whereas the local injection of macrophages can promote wound repair (Danon et al., 1989; Sorg et al., 2017).

ROS are generated during normal metabolic processes and take part in various physiological cell signaling processes, which are enhanced and centrally involved in the whole process of wound healing (Sen and Roy, 2008). Low concentrations of ROS are required to kill microorganisms and stimulate cell survival signaling; however, the excessive accumulation causes oxidative stress and impaired wound healing (Bryan et al., 2012; Dunnill et al., 2017). The production of ROS is dramatically elevated in aging individuals, leading to severe extracellular and intracellular oxidative stress, which damages the surrounding tissue and suppresses cell vitality, delaying wound healing (Cano Sanchez et al., 2018; Kurahashi and Fujii, 2015). Antioxidative enzymes exist abundantly and regulate reduction–oxidation homeostasis in the skin; many have been proven to be involved in wound healing (Kurahashi and Fujii, 2015). PRDX4, an
enzyme catalyzing the detoxification of hydrogen peroxide (H$_2$O$_2$), is a unique secreted member of the PRDX family (Yamada and Guo, 2018). In a series of previous studies, we reported that PRDX4 had important antioxidative anti-inflammatory roles and was important for the regulation of cell vitality, including proliferation and migration in chronic inflammatory diseases and cancer (Guo et al., 2019, 2012; Nabeshima et al., 2013; Zheng et al., 2020). A study showed that the mRNA expression of PRDX4 was increased on days 5–8 after injury, implying that it may be involved in the wound healing process (Kümin et al., 2007). However, the precise roles and the mechanism through which this enzyme is involved in wound healing remain unknown.

In this study, we subjected C57BL/6J (wild-type [WT]), human Prdx4–transgenic (i.e., hPrdx4+/+), Prdx4-knockout (i.e., Prdx4−/−) mice of three age groups (young, adult, and aged) to skin wound formation to investigate the effect of PRDX4 on aging-related wound healing. By harvesting fibroblasts from the normal skin of WT and hPrdx4+/+ mice of these three age groups, we observed the effect of the overexpression of PRDX4 on the proliferation and migration of these cells and on cell vitality under high oxidative stress in vitro. This study will give distinct insights that will improve the understanding of the control of proliferation, migration, and survival of fibroblasts and will hopefully lead to the development of therapeutic strategies to improve wound healing, especially in elderly individuals.

RESULTS
PRDX4 had crucial roles in the wound healing of adult and aged mice

In line with the results of an mRNA analysis (Kümin et al., 2007), the protein expression of PRDX4 in wound skin tissue was higher than that in normal skin tissue (Supplementary Figure S1a). After injury, the wound area on days 6–7 in adult hPrdx4+/+ mice and that on days 2–7 in aged hPrdx4+/+ mice were significantly smaller than that in the matched WT mice. However, in the young group, the wound area did not differ between the WT and hPrdx4+/+ mice (Figure 1a and b). Seven days after injury, H&E staining showed granulation tissues that were rich in cells and blood vessels in all young mice and in adult and aged hPrdx4+/+ mice. In contrast, fewer cellular and vascular components were observed in the granulation tissues of the adult and aged WT mice (Figure 1c). Moreover, severely impaired wound healing and high wound-related mortality were observed in the adult and aged Prdx4−/− mice (Supplementary Figure S1b and Supplementary Table S1). The three groups of mice showed no significant cutaneous phenotypic changes in histopathology or dermal thickness (Supplementary Figure S2a and b). Thus, PRDX4 may be a key molecule in wound healing.

PRDX4 overexpression improved oxidative stress during wound healing

The immunohistochemistry, western blotting, and ELISA results showed the high expression of hPRDX4 in wound skin tissue and serum in all hPrdx4+/+ mice (Figure 2a, b, and c). With aging, the ratio of 8-hydroxy-2′-deoxyguanosine–positive cells in the granulation tissue in WT mice was increased 7 days after injury, and in adult and aged hPrdx4+/+ mice, this ratio was reduced in comparison with that in the matched WT mice (Figure 2d). Moreover, 7 days after the injury, the thiobarbituric acid reactive substance levels in the serum of adult and aged hPrdx4+/+ mice were significantly lower than that in the matched WT mice (Figure 2e). Furthermore, in aged hPrdx4+/+ mice, a lower level of H$_2$O$_2$ was observed (Figure 2f). The overexpression of PRDX4 could improve the severe local and systemic oxidative stress induced by wounds and aging.

PRDX4 overexpression suppressed the inflammatory response in the adult and aged groups

The numbers of Gr1–positive neutrophils in adult and aged hPrdx4+/+ mice were significantly lower than those in the matched WT mice, whereas the numbers of Mac2–positive macrophages in aged hPrdx4+/+ mice were significantly higher than those in the aged WT mice (Figure 3a and b). The numbers of CD3–positive T cells did not differ between the WT and hPrdx4+/+ mice in each age group (Supplementary Figure S3). Similarly, the numbers of CD31–positive microvessels in adult and aged hPrdx4+/+ mice were significantly increased in comparison with those in the matched WT mice (Figure 3c). In line with these results, the IL-1β, TNFα, and NF-kB expression levels in aged hPrdx4+/+ mice were significantly reduced in comparison with those in the aged WT mice (Figure 3d). These data indicated that the local inflammatory response after skin wound was enhanced and prolonged with aging and that the overexpression of PRDX4 can suppress neutrophil infiltration and proinflammatory cytokines production, improving the prolonged inflammatory phase induced by aging.

PRDX4 overexpression promoted granulation tissue formation in adult and aged mice

In the adult and aged groups, the granulation area with α-smooth muscle actin–positive cells was significantly larger in hPrdx4+/+ mice than in WT mice (Figure 4a); similar results were observed regarding the number of α-smooth muscle actin–positive myofibroblasts per defined area (Figure 4b). Immunohistochemistry showed that the number of Ki-67–positive cells in granulation tissue decreased with aging in WT mice, and more Ki-67–positive cells were found in adult and aged hPrdx4+/+ mice than those found in the matched WT mice (Figure 4c), revealing that the proliferative capacity in adult and aged mice was higher than that in matched WT mice. Western blotting showed that cyclin D1 and vimentin expression levels were consistent with the results of Ki-67 staining (Figure 4d). Moreover, FGF2 levels in wound skin tissue and serum of aged hPrdx4+/+ mice were higher than those in aged WT mice; however, no difference was found in the adult group (Figure 4e and f). The TGFβ1 and PDGF-BB levels did not differ between WT and hPrdx4+/+ mice in each age group (Supplementary Figure S4). Next, we examined the expression of downstream genes related to the FGF2 pathway in the aged group. Western blotting showed that the extracellular signal–regulated kinase (ERK) pathways, which involve ERK1/2, phosphorylated cJUN, and phosphorylated cFOS, were highly activated in aged hPrdx4+/+ mice (Figure 4g). Furthermore, there was no significant difference between WT and hPrdx4+/+ mice in the length of the re-epithelialized epidermis and in the Ki-67–positive epithelial cells of the newly formed epidermis in
each of the age group (Supplementary Figure S5). In addition, the collagen expression levels did not differ among the three groups (Supplementary Figure S6). These results suggest that PRDX4 may promote granulation formation, especially through fibroblast proliferation and migration, by activating the FGF2/ERK signaling pathway in aged mice.

**In vitro,** PRDX4 overexpression reduced oxidative stress and promoted the proliferation and migration of skin fibroblasts. The high expression of hPRDX4 was confirmed in fibroblasts derived from the normal skin tissue of all hPrdx4+/+ mice (Figure 5a and b). The intracellular thiobarbituric acid reactive substance and H$_2$O$_2$ levels in fibroblasts increased with

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**Figure 1.** Adult and aged Prdx4– transgenic (i.e., hPrdx4+/+) mice but not young mice showed accelerated wound healing. (a) The skin wound healing process. Representative results of injury after 1 and 7 days are shown. Bar = 10 mm. (b) Changes in wound area as the percentage of the initial wound area for 7 days after the injury are shown. All values represent the mean ± SEM. **P < 0.01 versus WT mice; n = 15–18 mice per group. (c) Midline sections of the wound with H&E staining 7 days after the injury are shown. Bar in low power view = 1 mm; Bar in high power view = 100 μm; n = 8–10 mice per group. wk, week; WT, wild type; y, year.
aging and were lower in adult and aged hPrdx4+/+ mice than in matched WT mice (Figure 5c and d). In contrast, proliferative activity was decreased with aging, and significantly higher proliferative activity was observed in adult and aged hPrdx4+/+ fibroblasts than in matched WT fibroblasts (Figure 5e). The migration ability of these fibroblasts also decreased with aging, but the migration ability in all groups of hPrdx4+/+ fibroblasts was significantly higher than in the matched WT fibroblasts (Figure 5f). These findings were supported by western blotting to detect cyclin D1 and vimentin (Figure 5g).

Fibroblasts derived from hPrdx4+/+ mouse skin were more resistant to the cytotoxicity of H2O2

To identify the protective effects of PRDX4 on cell viability, skin fibroblasts derived from young mice were exposed to H2O2 of different concentrations. With an increase in the H2O2 concentration, cell vitality was gradually inhibited in the fibroblasts derived from WT skin; however, under the same H2O2 condition, higher vitality was observed in the fibroblasts derived from hPrdx4+/+ mouse skin (Figure 6a), suggesting that the inhibition of cell vitality was slowing down in these fibroblasts. Western blotting showed that the expression of cyclin D1 (Figure 6b) was consistent with the changes in cell vitality under different H2O2 concentrations. In contrast, the expression of cleaved caspase 3 increased as the H2O2 concentration increased in the fibroblasts derived from WT skin, and the lower expression of cleaved caspase 3 was found in fibroblasts derived from the skin of hPrdx4+/+ mice (Figure 6b), indicating that the overexpression of PRDX4 protected the fibroblasts from the cell apoptosis induced by high concentrations of H2O2. These results suggested that increased extracellular oxidative stress inhibited cell vitality and induced cell apoptosis and that PRDX4, as a secretory protein, was effective for protecting cells from the damage caused by extracellular oxidative stress.

DISCUSSION

In this study, the overexpression of PRDX4 accelerated wound healing in adult and aged mice, but no significant differences were observed in young mice. Similar results were also reported in superoxide dismutase 1 e-deficient and Prdx6-transgenic mice (Iuchi et al., 2010; Kümmer et al., 2006). This seems to suggest that delayed wound healing may be improved by enhancing the expression of these antioxidative enzymes. However, unexpectedly, the overexpression of catalase, an enzyme catalyzing the dismutation of H2O2, delayed wound healing in mice (Roy et al., 2006), suggesting that a certain level of ROS, especially of H2O2, is crucial for promoting the healing process and that excessive antioxidant use may have adverse effects. Moreover, the protein expression of PRDX4 was increased in skin wound tissue, and its deficiency could lead to death in adult and aged wound mice. These observations imply that PRDX4 may play a crucial role in resisting lethal factors during wound healing.

After an injury, neutrophils are first recruited to clean the wound. Neutrophil accumulation increases during the initial
inflammatory phase and quickly declines from day 4 after injury (Kim et al., 2008). On day 7 after injury, numerous neutrophils are still present at the wound site in aged WT mice, and inflammatory cytokines levels are higher in these mice than in young and adult mice. Studies show that early neutrophil infiltration increases with age owing to an evident reduction in neutrophil activity in association with aging (Sgonc and Gruber, 2013), which explains why more neutrophils were found in the aged WT mice. Neutrophil persistence and high proinflammatory cytokine levels will prolong the inflammatory phase and delay wound healing (Barrientos et al., 2008; Chen and Rogers, 2007). However, fewer neutrophils and reduced proinflammatory cytokine levels were observed in aged hPrdx4+/+ mice in comparison with those in aged WT mice, suggesting that the prolonged inflammatory response was improved at the wound site, probably promoting healing in these mice. Furthermore, excessive neutrophil accumulation can lead to extracellular oxidant stress, and neutrophil-generated ROS can directly trigger mitochondrial dysfunction (Zhang et al., 2018), damaging tissue and cells around the wound and further amplifying the inflammatory response. The overexpression of PRDX4 in wound skin significantly improved the local and systemic oxidative stress status to protect wound tissue from oxidative damage, probably leading to reduced neutrophil infiltration in aged hPrdx4+/+ mice.

Interestingly, in aged hPrdx4+/+ mice, macrophage numbers were increased in comparison with that in the aged WT group. A large improvement of oxidative stress caused by PRDX4 overexpression in aged mice may produce modified reduction–oxidation homeostasis, and alteration of ROS components may change capillary permeability (Sen and Roy, 2008) in skin wounds of these mice, facilitating monocyte infiltration. Moreover, aged mice showed delayed monocyte/macrophage infiltration in comparison with young mice (Sgonc and Gruber, 2013). The overexpression of PRDX4 may prevent the delay and allow monocytes to begin wound infiltration earlier, eventually leading to the accumulation of greater numbers of macrophages in the wound site in aged hPrdx4+/+ mice. To avoid further damage in the wound, these macrophages will phagocyte infiltrated neutrophils and cell debris, which may also be an important contributor to the reduction in the number of neutrophils in the wounds of aged hPrdx4+/+ mice.

FGF2 can accelerate granulation tissue formation by promoting fibroblast/myofibroblast proliferation and migration and angiogenesis (Behm et al., 2012). Delayed wound healing in aged mice is closely associated with the reduced

Figure 3. The inflammatory response was suppressed, and angiogenesis was promoted in adult and aged Prdx4–transgenic (i.e., hPrdx4+/+) mice. (a, b) Immunohistochemical analysis to detect (a) Gr-1, (b) Mac-2, and (c) CD31 7 days after the injury. Bar = 50 µm. All values represent the mean ± SEM. *P < 0.05 versus WT mice; n = 8–10 mice per group. (d) Western blotting to detect IL-1β, TNFα, and NF-κB in wound skin 7 days after the injury. HPF, high power field; wk, week; WT, wild type; y, year.
expression of FGF2 (Swift et al., 1999). In this study, a reduced FGF2 level was observed in the wound skin tissue of aged WT mice; however, surprisingly, the level of this GF was dramatically increased in the wound skin tissue and serum of aged hPrdx4+/− mice, which may be the main reason for the improvement in the delayed wound healing of these mice. It is unclear why FGF2 is elevated in the wound skin of aged hPrdx4+/− mice. Macrophages, a major cellular origin for FGF2, play important roles in granulation tissue formation and can promote wound healing by increasing FGF2 production (Laplante et al., 2017; Takehara, 2000). Thus, one possible explanation is that the massive aggregation of macrophages led to high local and systemic levels of FGF2 in aged hPrdx4+/− mice. Indeed, some key transcription factors related to the FGF2 signaling pathway, including ERK1/2, c-FOS, and cJUN (Cheng et al., 2007; Li et al., 2014; Xiao et al., 2012), were activated in these mice. Thus, PRDX4 may promote granulation tissue formation through FGF2/ERK1/2 pathway activation, leading to the improvement of delayed wound healing.

However, this hypothesis is probably oversimplified because although there were no significant differences in the expression of GFs between the adult WT and hPrdx4+/− mice, higher cellular density and proliferation activity of myofibroblasts in granulation tissue were observed in adult hPrdx4+/− mice with a faster healing process. In vitro, the viability of fibroblasts derived from the normal skin of adult and aged hPrdx4+/− mice was significantly higher than that in the matched WT group. Normally, intracellular ROS can be maintained at a low level by the regulatory role of the antioxidant system and can act as signaling molecules in cell proliferation, senescence, and apoptosis (Cui et al., 2012). ROS production increases with age (Sohal and Dubey, 1994), and high intracellular levels of
ROS (e.g., H$_2$O$_2$) are closely associated with cell senescence, wherein cell proliferation is arrested (Lu and Finkel, 2008). Increased antioxidant levels delay this cellular process (Itahana et al., 2003; Serre et al., 2003). The overexpression of PRDX4 may properly reduce the intracellular ROS/H$_2$O$_2$ levels and delay senescence, leading to the enhanced proliferation of fibroblasts in adult and aged $hPrdx4^{+/+}$ mice. Thus, the promotion of fibroblast proliferation by the overexpression of PRDX4 may be an important reason for the accelerated wound healing observed in adult $hPrdx4^{+/+}$ mice.

Furthermore, with an increase in the H$_2$O$_2$ concentration in the culture medium, cell vitality was obviously suppressed in WT fibroblasts; however, this suppression was improved in $hPrdx4^{+/+}$ fibroblasts (Figure 6a and b). Severe oxidative stress also induces cell apoptosis to suppress cell proliferation (Sinha et al., 2013). Thus, in aged $hPrdx4^{+/+}$ mice, PRDX4 overexpression not only promoted fibroblast proliferation but also inhibited the apoptosis of fibroblasts induced by high ROS/H$_2$O$_2$ levels.

Moreover, PRDX4 overexpression led to age-independent enhancement of the migration capability of fibroblasts in vitro. The association between PRDX4 and cell migration was also reported in other studies, but in different types of cells, it may regulate this cell behavior depending on different signaling (Wei et al., 2011; Zheng et al., 2020). The specific mechanism through which PRDX4 is involved in the regulation of fibroblast migration requires further investigation. However, during the wound healing process, in addition to the migration ability of cells themselves, various chemokines play important roles in fibroblast migration (Ridiandries et al., 2018). Thus, although the migration of skin fibroblasts was also significantly enhanced in young $hPrdx4^{+/+}$ mice, it did not affect the healing process in these mice.

In summary, the overexpression of PRDX4 significantly improved delayed wound healing by shortening the prolonged inflammatory phase, increasing GF production, and promoting fibroblast proliferation and migration in aged mice, whereas it only accelerated wound healing in the proliferative phase by upregulating fibroblast vitality in adult...
mice, suggesting that PRDX4 may be extremely beneficial to wound healing, especially in elderly individuals. Furthermore, in the young group, although faster wound healing was not found in hPrdx4+/+ mice, the overexpression of PRDX4, at the very least, did not impair wound healing, implying that the application of this antioxidant enzyme in wound treatment will not lead to worse outcomes in individuals with weaker oxidative stress.

**MATERIALS AND METHODS**

A detailed description of materials and methods is available online in the Supplementary Materials and Methods.

**Mice**

Male WT (C57BL/6), hPrdx4+/+, and Prdx4-/- mice aged 4 weeks (young), 12 weeks (adult), and 1 year (aged) were used for the experiments. All experiments were conducted in accordance with the guidelines of the Ethics Committee of Animal Care and Experimentation, Kanazawa Medical University, Uchinada, Japan (protocol code: 2020-77).

**Wound healing model**

After hair removal and disinfection and under anesthesia, a double 6-mm full-thickness wound was created at a dorsal site in all mice. Wound sites were digitally photographed daily after wound creation, and the wound area was measured using the ImageJ software program (National Institutes of Health, Bethesda, MD). Mice were killed 7 days after injury, and wound site skin tissue and serum were collected for experiments.

**Primary fibroblast culture and treatment**

A 1 × 1 cm piece of skin tissue was taken from the dorsum of the mice in all groups. The skin tissue was then cut into 1-mm square small pieces, placed on a culture dish with DMEM (FUJIFILM Wako)
Pure Chemical Corporation, Tokyo, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), and maintained in a humidified atmosphere at 37 °C. The fibroblasts that migrated out of the skin were harvested and cultured. These fibroblasts were exposed to 2.5, 5, or 10 μM H₂O₂ (FUJIFILM Wako Pure Chemical Corporation) for 24 hours, and then cell viability was measured.

Statistical analysis
Data were expressed as the mean ± SEM. An unpaired Student t-test was performed to compare the values between two groups. P-values < 0.05 were considered to indicate statistical significance.

Data availability statement
All relevant data for this article are contained within this manuscript, and any further inquiry can be made to the corresponding author.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This work was supported by Grant-in-Aid for Young Scientists (number 20K17363 to RY and number 19K16783 to XG) and Grant-in-Aid for Scientific Research (number 20K07454 to SY and 17K10803 to HU) from the Ministry of Education, Culture, Sports, Science and Technology (Tokyo, Japan). We thank Yuka Hiramatsu and Manabu Yamashita for their expert technical assistance.

AUTHOR CONTRIBUTIONS
Conceptualization: RY, XG, SY; Data Curation: RY, XG, SY; Formal Analysis: RY, XG, SY; Funding Acquisition: RY, XG, HU, SY; Investigation: RY, XG, JZhe, JZha, JH, AS, HU, TM, SY; Methodology: RY, XG, JZhe, JZha, JH, AS; Project Administration: RY, XG, SY; Resources: RY, XG, SY; Software: RY, XG, AS; Supervision: XG, SY; Validation: RY, XG, SY; Visualization: RY, XG, SY; Writing - Original Draft Preparation: RY, XG; Writing - Review and Editing: RY, XG, SY; Visualization: RY, XG, SY; Administration: RY, XG, SY; Resources: RY, XG, SY; Software: RY, XG, AS; Funding Acquisition: RY, XG, HU, SY; Investigation: RY, XG, JZhe, JZha, JH, AS, HU, TM, SY; Writing - Review and Editing: RY, XG, SY; Funding Acquisition: RY, XG, HU, SY; Investigation: RY, XG, JZhe, JZha, JH, AS, HU, TM, SY; Writing - Review and Editing: RY, XG, SY; Funding Acquisition: RY, XG, HU, SY; Investigation: RY, XG, JZhe, JZha, JH, AS, HU, TM, SY; Writing - Review and Editing: RY, XG, SY; Funding Acquisition: RY, XG, HU, SY; Investigation: RY, XG, JZhe, JZha, JH, AS, HU, TM, SY; Writing - Review and Editing: RY, XG, SY;

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.04.015.

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