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Original Article

Y-27632 preserves epidermal integrity in a human skin organ-culture (hSOC) system by regulating AKT and ERK signaling pathways

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ABSTRACT

Background: The human skin organ culture (hSOC) developed a century ago has been widely used to study various aspects of human skin development, differentiation, function, disease as well as skin appendages biology, however, maintaining the integrity of epidermal structure in long-term culture, has remained a challenge.
Objectives: Here we tried to establish a culture system using supplemented William's E medium in the presence of a ROCK inhibitor Y-27632 to maintain epidermal architecture in the long-term hSOC and to investigate the underlying mechanisms.
Methods: Human breast skins, cut into 5 mm × 5 mm pieces, were cultured in supplemented William's E medium in the presence of 30μM Y-27632. The cultured skin tissues were collected at different time points for analysis of epidermal cell proliferation and differentiation by real time qRT-PCR and immunofluorescence (IF) staining. The keratinocyte suspension assay and *in vivo* treatment of Y-27632 on mouse were also carried out to study that the regulation of Y-27632 on keratinocyte proliferation and differentiation.
Results: We found Y-27632 not only enhanced both basal cell proliferation and expression of suprabasal cell differentiation markers, but also maintained the balance of keratinocyte proliferation and differentiation through activation of AKT pathways on one hand and inhibition of ERK pathways on the other hand. The AKT inhibitor MK-2206 blocked the epidermal preservation effect of Y-27632, while the MEK/ERK inhibitor U0126 enhanced the preservation of epidermal structure in the hSOC.
Conclusions: Y-227632 can maintain skin epidermal integrity through regulation of AKT and ERK activity in the hSOC.

1. Introduction

Human skin organ culture (hSOC), known as an ex vivo model, has been considered to be closer native skin than that produced by *in vitro* culture. For this reason, hSOC has been widely used for in model studies relating to human skin diseases, pharmacology, physiology and toxicology [1,2]. However, hSOC as a laboratory model is rather infrequently used because it is a big challenge for researchers to preserve the skin intact structure for a sufficient time to perform relevant experiments. The epidermis, which is the outermost layer of skin and acts as a barrier, play a crucial role in maintaining skin homeostasis [3–5]. So, preservation of epidermal structure is crucial for effective skin-environmental interactions. Although long-term viability and histological preservation of the hSOC model has been studied over many decades using various culture media and techniques, little attention has been given to retention of epidermal structure and differentiation [2,6].

Rho-associated protein kinase (ROCK), consisting of two isoforms ROCK1 and ROCK2, originally was discovered to regulate cell shape and migration by acting on the cytoskeleton; however, it actually plays a wide role in modulating many biological processes

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such as cell survival, proliferation and apoptosis through multiple downstream targets [7,8]. Y-27632, a well-established ROCK inhibitor which blocks, both ROCK1 and ROCK2 activities, has been shown to significantly benefit the culture of a variety of pluripotent stem cell types including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) [9–13]. In the skin, Y-27632, has been shown to significantly enhance epidermal stem cell proliferation, and inhibit keratinocyte differentiation, processes which play a significant role in maintaining epidermal architecture [14–17]. Recently, we reported, in BRAF mutant melanoma cells, that Y-27632 could enhance both activation of AKT and ERK signaling pathway [18], which has been shown to play an important role in keratinocyte proliferation and differentiation [19–22]. The present study tests whether Y-27632 in the culture medium could preserve epidermal structure in the hSOC and whether Y-27632 effect on hSOC is through regulation of AKT or ERK signaling pathway. Indeed, we found that the addition of Y-27632 to William's E medium could significantly prolong the epidermal architecture of hSOC through regulation of the AKT and ERK pathways.

2. Materials and methods

The preparation and culture of hSOC followed the protocol described by Lu et al. [23]. Briefly, human female breast skins (range from 25 to 65 years old) were cut into 5 mm × 5 mm pieces, and were floated onto William's E medium with the epidermis up at air/liquid interface and the dermis down. The skin pieces of the experiment group were cultured with supplemented William's E medium in the presence of 30μM Y-27632 (Sigma Chem Co., St. Louis, MO, USA). The cultured skin tissues were collected at different time points as indicated in the figures for the following experiments.

The suspension assay followed previously described [24,25]; hSOC permeability barrier analysis were performed by the Lucifer Yellow dye (L453, Invitrogen) penetration assay followed previously described [26]; Procedures for histological analysis immunoblotting, immunofluorescence analysis were carried out as previously described [18,27,28]; And all detailed experimental procedures were described in Supplemental Materials and methods.

3. Results

3.1. Y-27632 stabilizes skin epidermal structure of hSOC

Williams E medium was shown to maintain intact skin structure for longer than DMEM medium with 10% FBS [23], therefore Williams E medium was used for all further studies. The skin tissues were cultured in William E medium plus/minus 30μM Y-27632, and harvested at 1 wk, 2, 3 and 4 wks for histological analysis. By light microscopy epidermis of the control skins became much thinner at 2 wks, with nearly one cell layer remaining at 3 wks (blue arrows); and complete loss of epidermis at day 28 (Fig. 1A, red arrows). In contrast, skins maintained in the medium with Y-27632, epidermal structure could be maintained for more than 2 wks, moreover several layers of epidermis remained at 4 wks (Fig. 1A). Interestingly, we didn't observed a significant difference of dermal structure between control and Y-27632 treated group even at 3 wks (Suppl. Fig. 1), suggesting that it is a main challenge to maintain the epidermal structure during hSOC, therefore the present study focused on the effect of Y-27632 on preservation of the structure of the epidermis. To further characterize the effect of Y-27632 on the epidermis, the allover thicknesses of the epidermis, which is from cornified layer to the basal layer indicated with double red arrows (Fig. 1B) grown under these two conditions were evaluated. As shown Fig. 1B, the epidermis treated with Y-27632 was significantly thicker than that of the control group. This result was corroborated by quantifying the number of epidermal cells (Fig. 1C), counted according to nuclei staining (DAPI, Fig. 1C), showing much more epidermal cells in Y-27632 treated epidermis at 3 wks. Together, it suggested that Y-27632 could stabilize the epidermis structure in the hSOC.

3.2. Y-27632 maintains the expression of suprabasal cell marker of the epidermis

A normal highly regulated keratinocyte differentiation program plays a crucial role in maintaining the epidermal integrity, which consists of a undiffereniatied basal cell layer, differentiated suprabasal cell layers and terminal differentiated stratum corneum layer [29]. To analyze the differentiation status of the epidermis, we first studied the expression of keratin 5 (K5), a basal cell marker [30], with immunofluorescence (IF) staining. IF staining of K5 and its quantification data were shown in Fig. 2A and B, we could see that K5 positive cells locate in the lower part of the epidermis in both control and Y-27632 treated groups (red, Fig. 2A). The majority of K5 positive cells were confined to the basal layer of the epidermis, however multiple layers of K5 positive cells were observed at 2 wks in both control and Y-27632 treated skin, but slightly, more K5 positive cells in Y-27632 treated group (Fig. 2B), which p value was 0.047 for the statistical analysis. Next, we analyzed the expression of the suprabasal cell marker keratin 10 (K10) [30], as shown in Fig. 2C-D the basal cells clearly didn't express K10 (Fig. 2C, red) in both groups. In the control group, K10 positive cells were gradually lost with culture time. At 2 wks, some suprabasal cells showed either lower expression or loss expression of K10 (white arrows, Fig. 2C). At 3 wks, only one single layer of K10 positive cells were observed (white arrowheads, Fig. 2C). In contrast, those skins cultured in Y27632 showed a strong and stable expression of K10 in suprabasal epidermal layers at 2 wks, in addition, several layers of K10 positive cells were still present at 3 wks (Fig. 2C and D), indicating Y-27632 maintaining the expression of suprabasal cell marker K10. Then, we checked expression of the terminal differentiation marker loricrin, a major component of the epidermal cornified envelope(CE) [31]. CE is crucial for skin barrier function, although the loricrin knockout mice presented a mild defect of barrier function mainly due to a compensatory mechanism [32,33]. We could see loricrin expression (green) in the most outer layer of epidermis in both groups (Fig. 2E). In the control group, the integrity of the loricrin-positive layer was disrupted even at 1 week, and some of loricrin positive cells contain nuclei, however the expression level of loricrin was not significantly different between two groups (Fig. 2F). The integrity of loricrin-positive layer was maintained in the Y-27632 treated skin even at 3 wks (Fig. 2F), which was supported by the Lucifer yellow dye penetration assay (Suppl. Fig. 2). It suggested the epidermal barrier function was maintained in hSOC in the presence of Y-27632 until 3 wks. Finally, we detected the expression keratin 6, a hyperproliferating cell marker, previously reported to be suprabasally expressed in the organ cultured skin [34]. Agreed to the previous report, K6 expression was detected in suprabasal layers of both control and Y-27632 treated epidermis (Suppl. Fig. 3) at 1 wk. And at 3 wks, K6 expression was nearly lost in the control epidermis, but maintained in the Y-27632 treated group. This data further supported Y-27632 can maintain the suprabasal layer in the hSOC. Taken together, we could conclude that Y-27632 preserves expression of keratinocyte differentiation markers, such as K5, K10 and loricrin, presented in the right place of epidermis during the organ-culture, especially, it enhances maintaining suprabasal layer, resulting in maintenance of the epidermal integrity in the hSOC.

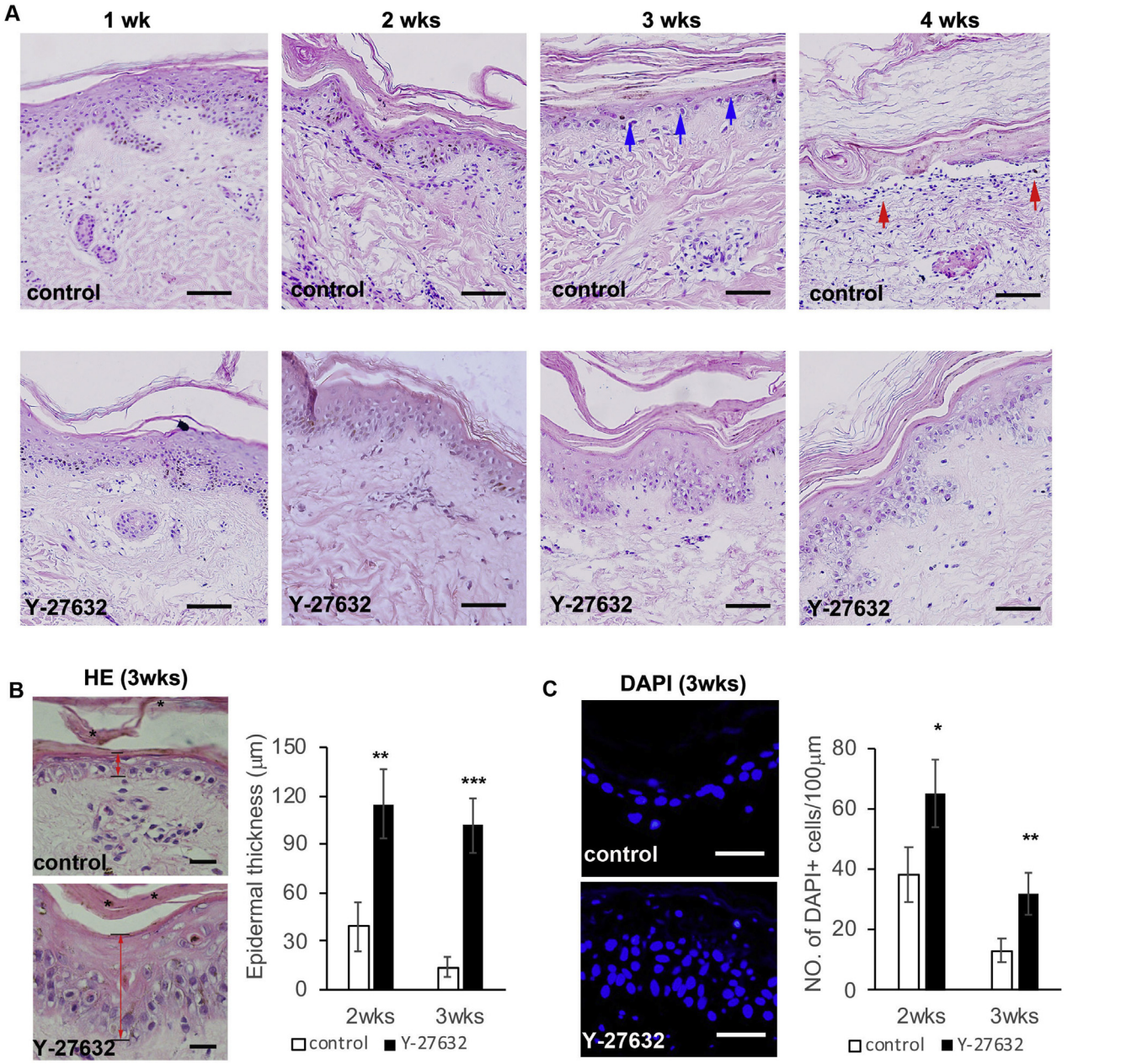

Fig. 1. Y-27632 enhances the preservation of hSOC epidermis.
A. The skin tissues were cultured with supplemented William's E medium with or without Y-27632 (30μM). The tissues were collected at different time points for histological analysis (H & E staining). Red arrows indicate loss of epidermal structure. Bars = 100μm. **B.** Left panel shows representative images of H & E staining of hSOC epidermis grown for 3wks with or without Y-27632. The measured epidermal thickness is indicated by red lines with double arrowheads from the cornified layer to the basal layer, which didn't include the detached layers labeled with * ; the average thickness of epidermis at 2wks and 3wks was shown in the right panel, Bars = 20μm. **C.** Left panel shows representative images of DPAI (nuclei) stained epidermis at 3wks with or without Y-27632; the number of DAPI positive epidermal cells was counted at 2wks and 3wks of culture; the total DAPI positive cells for 1 mm length of epidermis, and the average number per 100μm epidermis is shown in the right panel. Bars = 50μm. **B** and **C**: The experiment was repeated from three different donors, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ when compared with the control group.

3.3. Y-27632 enhances keratinocyte proliferation and expression of suprabasal differentiation markers in the skin organ culture

In order to understand how Y-27632 treatment maintain the integrity of epidermis during hSOC, we analyzed epidermal cell proliferation, apoptosis and differentiation in the early time points of culture. Using the keratinocyte proliferation marker Ki67, we found proliferating cells (green, red arrows, Fig. 3A) located in the epidermal basal layer of both control and Y-27632 treated group; more Ki67 positive cells were observed in Y-27632 group (red arrows, Fig. 3A). Quantification analysis showed that the percentage of proliferating cells in the control epidermis was reduced from 24 h to 72 h of culture, while that of the Y-27632 treated group showed an increase (Fig. 3B). The increased proliferation of epidermal cells in skin treated with Y-27632 was further confirmed by EdU incorporation assay (Fig. 3C and D), more EdU incorporated cells (green, red arrows, Fig. 3C) were found in Y-27632 treated group. Then we tested whether Y-27632 could reduce epidermal cell apoptosis to maintain the epidermal layers, the apoptotic cells were detected by cleaved caspase 3 staining (Fig. 3E), and no significant difference for the percentage of apoptotic cells between control and Y-27632 treated group,

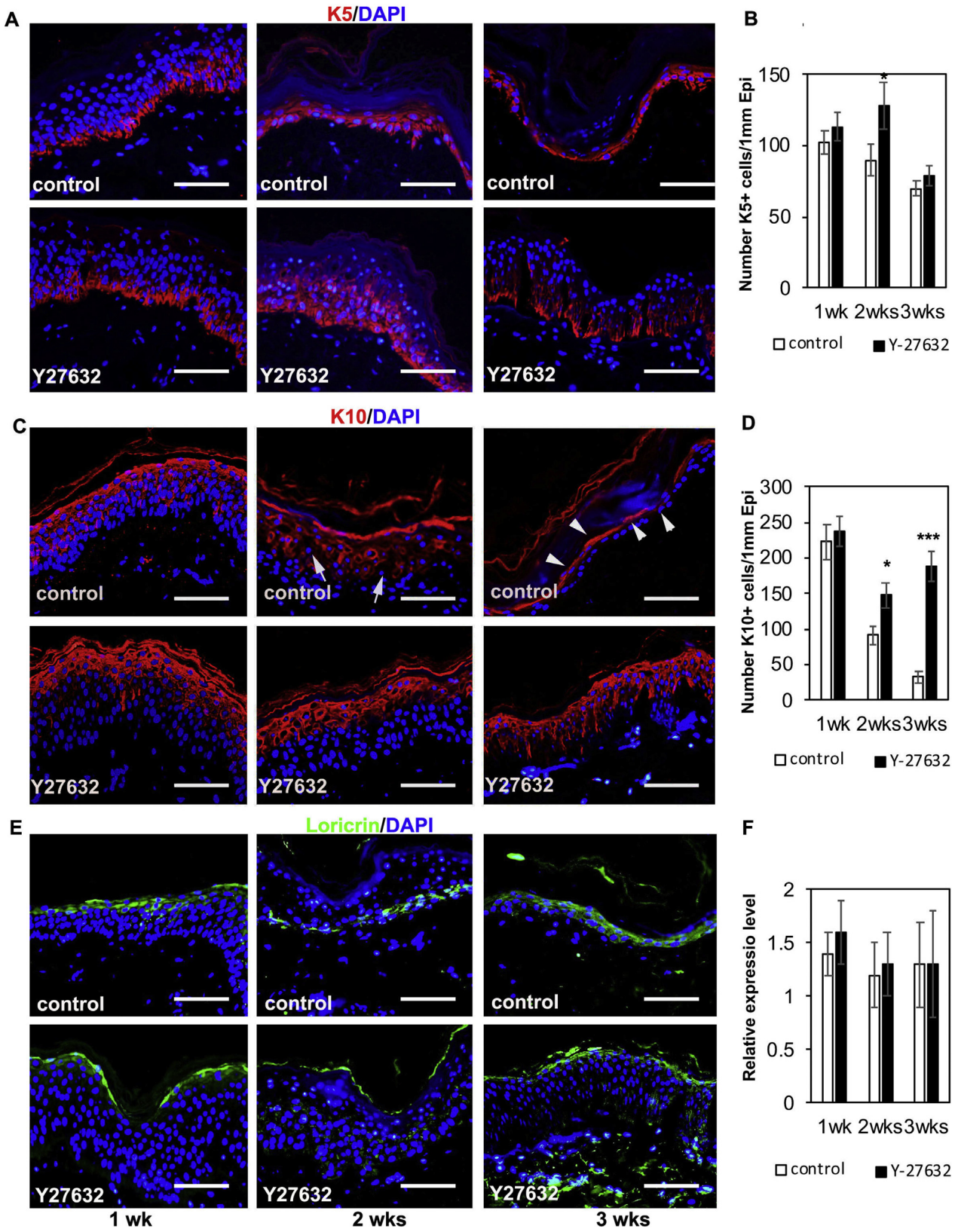

Fig. 2. In the presence of Y-27632 hSOC epidermis retained the differentiation layers for up to 3 wks.
A–F: Skin tissues were cultured in supplemented William's E medium with or without Y-27632. The tissues were harvested at different time points for assessing the presence of keratin 5 (K5, red) in **A**, keratin 10 (K10, red) in **C** and loricrin (green) in **E** by IF staining and DAPI (blue) staining of the nuclei. Arrows in **C** indicate K10 negative cells in suprabasal layer and arrowheads in **C** point a thin layer of K10 positive cells. All Bars = 100μm. **B**. Quantification of K5 positive (K5+) cells in **A** by accounting the number of K5+ cells per 1 mm length of epidermis; **D.** Quantification of K10 positive (K10+) cells in **C** by accounting the number of K10+ cells per 1 mm length of epidermis. **F**. Quantification of relative expression level of loricrin in **E**, which evaluated twice by two Independent persons. Evaluations were based on arbitrary units as follows: 0.5 for weak staining, 1 for intermediate staining and 3 for strong staining, and the mean value of the independent measurements was taken as the final score. **A–F**: All experiments were repeated from at least three different donors (n = 3); $^{*}p < 0.05$, $^{***}p < 0.005$.

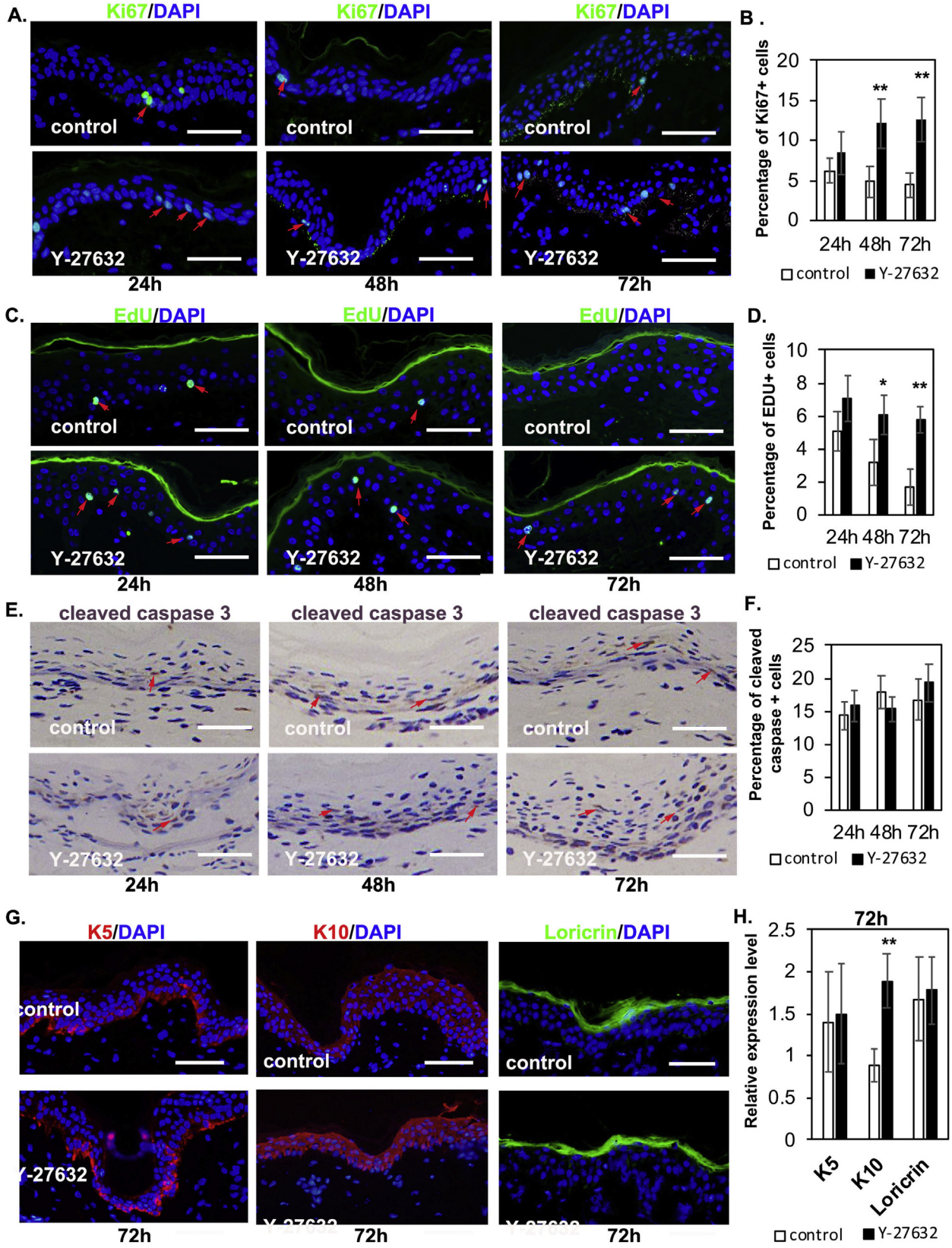

Fig. 3. Y-27632 increases keratinocyte proliferation and enhances expression of suprabasal differentiation marker K10 in hSOC.
A and **B**. Skin was cultured in supplemented William's E medium with or without **Y-**27632 as control. The tissues were collected at different time points as indicated to assess proliferation marker Ki67 (green, red arrows) in **A**. DAPI (blue) stained the nuclei. Quantification of Ki67 positive cells in **B** was performed by counting the number of Ki67 positive cells *versus* total number of basal cells (DAPI positive cells). Bars = 100μm. **C** and **D:** The skins were cultured as in **A,** then incubated with EdU solution for 2 h and were collected for EdU staining with Apollo® 488 (green, red arrows) and nuclei was stained with Hoechst 33342 (blue) in **C**, Quantification of EdU positive cells (red arrows) in **D** was performed by counting the number of EdU positive cells *versus* total number of basal cells (blue nuclei). **E** and **F:** The skins were cultured as in **A** were collected at different time points as

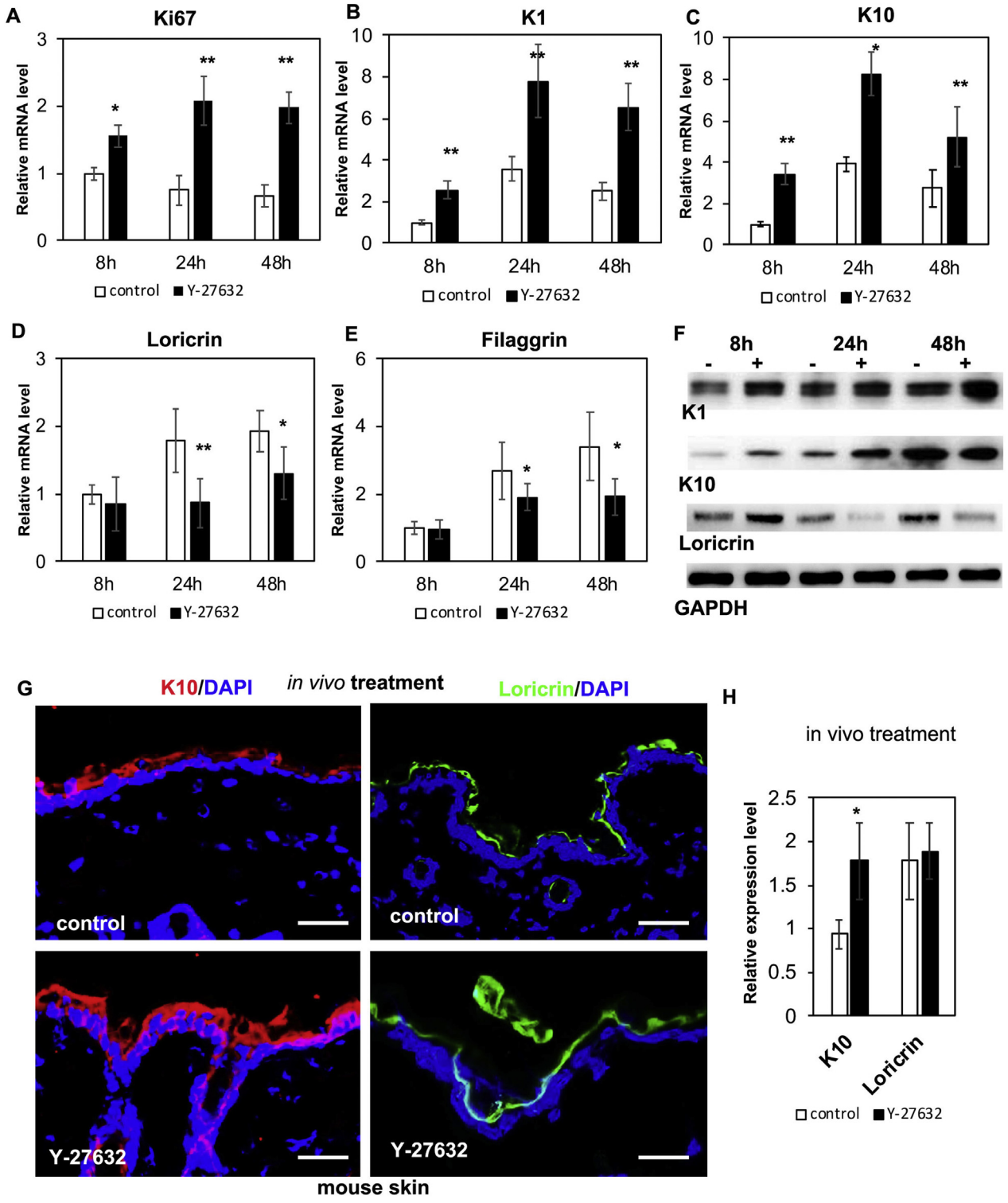

Fig. 4. Y-27632 increases the keratinocyte proliferation and expression of suprabasal cell differentiation markers *in vitro* suspension and *in vivo* mouse.
A–F. Primary keratinocytes were cultured in suspension with supplemented William's E medium in the presence and absence of Y-72632 (10μM). The cells were harvested at different time points as indicated. **A–E**: Shown in these panels are qRT-PCR analyses of proliferation marker Ki67, and differentiation markers K1, K10, Loricrin, and Filaggrin, **F:** Shown here are western-blot analyses of K1, K10 and Loricrin expression; GAPDH served as loading control. **G.** These back-skin tissues were collected from the mice treated with intraperitoneal Y-27632 or PBS (control) for 2 wks; shown are Immunofluorescence analyses of differentiation markers K10 (Red) in left panel, loricrin (Green) in right panel, and DAPI (blue). Bars = 100μm. **H.** Quantification of relative expression level in **G**, the evaluation method described in Fig. 2F legend. **A–E, H:** The experiments were repeated from three different donors (n = 3); $^{*}p < 0.05$, $^{**}P < 0.01$.

indicated to assess apoptotic cells with immunochemistry staining of cleaved caspase 3 (brownish, red arrows), Quantification of apoptotic cells shown in **F** was performed by counting the number of cleaved caspase 3 positive cells *versus* total number of cells per 1 mm length of epidermis. **G** and **H:** K5, K10 and loricrin of hSOC skin tissues collected after cultivation for 72 h in media with or without Y-27632 were measured by immunofluorescence antibody markers in **G**; the relative expression level was quantified in **H** as the method described in Fig. 2F. **A–H**: All experiments were repeated in triplicates (n = 3), $^{*}p < 0.05$, $^{**}p < 0.01$; Bars = 100μm.

indicating Y-27632 didn't reduce the epidermal cell apoptosis. Next, we analyzed the effect of epidermal differentiation by Y-27632 treatment, we carried out IF analysis of differentiation markers K5, K10 and loricrin at 72 h, and quantification analysis of IF staining in Fig. 3H, showing stronger staining of K10 in Y-27632 treated skin; there was no clear difference for K5 and loricrin expression level between control and Y-27632 treated skin. To further confirm Y-27632 enhance the expression of suprabasal markers such as K10, the real time qRT-PCR analysis for the expression of differentiation markers at 24 h, 48 h and 72 h after treatment of Y-27632 were performed (Suppl. Fig. 4). The PCR results showed increased expression of both suprabasal markers keratin 1 (K1) [30] and K10 in Y-27632 treated skin at all time points (Suppl. Fig. 4). There was an increased expression of terminal differentiation markers loricrin and filaggrin, a molecule linked to the cornified envelope [30,35], in Y-27632 treated skin at 24 h, but no clear difference at 48 h and 72 h (Suppl. Fig. 4), which supporting the staining result in Fig. 3G. Taking together, these data suggested that Y-27632 increases keratinocyte proliferation and expression of differentiation markers, K1 and K10, during the organ-culture.

3.4. Y-27632 promotes K1 and K10 expression of keratinocytes both in vitro *suspension culture and* in vivo

To further validate that Y-27632 treatment could enhance the expression of superabasal cell markers K1 and K10, two approaches were carried out. First, *in vitro* suspension assay, which is a frequently used method to induce differentiation besides modulating extracellular Ca^{2+} [24], was performed to test the effect Y-27632 on proliferation and differentiation on suspended cells. The suspended keratinocytes were collected for analysis at different time points by qRT-PCR analysis. As shown in Fig. 4A, Y-27632 increased Ki67 expression, and in Fig. 4B and C Y-27632 induced K1 and K10. These results agreed with the above observations of hSOC; however, we found that Y-27632 significantly decreased the expression of the terminal differentiation markers Loricrin and filaggrin in the suspended cells (Fig. 4D and E). The PCR results were confirmed by the western-blot analysis of keratinocytes lysed at different time points (Fig. 4F). Secondly, we repeated the study on *in vivo* skin preparations to validate the Y-27632 effects on expression of differentiation markers. The mouse skins treated with Y-27632 were collected for IF analysis, Fig. 4G and H shows stronger staining for K10 expression in the Y-27632 treated mouse skin, and the loricrin expression was not decreased by Y-27632 compared to the control mouse. The *in vivo* result agrees that of the organ-culture, suggesting hSOC is likely closer to in *vivo* system, compare to the suspension assay. Taken together, these data further supported that Y-27632 can enhance keratinocyte K1 and K10 expression in the skin both *in vitro* and *in vivo*.

3.5. Y-27632 activates AKT, but inhibits ERK activity in keratinocytes

To understand the Y-27632 mechanism affecting hSOC keratinocyte proliferation and differentiation, we analyzed the AKT and ERK pathways, which have been reported to play crucial roles in regulating keratinocyte proliferation and differentiation. To mimic the condition of hSOC, keratinocytes were cultured in suspension treated with or without Y-27632 and collected at different time points as indicated in Fig. 5A and B and then analyzed by western blot analysis for the phosphorylation forms of AKT and ERK (Fig. 5C). As shown in Fig. 5A after 6 h of suspension, the activity of AKT was significantly increased in the Y-27632 treated keratinocytes compared to the control cells (Fig. 5A). This higher expression of activated AKT persisted up to 72 h (Fig. 5B). In contrast, the activity of ERK decreased in Y-27632 treated cells starting at 1 h after suspension. It is notable that the difference of phospho-ERK expression between control and Y-27632 treated group diminished with time so that it was not apparent at 72 h (Fig. 5B). In the absence of Y-27632 (control), ATK activity decreased while ERK activity increased with culture time (Fig. 5C). The effect of Y-27632 on ERK activation was further confirmed by western-blot analysis of its downstream target p90RSK, and the pattern of p90RSK activation induced by Y-27632 was similar to that of ERK (Suppl. Fig. 5). Moreover, the increased AKT activity and inhibition of ERK by Y-27632 were further validated by staining of phospho-AKT and phospho-ERK in skin derived from hSOC at 1 wk (Fig. 5D, F) and corresponding quantification analysis (Fig. 5E, G).

3.6. Y-27632 maintains hSOC skin structure by regulating AKT and ERK activity

In order to verify whether Y-27632 regulates keratinocyte proliferation and differentiation through AKT and ERK pathway, we cultured keratinocytes in suspension with/without Y-27632 in the presence of MK-2206, which inhibits AKT activity or U0126, MEK inhibitor to block ERK pathway. At 48 h, cells were lysed for qRT-PCR analysis of proliferation and differentiation markers. Fig. 6A showed that the increased Ki67 expression induced by Y-27632 was blocked by MK-2206, but not by U0126, suggesting the increased proliferation by Y-27632 likely occurred through activation of AKT. The increased K1/K10 expression induced by Y-27632 could be blocked by both AKT and ERK inhibitors (Fig. 6B and C). The AKT inhibitor didn't significantly decrease the expression of terminal differentiation markers and couldn't rescue the inhibition of terminal differentiation induced by Y-27632, however ERK inhibitor did block induced differentiation of cells in suspension, regardless of the presence of Y-27632 (Fig. 6D–F). Then, we tested whether Y-27632 maintains hSOC epidermal structure by regulating AKT and ERK activation. Fig. 6G shows that at 1 wk the integrity of epidermis was maintained in all groups (Fig. 6G). At 3 wks, the epidermis was almost lost in the control group, which is similar to as shown in Fig. 1A, and most of epidermis in MK-2206 treated group was detached from the dermis (black arrows, Fig. 6G). Although epidermal structure in the U0126 treated group was better than control and MK-2206 treated group, it was still thinner than that of skin treated with Y-27632, suggested inhibition ERK alone might be not sufficient to maintain the integrity of epidermal structure. These results were corroborated by quantification analysis of epidermal thickness and number of viable epidermal cells (Fig. 6H). Taking together, the data suggest that Y-27632 maintains hSOC epidermal structure by regulating both AKT and ERK activation.

4. Discussions

An ideal culture system, should not only preserve epidermal viability, but it should also preserve proliferation and normal differentiation of epidermal cells [2]. In early organ-culture systems, serum was considered an essential component necessary to maintain the skin viability and integrity, but as serum is chemically undefined and often variable presents difficulties for reproducible skin culture studies [2]. Zeltinger et al. tested 11 types of culture media to grow fetal skin tissues for 3–4 wks and found that serum free medium DMEM/F12 was optimal, suggesting that defined serum free media was able to maintain long-term skin viability and integrity in hSOC [36]. Lu et al. applied supplemented serum-free William's E medium to culture adult human scalp skin and observed active hair shaft growth until day 16; however there was progressive thinning and hyperkeratosis of the interfollicular epidermis after day 5(23). By using the same medium, Kleszczynski and Fisher showed that epidermal proliferation and

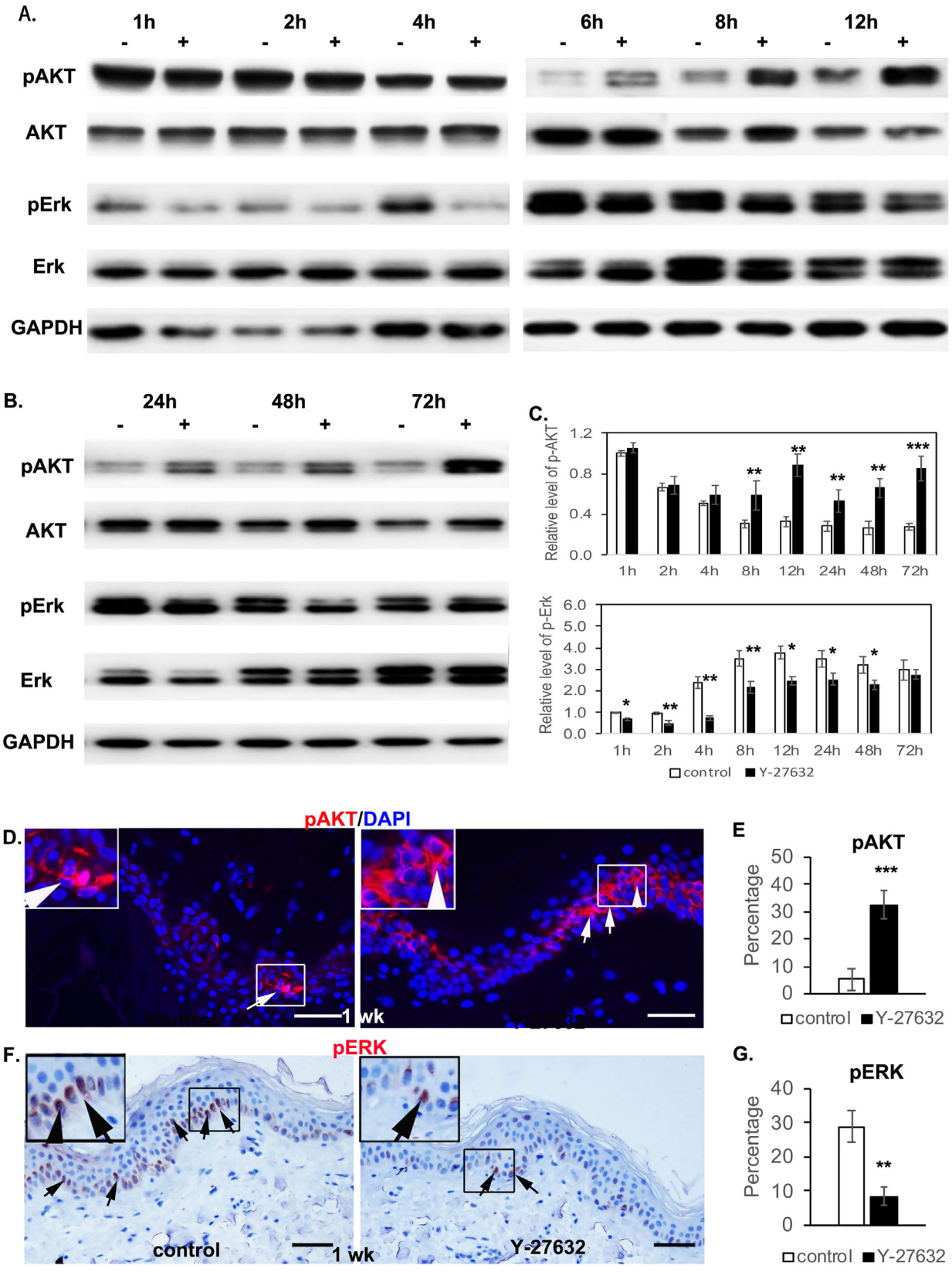

Fig. 5. Y-27632 increases AKT activation and inhibits ERK activity of keratinocytes cultured *in vitro* suspension assay.
A–C. Primary keratinocytes were cultured in suspension with supplemented William's E medium in the presence or absence of Y-27632 (10μM). The cells were collected at different time points as indicated. Western blot analysis shows the protein level of phosphorylated AKT, total AKT, the phosphorylated form of ERK and total ERK. Quantification of p-ATK or p-ERK in cells cultured with Y-27632 is given in **C;** the result is given as relative fold change compared to that of cells in cell culture for 1 h. **D–G:** IF analysis of pAKT in **D**, IHC analysis of pERK in **F** in skins at 1 week of hSOC. The arrows indicate either pAKT or pERK positive cells. The insert in upper-left corner of each image is a zoom of the square area as indicated. The quantification of percentage of pAKT positive cells in **E, or** pERK positive cells in **G**, was calculated by counting pAKT or pERK positive *versus* total number of cells (by nuclei) per 1 mm length of epidermis. **A–G:** All experiments were performed in triplicates (n = 3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

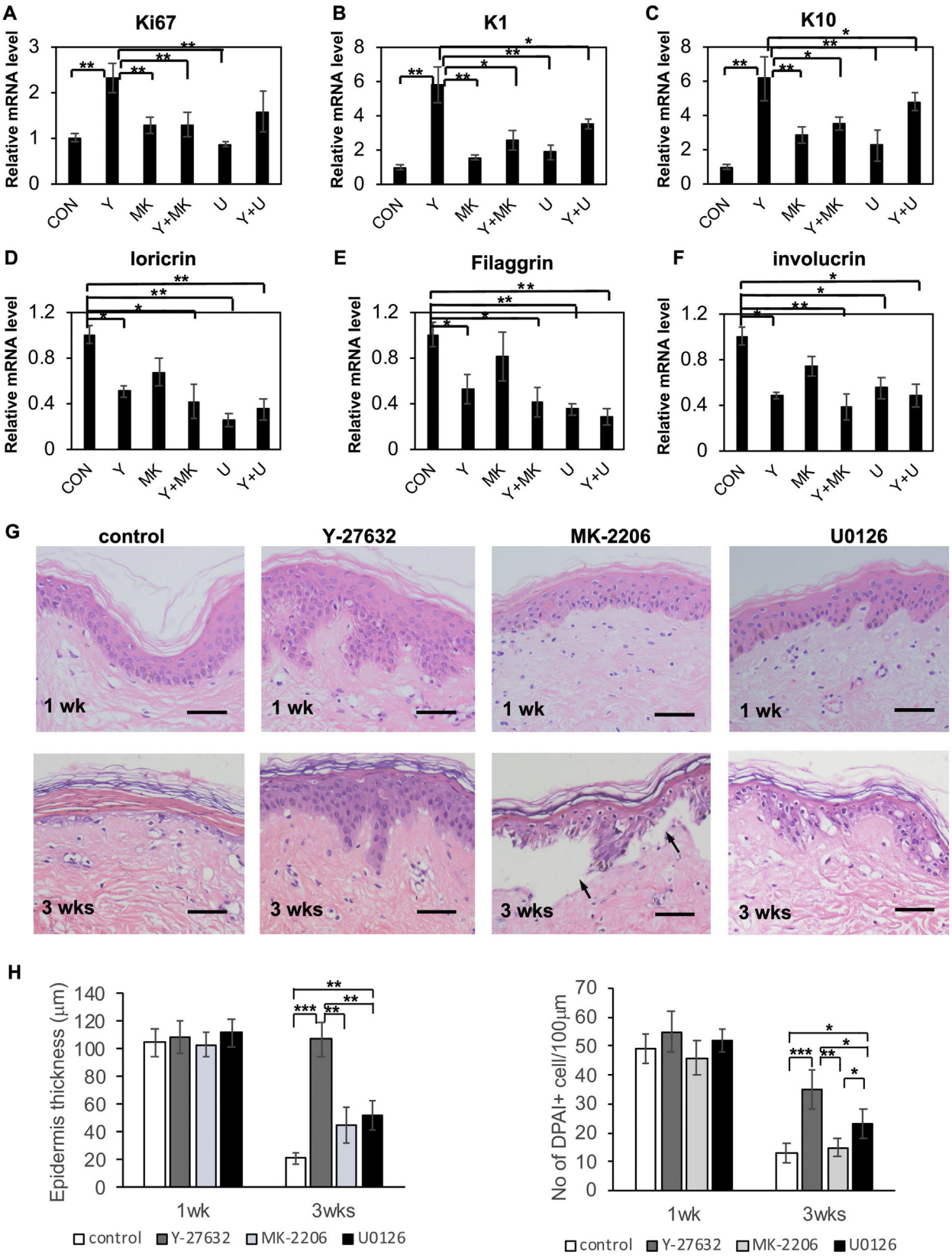

Fig. 6. Y-27632 maintains the integrity of epidermis in hSOC by regulating the PI3K/AKT and RAF/ERK pathways.
A–F: Primary keratinocytes were suspension cultured in supplemented William's E medium with different conditions as indicated: CON: control, Y: Treated with Y-27632, MK: MK-2206, Y + MK: Y27632+ MK-2206, U: U0126, Y + U: Y-27632+U0126. The cells were collected 48 h after in suspension for qRT-PCR analysis of Ki67, and of differentiation markers K1, K10, loricrin, filaggrin and involucrin. **G**: The histological analysis (HE staining) for hSOC tissues cultured under different conditions and harvested at 1 wk and 3 wks. Black arrows indicate the detachment of epidermis from the dermis. Bars = 100μm; **H**. In the left panel, the thickness of the epidermis from **G** was measured as described in Fig. 1**B**; the right panel showed the average number of DAPI positive cells per 100μm length of epidermis IF staining of section in **G,** counted as described in Fig. 1**C**. **A–F, H**: All experiments were repeated in triplicate from 3 different donors (n = 3), Student's *t*-test was used for statistical analysis, $^{*}p < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

structure of abdominal skin could be maintained for only 48 h [37], but that short-term cultures could be still be used for meaningful study of epidermal function [38]. Recently, Buckingham et al. used supplemented MEM to culture foreskins for 28 days and studied the autophagy response of human neonatal skin after infection with varicell-zoster virus (VZV); unfortunately the epidermal structure was not analyzed in this report [39]. Nevertheless, these studies did not address the issue of epidermal structure-function preservation. Here we carefully characterized the epidermal structure by both detailed histological analysis and immunofluorescence staining of epidermal differentiation status at different culture time points and discovered that the incorporation of the Rock inhibitor Y-27632 into the supplemented William's E medium significantly prolongs the preservation of skin epidermis architecture up to at least 3 wks and maintained keratinocyte proliferation and differentiation.

The normal keratinocyte proliferation and differentiation is highly regulated, plays a crucial role for maintaining epidermal integrity [29,40]. By analysis of differentiation and proliferation status, we found that Y-27632 preserves basal layer proliferation and suprabasal layer expression of K1 and K10 (Figs. 2C and D, 3, 4) during the culture. Interestingly, Y-27632 significantly inhibits the expression of loricrin and filaggrin in the suspension assay (Fig. 4D–F), but not in the hSOC (Figs. 3F and G, 4G and H). *in vivo* treatment of Y-27632 did not block keratinocyte terminal differentiation (in mouse), thus supporting the findings with hSOC. Importantly, our findings fully agreed with findings of previous reports [14,15]. It has been reported that Y-27632 could block terminal differentiation of loricrin mostly through inhibition of ROCK2 but increase early differentiation markers of K1 and K10 through inhibition of ROCK1 in the *in vitro* suspension assay [15]. However, it has been also reported that inhibition of ROCK actually induced terminal differentiation [41]. Therefore, the difference between *in vitro* suspension assay and the hSOC might be due to their different physiological microenvironments.

How Y-27632 regulates keratinocyte proliferation and differentiation has not been fully documented so far. We recently found Y-27632 could promote BRAF mutant melanocyte growth and migration through regulation of PI3K/AKT and RAF/ERK pathways [18]; both pathways have been shown to play a crucial role in regulating keratinocyte proliferation and differentiation [19,20,42–46]. Peng et al. reported that double knockout of AKT1 and ATK2 in mouse resulted in much thinner of skin layers, especially of the suprabasal (spinous) layer, compared to the control little mate, and they found that decreased proliferation of basal cells, and significantly reduced expression of differentiation markers such as K10 and filaggrin [45]. The study from Calautti et al. demonstrated that the PI3K/AKT pathway was activated during mouse keratinocyte differentiation, that active expression of AKT could promoted expression of differentiation markers K1 and loricrin, and that this effect could be blocked by AKT inhibitor MK-2206 [19]. Here we found Y-27632 could significantly increase AKT activity both in suspension and in the hSOC, and the AKT inhibitor MK-2206 could completely block both increased proliferation and differentiation induced by Y-27632 (Fig. 6). Therefore, we think that Y-27632 enhanced keratinocyte proliferation and increased the expression of K1/K10 differentiation in the hSOC by way of AKT activation. ROCK was shown to activate PTEN, which negatively regulate activity of PI3K [47], suggesting Y-27632 enhances AKT activation in keratinocytes probably through regulation of PTEN activation.

Besides the PI3K/AKT pathway, RAF-ERK signaling pathway is another mechanism for controlling cell survival, differentiation and proliferation in response to extracellular cues [21,44,48]. Our results showed that Y-27632 could significantly inhibit the activation of ERK in keratinocytes. It has been reported that RAF-MEK-ERK pathway could negatively regulate epidermal differentiation, and the activation of this pathway in epidermis will increase keratinocyte proliferation and block its differentiation [43,44], and that indicating downregulation of RAF-ERK activation would promote keratinocyte differentiation. Therefore, inhibition of ERK activity by Y-27632 could contributed to enhance keratinocyte differentiation. However, when we used U0126, a MEK inhibitor to block the ERK signaling pathway, we found that it actually blocked keratinocyte differentiation, and decreased the expression of terminal differentiation markers, such as loricrin, filaggrin, even in the presence of Y-27632. Recent publications showed that ERK inhibitors could block keratinocyte differentiation, just as we found here [22,49]. Therefore, the data suggest that Y-27632 possibly blocks keratinocyte differentiation through decreasing RAF-ERK activation, which to some extent, prevents a potential hyper-differentiation induced by the sustained ATK activation with the continuous treatment of Y-27632. Interestingly, the addition of U0126 into William's E medium prolongs the preservation of skin epidermal structure in the hSOC, which partially mimics the result from Y-27632 treatment (Fig. 6). It indicates that downregulation of ERK activity by Y-27632 is crucial for preserving epidermal integrity in the hSOC.

In summary, we report here that Rock inhibitor Y-27632 enhances the preservation of epidermal integrity in hSOC, very likely by increasing AKT and decreased ERK activities. A stable long-term hSOC system will definitely widen its future applications.

Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.10.006.

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