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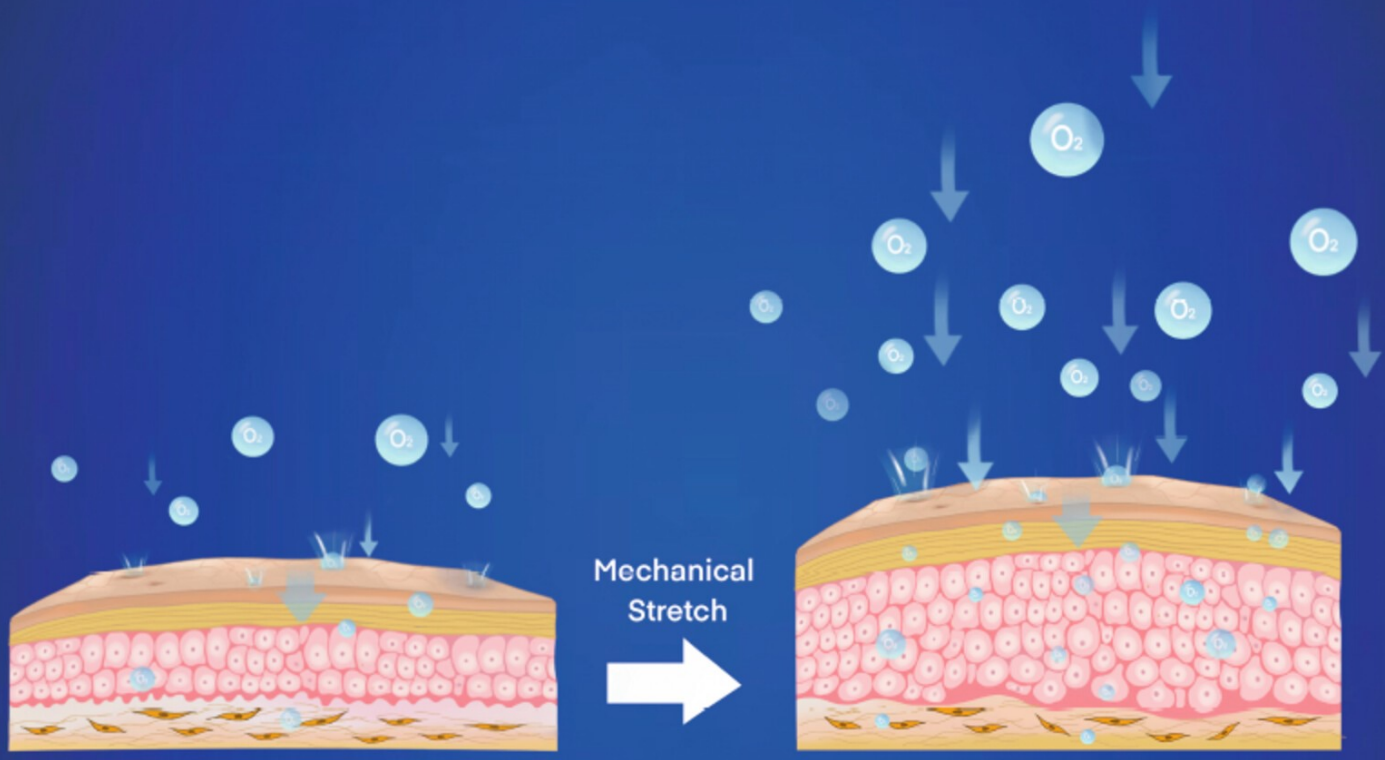

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RESEARCH ARTICLE

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Dynamics of cutaneous atmospheric oxygen uptake in response to mechanical stretch revealed by optical fiber microsensor

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Abstract

Skin expands and regenerates in response to mechanical stretch. This important homeostasis process is critical for skin biology and can be exploited to generate extra skin for reconstructive surgery. Atmospheric oxygen uptake is important in skin homeostasis. However, whether and how cutaneous atmospheric oxygen uptake changes during mechanical stretch remains unclear, and relevant research tools to quantify oxygen flux are limited. Herein, we used the scanning micro-optrode technique (SMOT), a non-invasive self-referencing optical fiber microsensor, to achieve real-time measurement of cutaneous oxygen uptake from the atmosphere. An in vivo mechanical stretch-induced skin expansion model was established, and an in vitro Flexcell Tension system was used to stretch epidermal cells. We found that oxygen influx of skin increased dramatically after stretching for 1 to 3 days and decreased to the non-stretched level after 7 days. The enhanced oxygen influx of stretched skin was associated with increased epidermal basal cell proliferation and impaired epidermal barrier. In conclusion, mechanical stretch increases cutaneous oxygen uptake with spatial-temporal characteristics, correlating with cell proliferation and barrier changes, suggesting a fundamental mechanistic role of oxygen uptake in the skin in response to mechanical stretch. Optical fiber microsensor-based oxygen uptake detection provides a non-invasive approach to understand skin homeostasis.

KEYWORDS
cutaneous oxygen uptake, mechanical stretch, optical fiber sensor, skin expansion, surgery

Abbreviations: ANOVA, analysis of variance; CCK-8, Cell Counting Kit-8; HEKs, human primary epidermal keratinocytes; OD, optical density; PFA, paraformaldehyde; SMOT, scanning micro-optrode technique.

Shengzhou Shan and Jiahao He contributed equally to this work.

1 | BACKGROUND

It has been observed since the nineteenth century that human skin can directly take up oxygen from the atmosphere. Human epidermis is almost exclusively supplied by atmospheric oxygen rather than the oxygen from dermal circulation.[1–3] Keratinocyte sensing of atmosphere oxygen contributes significantly to the mammalian systemic adaption to environmental hypoxia.[4] A recent study revealed that differential atmospheric oxygen level regulated the axis of keratinocytes proliferation-differentiation.[5] In addition, our previous work showed that atmospheric oxygen influx correlated with regeneration efficiency in a *Xenopus* wound healing model.[6] Thus, the atmospheric oxygen uptake has an essential role in maintaining epidermal homeostasis.

As the outmost surface of the human body, skin is constantly exposed to mechanical stress. Mechanical stress is involved in many cutaneous physiological and pathological process.[7,8] For example, mechanical stretch is a key factor in the pathogenesis of hypertrophic scars[9,10] and psoriasis.[11] Human skin can be stretched and enlarged remarkably during pregnancy or obesity, and also in skin expansion for skin grafts in plastic surgery up to tens of folds of area size. Epidermis responds to stretch at the single-cell level in skin expansion with distinct proliferation and differentiation subpopulations mediated by mechano-sensing, biochemical signalling and regulated transcriptomics.[12–15] However, oxygen uptake by the skin has not been studied when the mammalian epidermal homeostasis is disrupted during mechanical stretching.

The optical fiber technique has been clinically used in medical diagnosis and therapeutic monitoring since the 1960s.[16,17] An optical fiber can be placed non-invasively close to external organs such as the skin or cornea, providing a convenient and safe approach for tissue assessment. Because oxygen has a quenching effect on fluorescence, oxygen concentration can be indicated by detecting the rate of fluorescence decay of the fluorescent substance.[18] A new development of this optical sensing technique coupled with self-referencing gives a quantitative measurement of flux of oxygen with high spatial–temporal resolution.[6,19] Based on this novel approach, here we used a scanning micro-optrode technique (SMOT), a non-invasive self-referencing optical fiber microsensor, to determine spatial-temporal changes in oxygen uptake in a mechanical stretch-induced skin expansion model and assessed the epidermal proliferation and barrier function.

2 | METHODS

2.1 | Animal model

Animal procedures were approved by the Institutional Animal Care and Use Committees at University of California, Davis (protocol #20909). Female Wistar rats aged 4–6 weeks were obtained from Envigo (Indianapolis, IN, USA). Based on previous methods with modifications,[20,21] the skin expansion model was established to apply mechanical stretch to the skin. Briefly, under anaesthesia, 10 mL silicone expanders (Guangzhou Wanhe Plastic Materials Co., Ltd., Guangzhou, China) were implanted into the dorsum subcutaneously. Sterile physiological saline was injected into the expander after 7 days post-operation to 120 mmHg of the intracapsular pressure monitored with a modified sphygmomanometer.

2.2 | Oxygen flux measurement

The oxygen flux was measured by SMOT as previously described in detail.[19] In brief, hair on the dorsum was removed with hair remover cream (Veet) prior to measurements. During measurements (under anaesthesia), the tip of the optrode was brought to measurement position ~10 μm from interfollicular epidermis and at least three measurements were taken at each position (outside stretch area, and edge, middle and centre of stretch area; see Figure 3a). All procedures were conducted at room temperature 21.1–22.4°C. The rat body temperature was 36.0–36.4°C.

2.3 | Histology and Immunohistochemistry

Skin tissue were obtained, fixed and embedded in paraffin. Cross sections were stained with haematoxylin and eosin. For immunohistochemistry staining, sections were antigen repaired and blocked and incubated with primary antibody. Then, the sections were washed and incubated with corresponding HRP-conjugated secondary antibody. Slides were scanned using a slide scanner (Pannoramic SCAN II; 3DHistech Kft. Budapest, Hungary), and images were randomly captured by a slide viewer (CaseViewer; 3DHistech Kft. Budapest, Hungary). The quantitative analysis of positive expression was conducted using ImageJ (National Institutes of Health, Bethesda, MD, USA). The following primary and secondary antibodies were used: anti-Ki67 (1:200; Abcam, Cambridge, UK), anti-Loricrin (1:500; Abcam, Cambridge, UK) and Goat anti-Rabbit Secondary Antibody (1:500; Jackson Immuno Research Laboratories, West Grove, PA, USA).

2.4 | Cell culture and mechanical stretching

Normal human primary epidermal keratinocytes (HEKs) were purchased from ScienCell Research Laboratories. The HEKs were cultured in keratinocyte medium containing 1% keratinocyte growth supplement and 1% penicillin–streptomycin (all from ScienCell Research Laboratories, Carlsbad, CA, USA). HaCat cells were cultured in DMEM medium (HyClone) containing 10% FBS (Gibco, Australia) and 1% penicillin–streptomycin. The cultures were maintained in incubator at 37°C in a humidified atmosphere with 5% CO_2.

To apply mechanical stretch to HaCat cells and HEKs, cells were seeded on six-well flexible silicone rubber BioFlex plates (Flexcell

International Corporation, Hillsborough, NC, USA) at a density of 5×10^5 cells/well in 2 mL of medium. Cells were cultured for 24 h to reach 60%–80% confluence before mechanical stretch was applied. Cyclic mechanical stretch was applied in a sinusoidal pattern with 10% amplitude at 0.5 Hz for 24 h using an FX-5000T™ Flexcell Tension Plus device (Flexcell International Corporation, Hillsborough, NC, USA) as previously reported.[22] HaCat cells and HEKs cultured in the same plates but not stretched served as controls.

2.5 | Western blot

Cells were lysed, and protein fractions were run on SDS-PAGE gel and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked and then incubated with primary antibodies against Loricrin (1:1000; Affinity Biosciences, USA) and GAPDH (1:10000; Bioworld, Minnesota, USA). The blots were then incubated with HRP-conjugated secondary antibodies and visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA). Quantitative analysis was performed for immunoreactive bands using ImageJ software.

2.6 | Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) was used to assess cell proliferation. After stretching, cells were reseeded in 96-well plates at a density of 1×10^4 cells/well. After culturing for 24 and 48 h in complete medium, each well received 90 μL medium mixed with 10 μL CCK-8 reagent and was further incubated at 37°C for 2 h. Subsequently, the optical density (OD) at 450 nm (630 nm as reference) was measured using an Infinite M200 PRO microplate reader (TECAN, Switzerland).

2.7 | Statistical analyses

Data are presented as mean ± SD. A two-tailed Student's *t*-test or analysis of variance (ANOVA) was used to assess the statistical significance. The differences were considered statistically significant when $p<0.05$.

3 | RESULTS

3.1 | Rat skin takes up atmospheric oxygen

We first determined cutaneous uptake of atmospheric oxygen by unstretched skin in anesthetized rats. Oxygen concentration at the near and far positions close to the skin surface was detected during the excursion of the micro-optrode (Figure 1A). The tip of the micro-optrode is coated with oxygen sensitive fluorophore. The quenching effect of oxygen allows the measurement of the oxygen level (Figure 1B). To eliminate the possible effects of hair remover on oxygen flux, we detected the oxygen flux on footpad (no hair) pre- and post-hair remover treatment (Figure S1A). The results showed that hair remover did not significantly affect the oxygen flux ($p>0.05$) (Figure S1B). Freshly isolated skin ex vivo had similar oxygen influx (-48.7 ± 11.03 pmol cm^{-2} s^{-1}) as that of the in vivo skin (-47.32 ± 8.79 pmol cm^{-2} s^{-1}), which supports the notion that atmospheric oxygen can be taken up directly by the skin. In contrast, after the skin was fixed in 4% paraformaldehyde (PFA), oxygen influx returned to a value near zero (-6.32 ± 3.98 pmol cm^{-2} s^{-1}) ($p<0.001$) (Figure 1C,D). This indicates that dead skin lost the ability to take up atmospheric oxygen.

3.2 | Mechanical stretch increases cutaneous atmospheric oxygen influx in a time-dependent manner

To detect the cutaneous atmospheric oxygen influx in response to mechanical stretch, we measured the oxygen flux at the centre area of stretched skin at 6 hours, 1, 3 and 7 days after expander inflation (Figure 2A). The oxygen flux of non-stretched skin was also measured as control. The results showed that there was no significant increase in oxygen influx at 6 hours post-inflation. However, oxygen influx was significantly increased after 1 day ($p<0.05$) and 3 days ($p<0.001$) stretching respectively, compared to non-stretched rat skin. Interestingly, the oxygen influx decreased to baseline after 7 days ($p>0.05$) stretching, which showed no significance compared to the non-stretched skin (Figure 2B). Taken together, these data show that mechanical stretch increases the epidermal oxygen uptake in a time-dependent manner, and we chose 3 days post-stretching for subsequent experiments.

3.3 | Spatial characteristic of oxygen influx at stretched skin

To define the spatial characteristic of the atmospheric oxygen uptake in rat skin expansion, we measured the oxygen flux at different sites in the rat skin expansion model after 3 days stretching. According to the shape contour of the expanded area, points A-D were selected to map the spatial characteristic of oxygen influx in skin expansion (Figure 3a). Point A is located outside the stretched area. Points B, C and D located in the stretched area. Point D is at the centre of the stretched area, point B is at the edge of the stretched area and point C is at the midpoint between B and D. The overall results showed a significant increase of oxygen influx at point D compared to point A. For some rats (rat 1, 3 and 5), points B and C also showed an increase of oxygen influx compared to point A. However, there is no significant difference in points B, C and D on the whole (Figure 3b). Taken together, these findings indicate that rat skin, especially in the centre site of the expanded area, takes up more atmospheric oxygen after stretching.

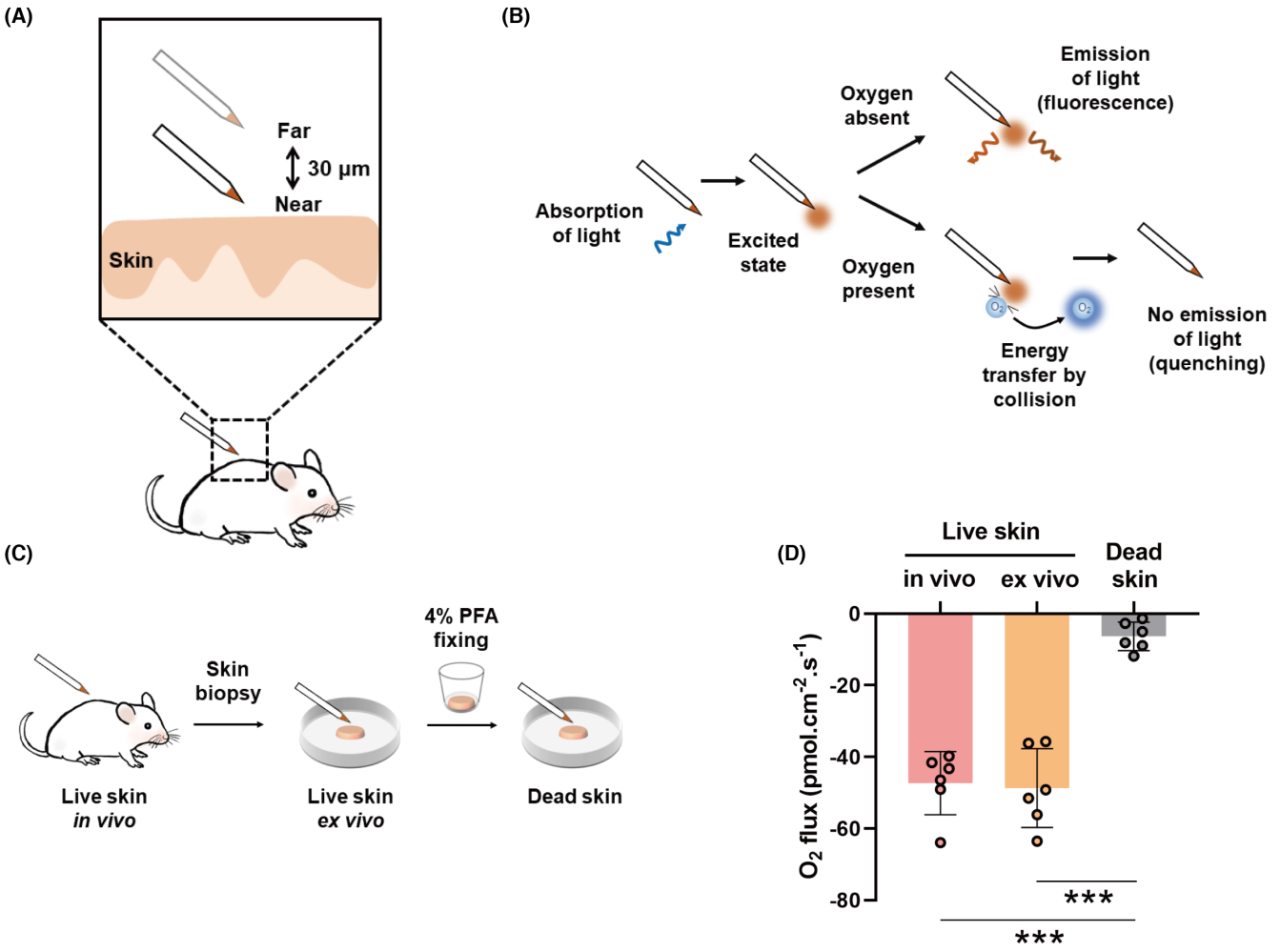

FIGURE 1 Atmospheric oxygen flux at rat skin. (A and B) Schematic of the fluorescence quenching-based oxygen measurement using the scanning micro-optrode technique (SMOT). (C) Schematic of measurements in the live/dead rat skin. (D) Results of the oxygen flux in the live/dead rat skin. Data are presented as means ± SD. Each dot represents one rat. ***$p < 0.001$.

3.4 | Mechanical stretch impairs epidermal barrier and increases epidermal keratinocytes proliferation in vitro and in vivo

We further investigated the reasons why oxygen influx increased in the stretched rat skin. It is likely that stretch induced epidermal barrier breaking leading to the increased atmospheric oxygen sink. We detected the epidermal barrier function in skin expansion by staining loricrin, a major component of cornified cell envelope, in the rat skin. The results showed that loricrin decreased significantly in 3-day-stretched rat skin compared to the non-stretched rat skin (Figure 4A,D). Meanwhile, the in vitro cell stretching assay also showed decreased loricrin expression level in HEKs and HaCat cells (Figure 4G) after mechanical stretching for 24 h. These data indicate that impaired epidermal barrier after mechanical stretching might give rise to the increased oxygen influx in skin expansion. In addition, we detected the state of epidermal cell proliferation after stretching, because cell proliferation increased demand for oxygen. Ki67-positive cells were counted in the 3-day-stretched rat skin. The results showed that epidermal basal cell proliferation increased significantly in 3-day-stretched rat skin compared to the non-stretched rat skin (Figure 4A,C). Next, H&E staining showed increased thickness of epidermis in the 3-day-stretched rat skin compared to the non-stretched rat skin (Figure 4A,B), which may result from the increase of hyperproliferative epidermal cells in response to stretch. Moreover, the in vitro cell stretching assay also showed increased proliferative capacity in HEKs (Figure 4E) and HaCat cells (Figure 4F) after mechanical stretching for 24 h. This enhanced capacity to proliferate lasted at least 48 h even when detached from mechanical stretch stimulation. Together, these data illustrate that mechanical stretch-induced cell proliferation and epidermal barrier impairment might be correlated to the increased oxygen influx in skin expansion.

4 | DISCUSSION

Skin homeostasis maintenance is a complex process. In response to extrinsic mechanical stimuli, intrinsic physiological reactions are orchestrated to restore the cutaneous homeostasis. In this context,

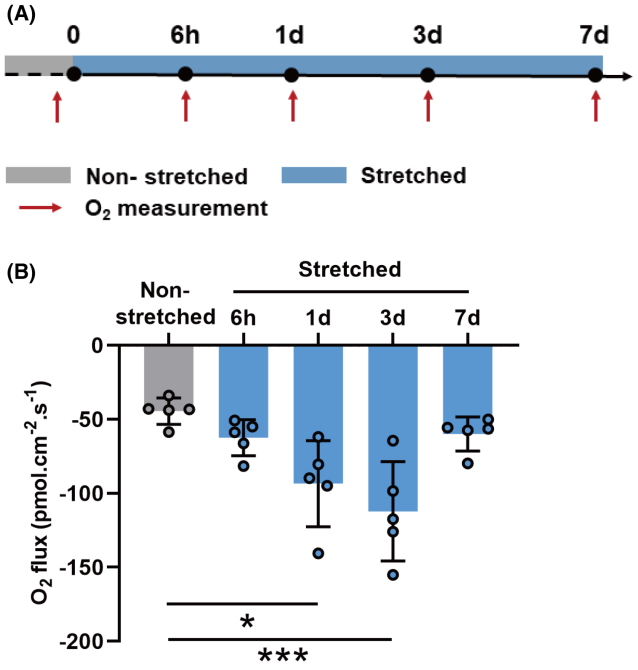

FIGURE 2 Atmospheric oxygen flux in stretched rat skin at different time points. (A) Schematic timecourse of measurements in rat skin expansion. (B) Results of the oxygen flux in rat skin. 'Control' represents non-stretched rat skin. '6h, 1d, 3d, 7d' represent stretched rat skin for different periods in hours and days. Data are presented as means ± SD. Each dot represents one rat. $^{*}p < 0.05$. $^{***}p < 0.001$.

the cutaneous oxygen uptake alterations in response to external mechanical stretch appear very interesting and remain to be elucidated. In our study, a real-time non-invasive SMOT was utilized to directly quantify the cutaneous atmospheric oxygen flux. We demonstrated that rat skin in vivo and ex vivo could directly take up atmosphere oxygen. Moreover, the cutaneous oxygen uptake increased significantly in response to mechanical stretch during skin expansion and was associated with enhanced epidermal cell proliferation and impaired epidermal barrier.

Cutaneous respiration has been known for a long time.[23,24] According to a review from Fitzgerald et al., the absorption of atmospheric oxygen through skin surface is approximately 30 to 100 $mL\,m^{-2}\,h^{-1}$.[24] Stücker et al. (2002) developed non-invasive measurements of transcutaneous oxygen flux with an oxygen fluxoptode consisting of multiple layers of membrane. They reported the oxygen influx from the atmosphere at $0.53 \pm 0.27\,mL\,m^{-2}\,min^{-1}$ at normal human skin surface.[1] Based on the same measuring principle of oxygen-induced fluorescence quenching, we used the SMOT to detect the oxygen influx in vivo in an improved non-invasive self-referencing (near and far positions) mode without direct contact with the skin. The value we detect at rat skin under resting conditions varies in the range of $-47.32 \pm 8.79\,pmol\,cm^{-2}\,s^{-1}$. Interestingly, we found that the oxygen influx in rat palm (foot) skin was less than the dorsal skin. One possible explanation is that the palm is more cornified than the dorsal skin which leads to the thickness barrier and thus lower oxygen diffusion.

Effective tissue regeneration requires an adequate supply of oxygen, which regulates cell proliferation, differentiation and angiogenesis.[5,25,26] Continuous delivery of dissolved oxygen has been reported to significantly improve chronic wound healing.[27] The use of hyperbaric oxygen therapy for rapid skin expansion can effectively increase capillary blood flow to the expanded skin and skin growth.[28] Skin expansion usually involves stages including expander inflation, stretching, skin growth and relaxation. During this sequential process, mechanical stretch triggers a series of morphological and physiological changes in the epidermis and dermis,[29] while the atmospheric oxygen influx conditions are not well known. Here, our data reveal a spatial-temporal pattern of atmospheric oxygen influx during skin expansion. We observed that the oxygen influx increased at 1 to 3 days post-expansion and then returned to the control level at 7 days post-expansion. One possibility for this is relaxation of skin over time, because of the viscoelastic properties and skin growth which diminishes the pressure inside the expander. It is reported that after 7 days, there was no measurable pressure inside the expander.[30] Thus, the decreased atmospheric oxygen influx after 7 days stretching might result from the decreased strain that the expander imposed on the skin. Next, we observed the spatial characteristics of cutaneous atmospheric oxygen influx during stretch-mediated skin expansion. After stretching for 3 days, the overall spatial distribution trend showed enhanced cutaneous atmospheric oxygen uptake in the stretched area, especially the centre portion of the area. Meanwhile, we noticed that the differences of atmospheric oxygen influx existed between different sites of stretched area in one rat. Adrián Buganza Tepole et al. showed that spatial-temporal evolution of the grown skin is co-related to the shape of the expander.[31] Besides, Marquardt et al. reported that the intracutaneously measured tissue oxygen partial pressure exhibited a linear correlation with the force.[32] Therefore, we presumed the geometric shape of expander, and thus, spatial strain distribution difference may be a factor in the differential oxygen influx in the stretched area. However, an accurate stress profile of the expander has not been quantified. Some studies have used finite element analysis to model tissue expansion, providing a method to map the stress distribution of the skin during expansion.[33–35] Additionally, we observed strong inter-individual fluctuations of atmospheric oxygen influx. In our experiment, animal age, gender and skin humidity have been controlled. On one hand, this may be explained by the fact that anaesthesia condition, the breath triggered body movement and skin temperature inconsistencies could be the interference factors upon in vivo detection. On the other hand, the amount of skin deformation of each rat may vary due to the differential skin texture and elasticity in response to stretch, although we have controlled the intracapsular pressure of expander when expanding.

To understand the possible reasons involved in mechanical stretch-induced atmospheric oxygen influx peaking at 3 days post-expansion, we next investigated the epidermal barrier and cell proliferation conditions from the standpoint of oxygen flux following

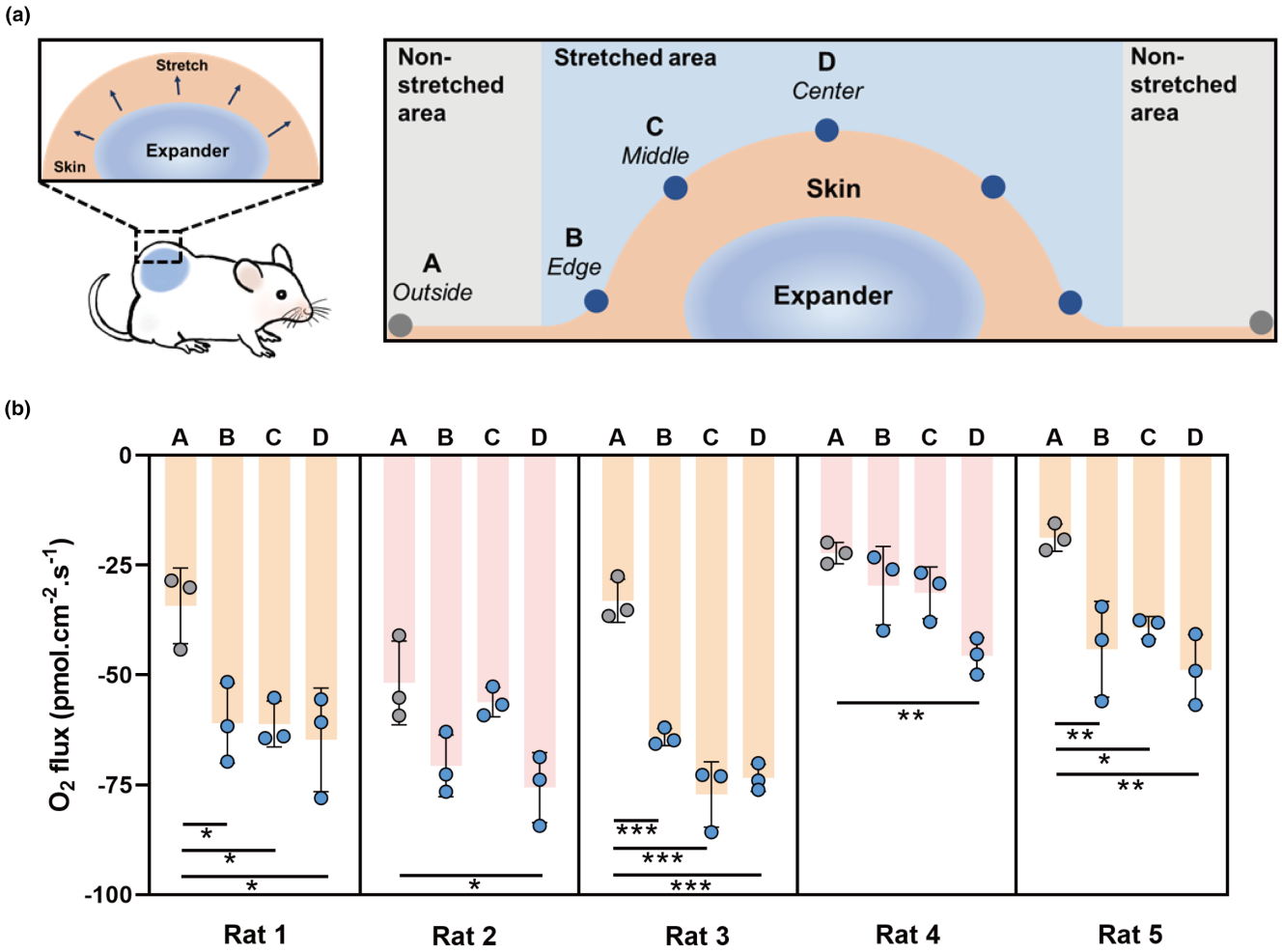

FIGURE 3 Spatial characteristic of atmospheric oxygen flux in rat skin expansion model. (a) Schematic of skin expansion model and measuring points A–D. Point A is located outside the stretched area. Point D is at the centre of the stretched area, point B is at the edge of the stretched area and point C is at the midpoint between B and D. (b) Results of the oxygen flux in rat skin. 'A, B, C and D' represent points A–D for different sites as shown above. Data are presented as means ± SD. Each dot represents one experiment. $^{*}p < 0.05$. $^{**}p < 0.01$.$^{***}p < 0.001$.

passive diffusion in a supply–demand way. Atmospheric air contains about 20.9% oxygen, higher than epidermis (0.2–0.8%) and dermis (>7%),[3] which would theoretically result in the atmospheric oxygen flowing down a concentration gradient when the epidermal barrier is compromised. The stratum corneum is a significant epidermal barrier to gas diffusion from the atmosphere. When the epidermal barrier is disrupted, the skin permeability as well as oxygen diffusion is increased.[36–38] Stratum corneum components, including loricrin, filaggrin and involucrin, are functionally important to accomplish the barrier function.[39,40] Qiao et al. reported that down-regulation of loricrin, filaggrin and involucrin level in HaCat cells after mechanical stretch may contribute to the psoriasis progression.[11] Also, our previous work showed cyclic mechanical stretch inhibited loricrin expression in keratinocytes.[22] Moreover, human epidermal stem cells seeded on stiff substrates showed less expression of loricrin and filaggrin than that on soft substrates.[41] These indicate that skin barrier function is affected by mechanical stimulation. In accordance with these works, here we found that the epidermal barrier was impaired after stretching characterized by down-regulation of loricrin in vitro and in vivo. This might give an explanation for the robust atmospheric oxygen influx after stretching.

As determined by our experiments, mechanical stretch promoted epidermal keratinocyte proliferation in vitro and in vivo, in general agreement with previous reports.[13,22,42] Cell hyperproliferation consumed a large amount of oxygen. This might result in an aggravated hypoxic microenvironment in the epidermis. Our previous work verified higher levels of HIF-1α expression in stretched skin compared to non-stretched skin in a rat skin expansion model,[43] and the canonical HIF-1a pathway functioned as a predominant pathway activated by tissue expansion.[44] These data indicate the epidermal hypoxic microenvironment in skin expansion. Thus, we presumed that the atmospheric oxygen of relatively higher concentration sank down to the epidermis because the diffusion gradient is a key factor in oxygen delivery. Our previous work on a *Xenopus* tail model showed peak oxygen influx at 24 h post amputation, which also indicated the increased oxygen demand due to cell proliferation.[6]

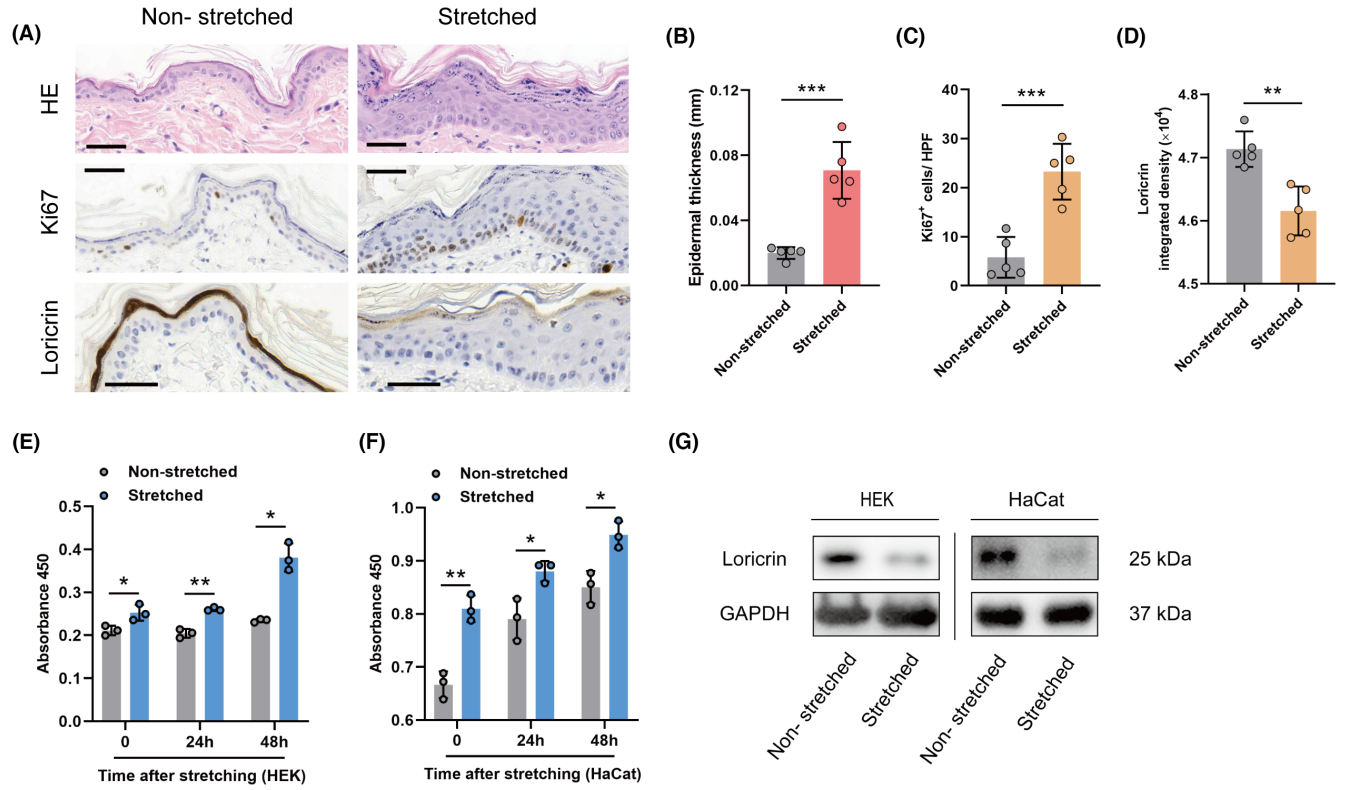

FIGURE 4 Assessment of epidermal barrier function and cell proliferation in vitro and in vivo. (A) H&E, Ki67 and Loricrin staining in non-stretched rat skin and 3-day-stretched rat skin. Scale bars: 50 μm. (B) Quantification of epidermal thickness. (C) Quantification of Ki67 positive cells per HPF. (D) Quantification of loricrin. (E and F) CCK-8 assay of human primary epidermal keratinocytes (HEK) and HaCat cells after stretching for 24 h. (G) Western blot of loricrin in HEK and HaCat cells after stretching for 24 h. Data are presented as means ± SD. Each dot represents one experiment. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

There are some limitations to our study. First, because of the fine tip of the micro-optrode, we choose interfollicular epidermis as the detecting points, while the follicular sites are not detected in our current experiment. Secondly, our data now enable us to know mechanical stretch increased cutaneous atmospheric oxygen uptake; we further hope to map the dynamic changes of strain distribution and specify the strain-oxygen influx relationship during the expanding process. Moreover, the specific mechanism of oxygen influx in the stretch-mediated skin expansion, especially the epidermis homeostasis maintenance, remains to be investigated. It is of great interest to further compare the oxygen uptake volume of epidermis alone and dermis alone with the advanced micro-optrode technique. Finally, translational studies on the oxygen uptake in pathological skin and the related effect of therapeutic agents in the view of oxygen influx will continue.

5 | CONCLUSION

In conclusion, this study used a new optical sensing technology to measure oxygen uptake at skin, and provides the first evidence (to our knowledge) that skin responds to mechanical stretch by increasing atmospheric oxygen uptake with a spatial-temporal pattern, which correlates with down-regulation of barrier (e.g. loricrin) and increased keratinocyte proliferation. Such homeostasis responses are important for the physiology of highly stretchability that is characteristic of the skin more than other tissues. The self-referencing optical fiber microsensor provides a practical tool for monitoring dynamics of cutaneous oxygen uptake with superior spatial-temporal resolution and may be used in cutaneous disorder assessment and therapy.

AUTHOR CONTRIBUTIONS

Conceptualization, M.Z. and Q.L.; methodology, validation and formal analysis, S.S., J.H. and Q.S.; resources, B.R.; data curation, K.Z and Y.L.; writing—original draft preparation, S.S.; writing—review and editing, B.R., M.Z. and K.Z.; supervision, project administration and funding acquisition, M.Z and Q.L. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1 Oxygen flux on footpad pre- and post-hair remover treatment. (A) Schematic of measurements pre- and post- hair remover treatment at footpad skin. (B) Results of the oxygen flux at footpad skin. Data are presented as means ± SD ($n = 3$). Each dot represents one rat. n.s., no significant differences.

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