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"Oxyregeneration: Tissue oxygenation regenerates aged skin"

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1. Introduction

Starting from the innermost layer to the outermost layer, the epidermis is composed of the basal layer, spinous layer, granular layer, and stratum corneum. Undifferentiated keratinocyte stem cells (KSCs) localized in the basal layer divide and migrate outward while differentiating into basal, spinous, granular, and stratum corneum cells, in a process referred to as the epidermal turnover. Normal epidermal turnover helps to maintain skin health by enabling the epidermis to perform its barrier function efficiently, retain moisture, and prevent foreign substances from entering the skin. However, with aging, a delay in the epidermal turnover, which originates from KSCs, results in thinning of the epidermis and loss of function [1,2]. Currently, the incidence of aging population is increasing in developed countries. Therefore, the deterioration of skin function resulting from the age-related delay in epidermal turnover is a significant issue that hinders the maintenance of a good quality of life.

Age-related reduction in the number of KSCs is one of the known causes of delayed epidermal turnover [3,4]. However, the changes in the functions as well as the number of KSCs might be related to the age-related delay in epidermal turnover. In general, the functions of the cells that build up the various tissues of the human body are regulated by a 24-h cycle known as the circadian rhythm. The circadian rhythm efficiently regulates cell proliferation, differentiation, and energy metabolism [5,6]. The circadian rhythm is also known to regulate the division and differentiation of KSCs and basal cells in the epidermis. In particular, the division of the KSC, which is the starting point of the epidermal turnover, has been reported to take place during the night [7,8]. Therefore, for proper epidermal turnover, KSCs are expected to respond to circadian rhythms and perform their function by altering energy metabolism, i.e., ATP (adenosine triphosphate) production. However, the relationship between the delay in epidermal turnover with aging and the energy metabolism and circadian rhythms involved in KSC function remains unclear since the changes in energy metabolism between the day and the night in KSCs have not yet been clarified.

This study aimed to elucidate the mechanism of age-related delay in epidermal turnover from the aspect of energy metabolism of KSCs and to establish a new solution to ameliorate the decline in epidermal function to improve the quality of life of the elderly population. Specifically,

the focus of the study was on the changes in the energy metabolism of KSCs regulated by circadian rhythms. We also analyzed the importance of oxygen-dependent ATP production by KSCs in epidermal turnover. Next, the relationship between hypoxia in the skin due to aging and epidermal turnover and the ways to improve the oxygen environment in the skin were examined.

2. Materials and Methods

Culture of the KSCs

Human epidermal keratinocytes (HEKs) (KURABO, Japan) were grown in KG2 medium (KURABO). The cells were trypsinized and then were first incubated with anti-CD271 antibody (Acris Antibodies, MD, USA) for 30 min at 4°C, which was followed by incubation with AlexaFluor594-conjugated anti-mouse IgG antibody for 30 min at 4°C. After subsequent washing with phosphate-buffered saline (PBS), a cell sorter (FACS Melody, BD bioscience, CA, USA), was used to isolate CD271-positive KSCs. For stimulating circadian rhythms, the KSCs were cultured for 12 h under nutrient starvation conditions to synchronize the cell cycle and the medium was later replaced with complete medium. The BIONIX-1 hypoxia cell culture kit (Sugiyamagen, Tokyo, Japan) was used to generate culture conditions that reflect oxygen levels in the skin [9,10]. The oxygen concentrations were set to 3% for the physiological condition (physioxia) and 0.5% for the hypoxic condition (hypoxia) in accordance with the manufacturer's instructions.

qRT-PCR

A StepOnePlus Real-time RT-PCR system (Applied Biosystems) was used to perform qRT-PCR (quantitative reverse transcriptase polymerase chain reaction) with SYBR Select Master Mix (Applied Biosystems, Tokyo, Japan) in accordance with the manufacturer's protocol. The sequences of primers used were as follows: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-TCTTCTGGGTGGCAGTGATG-3'; BMAL1 forward, 5'-ACTGTGCTAAGGATGGCTGTTC-3' and reverse, 5'- GTTGGTTTGTAGTTTGCTTCTGTGT-3'; PER2 forward, 5'-GCAGCTACAGCAGCACCATC-3' and reverse, 5'-GCATCTTCCACCATCTCTAACTCC-3'; HK2 forward, 5'-AGATTGAGAGTGACTGCCTG-3' and reverse, 5'-ACACCTCCTTAACAATGATGCT-3'; CS forward, 5'-GAATGCCAGAAACTGCTACCC-3' and reverse, 5'-GAGAGCCAAGATACCTGTTCCT-3'; BMP4 forward, 5'-GAGCCATTCCGTAGTGCCAT-3' and reverse, 5'-TCTTCCCCGTCTCAGGTATCAA-3'; VEGFA forward, 5'-CTCTCTCCCTGATCGGTGACA-3' and reverse, 5'-GGAGGGCAGAGCTGAGTGTT-3'; VEGFB forward, 5'-CCCTGTCTCCCAGCCTGAT-3' and reverse, 5'-GCGCGAGTATACACATCTATCCAT-3'; FGF2 forward, 5'-TGGTATGTGGCACTGAAACGAA-3' and reverse, 5'-TTCTGCCCAGGTCCTGTTTT-3'.; Amplification was normalized to the housekeeping gene GAPDH, and differences between samples were quantified based on the $\Delta\Delta$Ct method. Melting curve analysis was performed to check all PCR products to exclude the possibility of multiple products or an incorrect product size.

Immunostaining

A solution of 4 % paraformaldehyde was used for fixing the KSCs. The cells were washed with PBS, blocked with 1% bovine serum albumin for 1 h, and then incubated with primary antibodies against Geminin and CDT1 overnight at 4°C. This was followed by incubating the cells with Alexa Fluor 488 - or Alexa Fluor 594-conjugated secondary antibodies. Nuclear staining was performed with 4',6-Diamidino-2-phenylindole (DAPI).

Mitochondrial membrane potential (MMP)
The MT-1 MitoMP Detection Kit (DOJINDO, Japan) was used for monitoring MMP. After incubation with the MT-1 fluorescent dye, the KSCs were observed under a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan).

Measurement of oxygen consumption rate (OCR) and intracellular ATP level
The extracellular OCR plate assay kit (DOJINDO) and ATP assay kit-luminescence (DOJINDO) were respectively used to measure the OCR and intracellular ATP levels of KSCs in accordance with the manufacturer's instructions. The cells were then seeded onto a 96-well plate at a density of 3.0×10^4 cells/well. Oligomycin was added to the culture three hours before measurement.

Cell proliferation assay
KSCs were seeded onto a 96-well plate at a density of 2.5×10^3 cells/well and cultured under physconic or hypoxic conditions. After 48-h, a cell count normalization kit (DOJINDO, Kumamoto, Japan) was added to the cultured cells along with a fresh medium, and the absorbance was measured at 450 nm. The relative cell proliferation rate of the KSCs was then calculated.

LC-OCT-based epidermal SC analysis
LC-OCT: Line-field Confocal Optical Coherence Tomography (DAMAE Medical, Paris, France) was used to acquire confocal continuous images of the cheek, and three-dimensional processing was performed for analyzing the internal structure of the living skin [11-14]. Thirty-five volunteers (mean age 62.7 ± 8.6 years) were enrolled for the purpose after they provided written informed consent. The local Ethics Committee approved the clinical trials.

Preparation of a three-dimensional reconstructed skin model (3D skin model) equipped with a capillary
For creating the 3D dermis model, type I collagen solution (Nitta Gelatin, Osaka, Japan) containing 1.0×10^5 cells/mL of human dermal fibroblasts was poured into the stretch chamber. A gelatinized 3D dermis model was obtained after incubation at 37°C. CellTracker Red fluorescent probe-labeled human umbilical vein endothelial cells (HUVECs) were seeded onto the model, and after confirming adhesion, another 3D dermis model was created on top of it. Final seeding was performed with KSCs using expansion medium CNT-07 (CELLnTEC, Berne, Switzerland) at 5×10^5 cells per chamber. The medium was subsequently changed to a three-dimensional medium (CNT-02-3DP5, CELLnTEC) to commence induction of epidermal differentiation. The 3D skin model was cultured for five days to obtain a 3D skin model with capillaries. The cyclic mechanical stretching experiments were performed using an ST-1400 cell stretcher system (Strex Inc., Osaka, Japan).

Statistical analysis
Pearson's product moment correlation was used for testing the correlation. Student's t-test or Tukey's multiple comparison test was used for testing the statistical differences. p values < 0.05 were considered statistically significant.

3. Results

Increased energy metabolism of KSCs and cell division at night is important for the progression of epidermal turnover

BMAL1 regulates circadian rhythms and is also known to positively regulate the expression of PER2 and CRY, which indicates that BMAL1 expression is negatively regulated by PER and CRY (Fig. 1A). Such a rhythm is also observed in human epidermal tissues, where BMAL1 expression is high at night (Fig. 1B) [15]. We attempted to synchronize the cell cycle of the KSCs to reproduce the circadian rhythm. The results demonstrated elevated BMAL1 expression and lower PER2 expression from 12 to 20 hours following synchronization. Conversely, BMAL1 expression was low and PER2 expression was high 4 and 28 hours after the synchronization (Fig. 1C). Based on these results, it was determined that the timing of high BMAL1 expression at 12 to 20 hours after synchronization corresponded to the night. Therefore, in subsequent experiments, 4 hours after synchronization was considered as “Daytime” and 12 hours after synchronization was considered as "Night”.CDT1-positive cells specific to the G1 phase were observed during the daytime, while several Geminin-positive cells specific to the S/G2/M phase were observed at night (Fig. 1 D). These results indicate that KSCs undergo cell division at night and that proper proliferation of KSCs at night is required for epidermal turnover to proceed without delay.

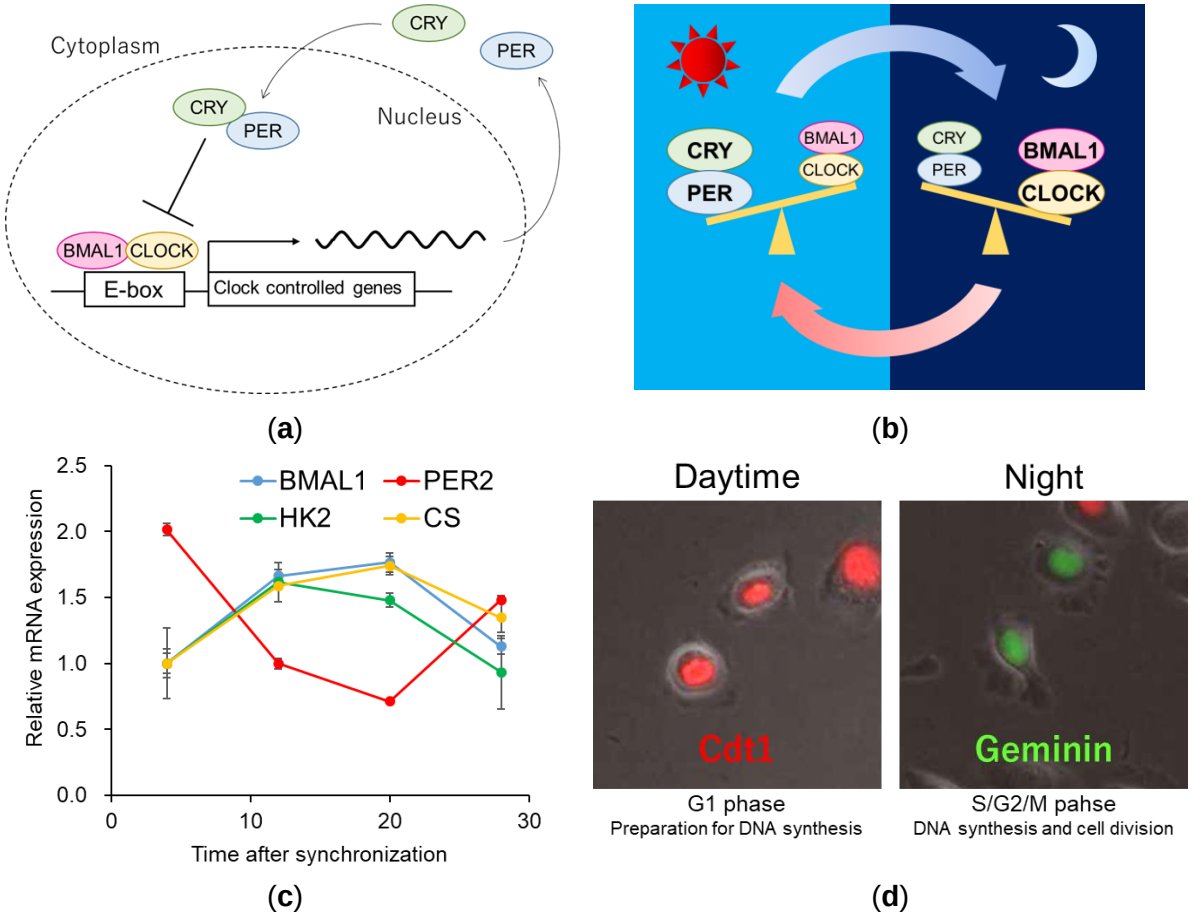


Figure 1. Reproduction of circadian rhythms in KSC cultures: (a) Schematic diagram of the regulatory mechanisms of circadian rhythms. (b) The BMAL1/CLOCK complex is predominant in KSCs at nighttime. (c) Analysis of the gene expression of KSCs by qRT-PCR following synchronization of the cell cycle. (d) Analysis of the expression of cell cycle-related proteins during the day (4 hours after synchronization) and at night (12 hours after synchronization) by immunostaining. Data shown are the mean ± SD (n = 3). (qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; SD: standard deviation)

Glycolysis, tricarboxylic acid (TCA) cycle, and oxidative phosphorylation coupled to the electron transport chain in the mitochondria are the most well-known and important processes in cellular energy metabolism (Fig. 2a). It is important to have a high level of oxidative phosphorylation, an oxygen-dependent energy metabolism, to efficiently produce a large amount of ATP from a single molecule of glucose. Analysis of the energy metabolism of the KSCs during the daytime and at night demonstrated that the expressions of both Hekisokinase (HK2) and Citric acid synthase (CS), the rate-limiting enzymes of the glycolysis and TCA cycle, were higher at night (Fig. 1c). The OCR and MMP, indicators of mitochondrial function, were higher at night compared with that observed in the daytime (Fig. 2b and c). Furthermore, the intracellular ATP levels were higher at night compared to the daytime, and were markedly suppressed by the inhibitor of oxidative phosphorylation, oligomycin (Fig. 2d). These results indicate that KSCs are more active in oxygen-dependent energy metabolism at night, which further indicate that an appropriate oxygen environment surrounding KSCs is vital for the proliferation of KSCs at night to proceed with epidermal turnover.

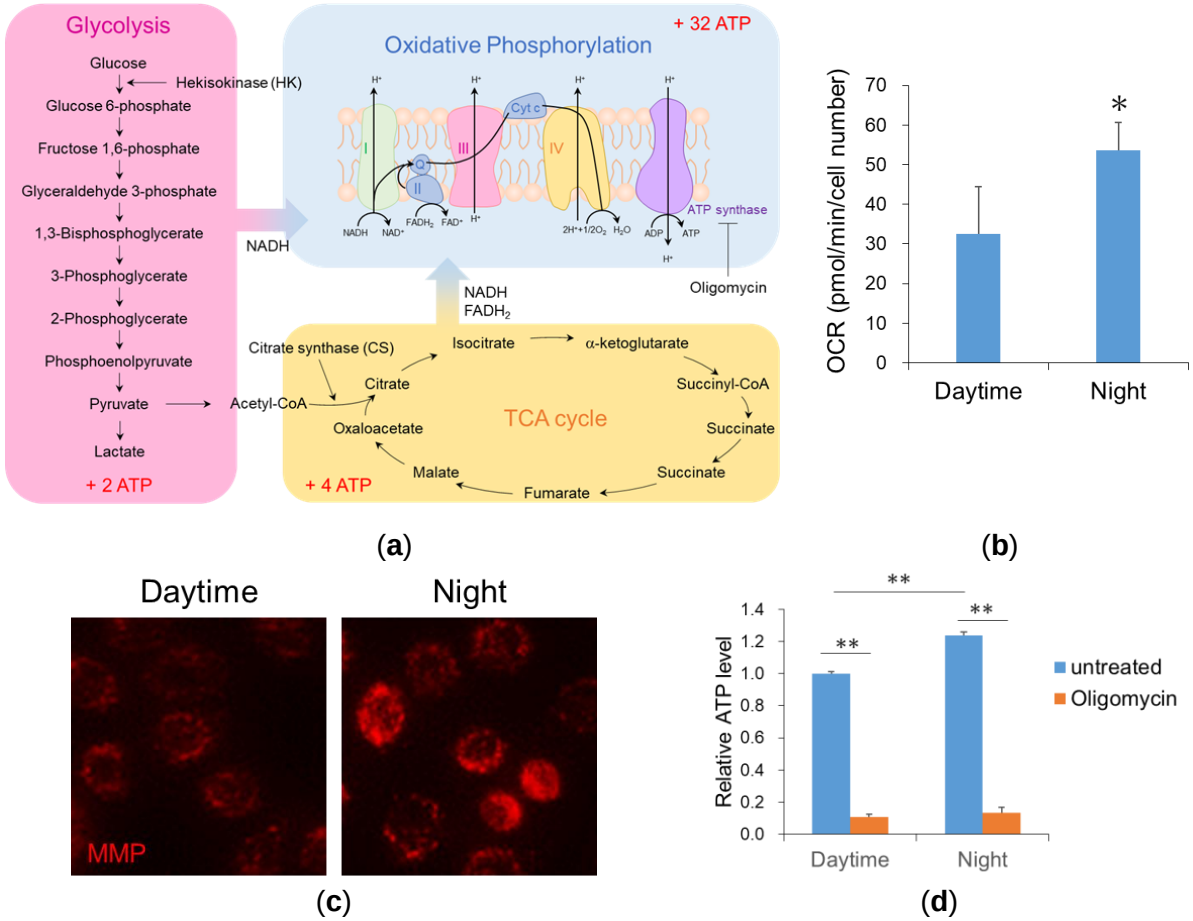


Figure 2. Changes in the energy metabolism by circadian rhythms in KSC: (a) Glycolysis, Tricarboxylic Acid Cycle, and Oxidative Phosphorylation; (b-c) Changes in OCR (b), mitochondrial membrane potential (c), and intracellular ATP levels (d) in KSC due to circadian rhythms. Data shown are the mean ± SD (n = 3). *P < 0.05; **P < 0.01. (OCR: oxygen consumption rate; MMP: mitochondrial membrane potential; ATP: adenosine triphosphate)

Hypoxic environments reduce the growth of KSCs

The oxygen concentration of 21% (Normoxia) commonly used for cell culture, reflects the oxygen concentration in the atmosphere, and does not require specialized cell culture

equipment. It does not reflect the physiological oxygen environment in the tissue. The oxygen concentration in the epidermis to the papillary dermis has been reported to be approximately 1%–3% [16] and 0.5% in partially more poorly oxygenated areas [17]. KSCs were cultured under physiological oxygen concentration conditions of 3% (physioxia) and hypoxic conditions of 0.5% (hypoxia). As a result, a reduction was observed in the cell proliferation of KSCs in hypoxic conditions compared to the physioxic conditions (Fig. 3a). Compared to the physioxic conditions, MMP and ATP levels were lower under the hypoxia at night (Fig. 3b and c). Furthermore, 3D epidermis models cultured under different oxygen concentration conditions demonstrated that the epidermal thickness became thinner with decreasing oxygen concentration (Fig. 3d). In particular, the layer structure of the epidermis was markedly dysplastic in hypoxic conditions.

These results indicate that epidermal turnover is delayed under hypoxia because of decreased cell proliferation associated with decreased energy metabolism in KSCs.

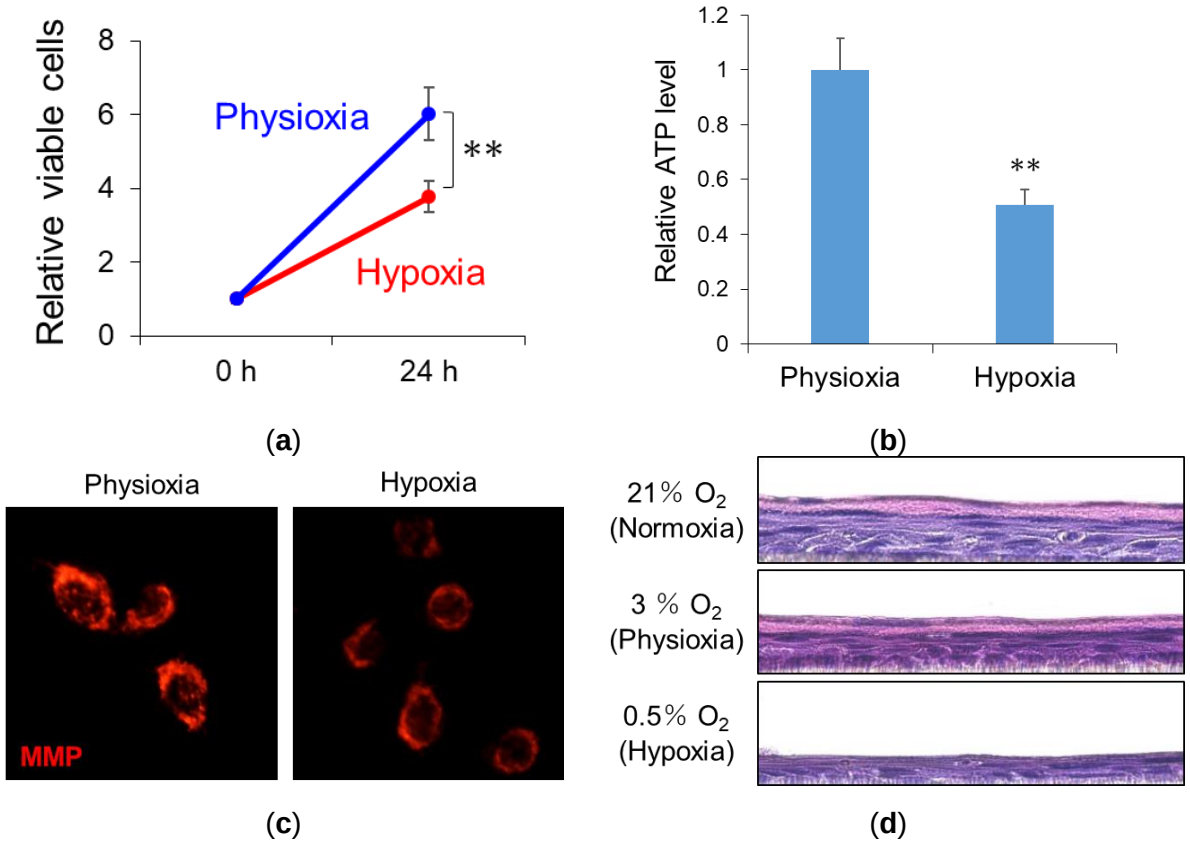
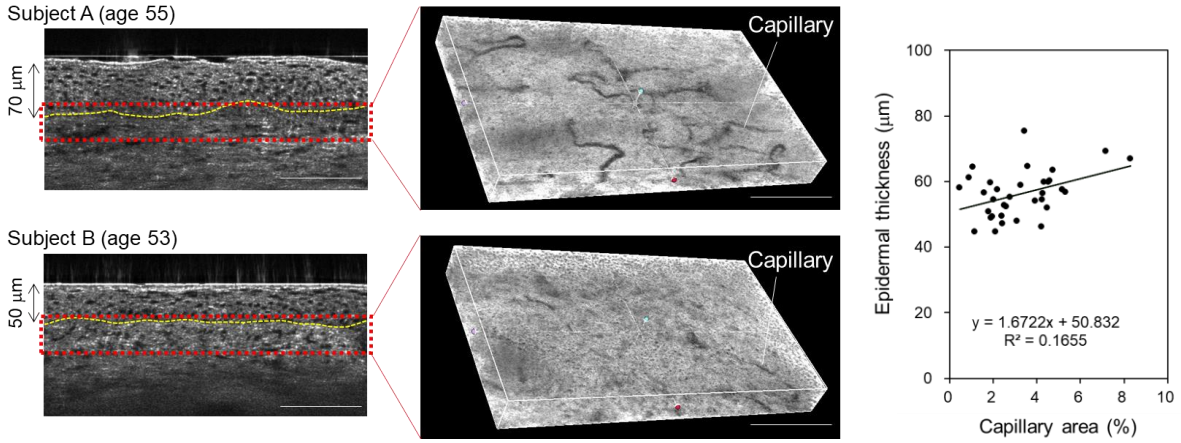


Figure 3. Effects of hypoxia on the proliferation and energy metabolism of KSCs: (a) KSCs were cultured under 3% oxygen (physoxia) and 0.5% oxygen (hypoxia), and relative cell numbers were analyzed; (b and c) Changes in the intracellular ATP (b) content and MMP (c) in KSCs induced by hypoxia. (d) Hematoxylin-eosin-stained images of the three-dimensional epidermis models cultured under different oxygen concentration conditions. Data shown are the mean ± SD (n = 3). **P < 0.01.

The amount of capillaries in the skin is related to the epidermal function.

Delayed epidermal turnover due to aging is known to cause epidermal thinning [2]. However, the relationship between the oxygen environment within the human skin and epidermal turnover remains unknown. It is therefore necessary to analyze the relationship between the

oxygen concentration in the skin and the epidermal thickness. However, currently there is no standard technique for the noninvasive measurement of the oxygen concentration in the skin. Therefore, the capillaries supplying oxygen to the skin and the thickness of the epidermis were analyzed using LC-OCT (line-field confocal optical coherence tomography) and it was found that in female subjects in their 50s to 70s, the epidermal thickness correlated with the number of capillaries in the papillary dermis layer (Fig. 4a and b). This suggests that a deteriorating oxygen environment in the skin leads to epidermal dysfunction due to delayed epidermal turnover. In other words, stimulation of angiogenesis could enhance the oxygenation within the skin, thereby improving epidermal turnover.



(**a**) (**b**)

Figure 4. Epidermal thickness and capillary correlation: (a) Representative images of internal skin structures analyzed by LC-OCT. Capillaries found within the area surrounded by the red dotted line were observed. The yellow broken line indicates the basement membrane. Scale bar = 100 µm. (B) Analysis of the correlation between epidermal thickness and capillary area (LC-OCT: Line-field Confocal Optical Coherence Tomography)

Angiogenesis by Cyclic Mechanical Stimulation (CMS)

Mechanical stretching promotes tissue regeneration [18]. This study investigated whether CMS can stimulate angiogenesis to ameliorate delayed epidermal turnover caused by hypoxia. When a 3D skin model equipped with capillaries was subjected to CMS for 1 hour, the development of the capillary network formed by fluorescent probe-labeled HUVECs was found to be enhanced 48 hours after CMS stimulation (Fig. 5a and b). Besides, an upregulation of the gene expression of the angiogenesis-promoting factors BMP4, VEGFA, VEGFB and FGF2 at peak levels was also observed 2 hours after CMS (Fig. 5c). To clarify the source of these factors, CMS was applied to the 3D epidermis model as well as the 3D dermis model. An enhanced expression of the angiogenesis-promoting factors was observed only in the 3D epidermis model (Fig. 5d). These studies were conducted under normoxic conditions; however, CMS was confirmed to promote the formation of capillary networks under physioxic and hypoxic conditions as well (Fig. 5e and f).

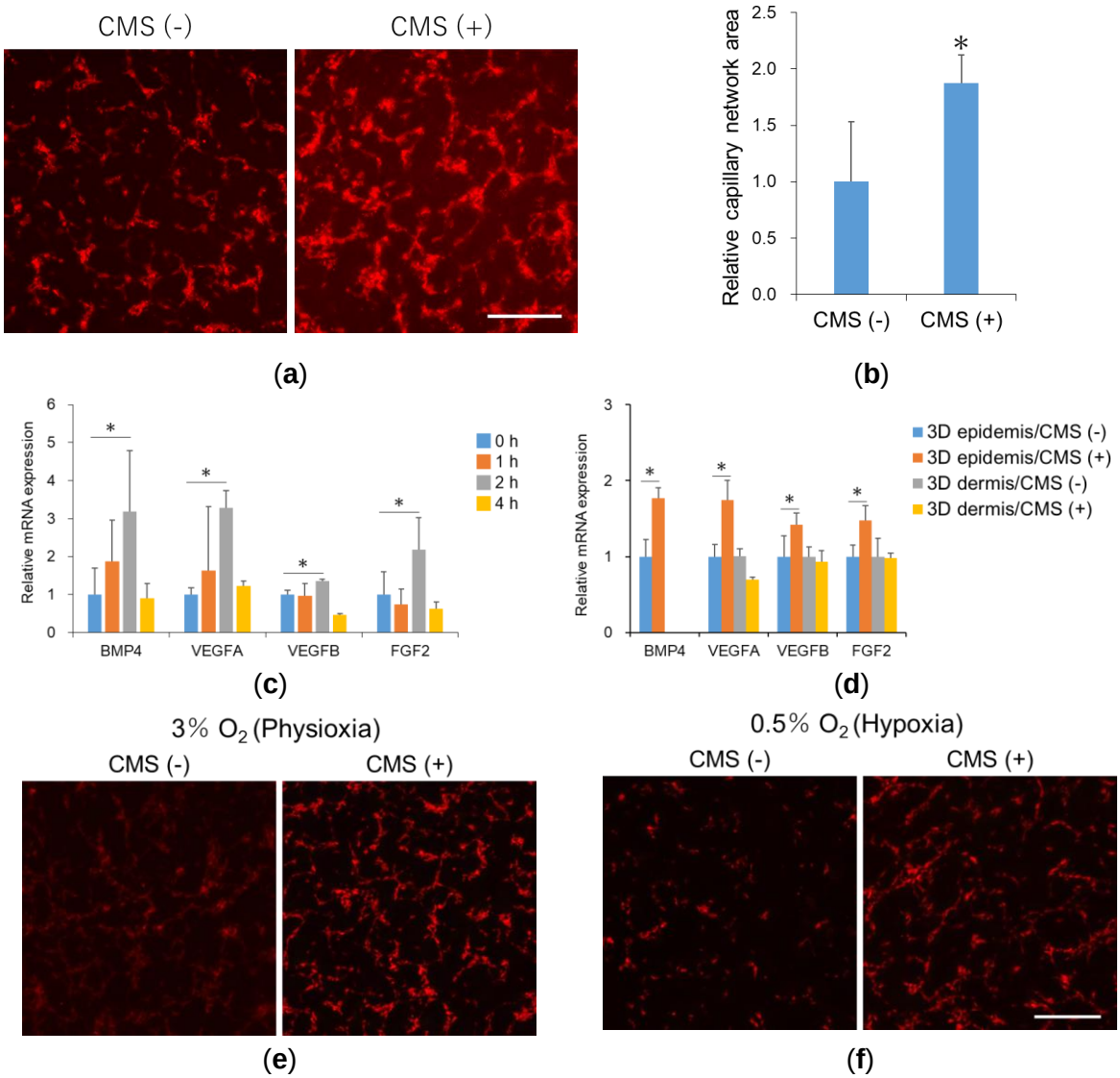


Figure 5. CMS-induced angiogenesis in the 3D skin model: (a and b) CMS was applied to the 3D skin model and the formation of the capillary network was observed after 48 hours (a), The area of the network was analyzed using image analysis software (b). (c) Changes in the gene expression of angiogenesis-promoting factors in the 3D skin model by CMS were analyzed by qRT-PCR. (d) Changes in the gene expression of angiogenesis-promoting factors in the 3D epidermis model and 3D dermis model after 2 h of CMS were analyzed by qRT-PCR. (e and f) 3D skin models were incubated in physconia and hypoxia conditions for 24 hours after which, CMS was applied. The skin models were incubated in their respective oxygen environments for additional 48 h. The formation of capillary networks was observed. Scale bar = 300 μm. Data shown are the mean ± SD (n = 3). *P < 0.05. (CMS: cyclic mechanical stimulation)

4. Discussion

Previously conducted studies have demonstrated that KSC function is regulated by circadian rhythms [7,8]. This study demonstrated the enhancement of energy metabolism during the night when KSC division initiates epidermal turnover, i.e., oxygen-dependent ATP production is enhanced (Figs. 1 and 2). However, the impaired KSC function was observed in experiments

in which KSCs were cultured under hypoxic conditions, which is a characteristic of aging skin (Fig. 3). This indicates that reduced oxygen supply causes hypoxia in the skin, which in turn impairs KSC function and delays epidermal turnover. Capillaries play an important role in supplying oxygen to the skin; however, the number of capillaries in the skin is known to decrease with age [19]. Therefore, the relationship between the thickness of the epidermis and the capillaries in the papillary dermis layer, that are involved in supplying oxygen to the epidermis was analyzed and a positive correlation was observed (Fig. 4). This indicates that a reduction in the number of capillaries causes hypoxia in the skin, which in turn delays epidermal turnover due to insufficient oxygen supply to the KSCs. Therefore, increasing the number of capillaries in the skin is considered essential to maintain sufficient oxygen supply to the KSCs to improve the delayed epidermal turnover associated with aging. Since mechanical stimulation is known to promote tissue regeneration, we focused our attention on CMS, since facial massage with cosmetics can be considered as a means of increasing capillaries. In this study, CMS was observed to stimulate angiogenesis in a 3D skin model equipped with capillaries (Fig. 5). The reason for this was attributed to angiogenesis-promoting factors such as BMP4 and VEGF, the expression of which was increased in the epidermis following CMS. Facial massage can easily be performed even at home by oneself in addition to places such as aesthetic salons, and does not require special equipment. Thus, facial massage is a skin care method that can be easily adopted by everyone on a daily basis. It might be beneficial for improving the quality of life of the elderly by improving the epidermal turnover. However, the amount of mechanical stimulation that can be applied to the human face to stimulate angiogenesis without damaging the skin is not clear.

Thus, a combination of previous reports and the findings of this study indicate that the delay in epidermal turnover due to aging is associated with not only a decrease in the number of KSCs but also a decline in their function, which is caused by tissue hypoxia resulting from a decrease in capillary vessels (Fig. 6). In contrast, if oxygenation of the skin tissue can be achieved by the promotion of angiogenesis through methods such as CMS proposed in this study, KSCs can be expected to function properly resulting in the regeneration of the epidermal tissue. In the future, we would like to verify the usefulness of this approach, i.e., oxygen regeneration, in human skin tissue.

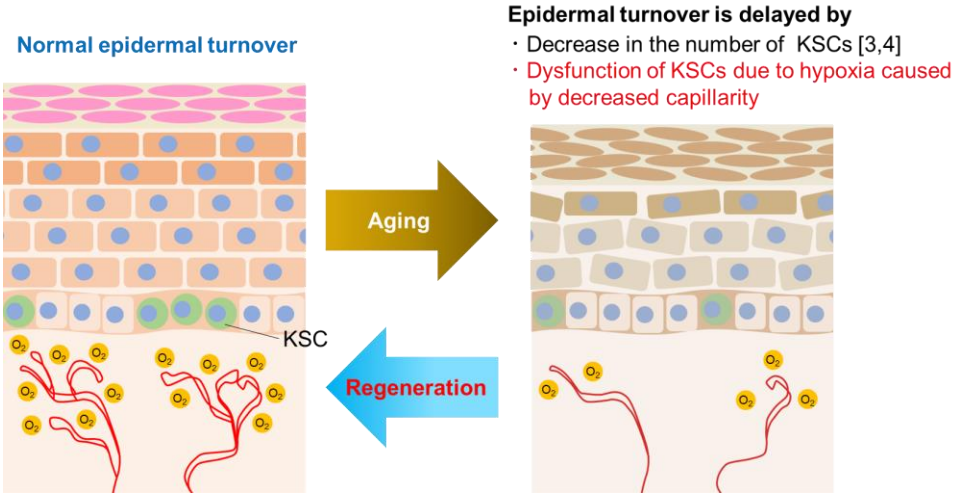


Figure 6. Schematic diagram of the age-related delayed epidermal turnover and a proposal of “oxyregeneration” as a its solution.

5. Conclusion

This study has demonstrated the need for a high energy metabolism of KSCs at night for normal epidermal turnover. However, KSCs in the aging epidermis do not receive sufficient oxygen due to decreased capillary vascularity. In contrast, by stimulating angiogenesis through moderate physical stimulation such as massage, the epidermis can be “oxygenated” and “regenerated,” which is expected to improve the delay in epidermal turnover due to aging.

6. References

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