Video Article

# Isolation and Culture of Primary Mouse Keratinocytes from Neonatal and Adult Mouse Skin

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## Abstract

The keratinocyte (KC) is the predominant cell type in the epidermis, the outermost layer of the skin. Epidermal KCs play a critical role in providing skin defense by forming an intact skin barrier against environmental insults, such as UVB irradiation or pathogens, and also by initiating an inflammatory response upon those insults. Here we describe methods to isolate KCs from neonatal mouse skin and from adult mouse tail skin. We also describe culturing conditions using defined growth supplements (dGS) in comparison to chelexed fetal bovine serum (cFBS). Functionally, we show that both neonatal and adult KCs are highly responsive to high calcium-induced terminal differentiation, tight junction formation and stratification. Additionally, cultured adult KCs are susceptible to UVB-triggered cell death and can release large amounts of TNF upon UVB irradiation. Together, the methods described here will be useful to researchers for the setup of *in vitro* models to study epidermal biology in the neonatal mouse and/or the adult mouse.

## Video Link

The video component of this article can be found at https://www.jove.com/video/56027/

## Introduction

The skin is the largest organ in the body with the epidermis as the outer most layer. The epidermis plays a critical role in forming an intact epidermal barrier to separate the body from the environment, and thus prevents water loss and provides protection from environmental insults, such as allergens, pathogens and UVB exposure. The epidermis develops from a single layer of undifferentiated basal keratinocytes (KCs) into a multi-layered stratified epithelium consisting of a basal layer, followed by a spinous layer, granular layer, and stratum corneum. Basal KCs, consisting of both epidermal stem cells and transit-amplifying cells, are proliferative and undifferentiated. As basal KCs exit the cell cycle, the cells commit to differentiation and gradually migrate towards the surface of the epidermis, accompanied by the maturation of cell-cell junctions and formation of an epidermal permeability barrier (EPB). The KCs at the spinous layer express early differentiation markers such as Keratin 10 (K10); as the KCs migrate to the granular layer, the cells express late differentiation markers such as Filaggrin (FLG), Loricrin (LOR) and Involucrin (INV). At the stratum corneum, the KCs become terminally differentiated corneocytes, which are eventually shed off through desquamation as new cells replace them.

Calcium is considered the most physiological agent in the epidermis and triggers differentiation *in vitro* and *in vivo* in a similar manner. In normal skin epidermis, calcium ions form a characteristic "concentration gradient", increasing in concentration towards the skin surface[1,2,3]. The calcium concentration rises from low levels in the lowest sublayers (basal and spinous layers) to a peak in the upper granular layer and then drops to negligible levels in the most superficial layer (stratum corneum). The calcium gradient also develops coincidently with the emergence of a component permeability barrier, which supports that calcium signaling plays a critical role of KC differentiation. *In vitro*, low calcium (0.02-0.1 mM) maintains the proliferation of basal KCs as a monolayer, whereas high calcium (>0.1 mM) induces a rapid and irreversible commitment of the cells to terminal differentiation as demonstrated by tight-junction formation and induction of LOR and INV upon high calcium treatment to the basal KCs[4,5].

In addition to barrier formation, epidermal KCs are also an important component of the skin's innate immune system. In response to pathogens or damaged-associated molecular patterns (DAMPs) released upon UVB irradiation or injury, KCs can produce large amounts of inflammatory cytokines, such as TNFα, IL6 and IFNβ, leading to immune system activation[6,7,8,9]. Although proper inflammatory signaling from KCs is required for pathogen clearance, uncontrolled inflammatory response may trigger the development of auto-inflammatory skin diseases, such as psoriasis and rosacea[6,8].

Overall, KCs play a vital role in maintaining the intact skin barrier and initiating an immune response upon pathogen invasion or environmental insults. Therefore, primary culture of epidermal KCs is a useful technique to study the epithelial biology, KC differentiation, as well as KC-stimulated innate immune responses. The isolation and culture of primary mouse epidermal KCs can be a challenging process due to KC's susceptibility and sensitivity to various external stimulants. Here we describe a method to isolate and culture KCs from either neonatal mouse

skin or adult mouse tail skin. For adult KC isolation, we do not use mouse dorsal skin because isolating sufficient quantities of viable KCs from this tissue can be difficult for the following reasons: First, the adult dorsal skin at the resting phase of the hair cycle (telogen) consists of a thin epidermis with only 1-2 layers of cells, leading to a low cell yield and inefficient separation of the epidermis from the dermis, which is the critical step for successful KC isolation. Second, the high hair follicle density that is present on adult dorsal skin further contributes to the difficulty in separating epidermis from the dermis. Instead, we routinely use tail skin as the source for adult mouse KCs as this epithelium is thicker with 3-5 layers of epidermal KCs. It also has a lower hair follicle density, which does not interfere with the epidermal separation, thus allowing KC isolation from any adult mouse tail skin regardless of the age and hair cycling stage of the mouse. The isolated neonatal KCs are seeded to gelatin-coated culture dishes, whereas collagen-coated dishes are used to seed isolated adult KCs due to the impaired ability of the adult cells to adhere compared to their neonatal counterparts. To culture mouse KCs, low calcium basal medium is supplemented with dGS, which contains epidermal growth factor (EGF), bovine transferrin, insulin-like growth factorc1 (IGF1), prostaglandin E2 (PGE2), bovine serum albumin (BSA) and hydrocortisone. Between 2-4 days after the initial plating, most of the differentiated KCs can be washed away during daily medium changes, and the remaining adherent cells show typical cobblestone morphology[4], are proliferating, and do not express the early differentiation marker K10.

## Protocol

All animal experiments are approved by the UCSD Institutional Animal Care and Use Committee.

# 1. Primary Mouse KC Isolation and Culture from Neonatal Skin

1. Sacrifice the post-natal day 0-2 neonates from the C57B/6 wildtype mouse strain by decapitation using scissors. Cut off the limbs just above the wrist and ankle joints, then cut off the tail completely, leaving a small hole.
2. **To peel off the whole skin, first insert sharp scissors through the hole at the tail and cut the skin along the dorsal midline of the body to the opening on the neck. Next, use one forceps to grasp the exposed body and the other forceps to grasp the skin, and gently peel the whole skin off the body and over the leg stumps with one continuous motion.**
    1. Peel off the skin as one intact piece and be careful not to break the skin into pieces as this will result in cell loss during the dispase step.
3. **Rinse the peeled skin with 15 mL of sterile PBS in a 10 cm Petri dish, then place the skin from each pup into a 2 mL tube filled with ice cold dispase digestion buffer, which contains 4 mg/mL dispase in KC growth medium (KC basal medium has 0.06 mM $CaCl_2$, dGS, and antibiotics/antimycotics).**
   NOTE: Multiple skin isolates can be combined and incubated in one 15 mL tube (up to 5 skins per tube) containing 12 mL of dispase solution. Incubate the skin tube overnight in a 4 °C refrigerator on a rotator.
    1. Make sure that the skin is not folded in the tube as this will result in insufficient exposure of the folded region to the dispase solution.
4. On the next day (after 12-18 h in dispase), transfer the skin together with the dispase solution into a Petri dish. Transfer each tissue piece to a new Petri dish with 15 mL sterile PBS to wash away excess dispase.
5. Using two pairs of forceps, grasp the skin from PBS, lift the skin, and transfer to a new Petri dish with the epidermal side down and the dermis side up. Carefully stretch the skin folds so that it is fully extended on the Petri dish.
6. **Place 500 µL of a trypsin-based digestion solution for each skin in a new Petri dish. Using forceps, slowly lift the dermis up and away from the epidermis, while holding the epidermis down. Dispose the dermis as biohazardous material. Transfer the separated epidermis and float on each drop of trypsin solution with the basal layer downward.**
    1. If the epidermis is folded on the trypsin solution, remove any folds using forceps to ensure efficient digestion of basal KCs from the epithelium.
7. Incubate the skin on a Petri dish in trypsin solution at room temperature (with the lid covered) on a horizontal shaker with gentle agitation for 20 min.
8. **Add 2 mL of supplemented KC growth medium per epidermis (about 1 square inch in size per epidermis) to the Petri dish. Grasp the epidermis using forceps, and vigorously rub the epidermis back and forth to release single cells from the epidermal sheet.**
    1. Watch the medium turn increasingly turbid as cells are detached from the epidermis. Tilt the Petri dish to collect and transfer the cell suspension to a new tube leaving the remaining epidermal sheet on the dish.
9. Repeat two more times the rubbing of the epidermal sheet after adding 2 mL KC growth medium, and combine the cell suspensions in the same tube.
10. Pipet the cell solution up-and-down gently a few times to break any cell clumps using the appropriate serological pipette, then pass it through a 100 µm filter to a new 50 mL centrifuge tube.
11. Centrifuge the filtered cells for 5 min at 180 x g.
12. Aspirate off the supernatant and gently resuspend the cell pellet in 1 mL cold KC growth medium/epidermis.
13. Count the cells by a hemocytometer.
    NOTE: The KCs can spontaneously differentiate in suspension, so the cells should be plated as soon as possible after isolation to ensure the optimal performance of the cells. We recommend placing the cells on ice while counting as this will reduce the rate of KC spontaneous differentiation.
14. **Seed the cells at a density of 5 x $10^4/cm^2$ in KC growth medium in culture dishes pre-coated with an extracellular matrix (ECM) product that promotes cell attachment.**
    NOTE: We use a commercial available (e.g. attachment factor, which is a gelatin-based coating material (see the Table of Materials for details). Cells were cultured in humidified incubator with 5% $CO_2$ at 37 °C, and fresh medium were replenished every other day.
    1. For coating, coat the culture dishes with the cell attachment coating material warmed to room temperature for 10 min to 2 h prior to the cell seeding. Remove the attachment factor just before adding the cell suspension.

15. Change the medium 24 h after the initial plating to remove unattached cells, and change the medium daily until the cells reach the desired confluency prior to the experiment.

## 2. Primary Mouse KC Isolation from Adult Tail Skin

1. Sacrifice an adult C57BL/6 mouse (preferably between 6-15 weeks of age; either male or female) according to the animal facility regulations. Cut off the tail off the body from the tail base, and cut off ~2 mm of the tail tip so that there is a visible hole at the tip.
2. To peel the tail skin off, first use a sharp blade to cut along tail skin from the base to the tail tip. Next, use one forceps to grasp the exposed tail bone and the other forceps to grasp the skin, and gently peel the tail skin off the bone with one continuous motion. Cut the peeled skin from the mid-line so that each piece is less than 2-3 cm long.
3. **Rinse the peeled skin with 15 mL of sterile PBS in a 10 cm Petri dish, then place the skin from each tail into a 2 mL tube filled with ice cold dispase digestion buffer (4 mg/mL dispase in KC growth medium).**
   NOTE: Multiple skin pieces can also be combined and incubated in one 15-mL tube (up to 5 tails per tube) containing 12 mL of dispase solution.
   1. Incubate the skin tube overnight in a 4 °C refrigerator on a rotator.
4. Perform steps 1.4 to 1.13 to isolate the single cell suspension from the tail epidermis.
5. Seed the adult cells at a density of 10 x $10^4$/$cm^2$ (counted by a hematocytometer) in KC growth medium in culture dishes pre-coated with collagen.
   NOTE: We use a commercially available collagen-based coating kit (see the **Table of Materials** for details). Cells were cultured in humidified incubator with 5% $CO_2$ at 37 °C, and fresh medium were replenished every other day. In general, the culture dishes should be coated with collagen for 30 min to 1 h prior to the cell seeding. We observe that there is a significant enhancement of adult KC attachment to the dish when coated with collagen compared to the attachment factor, which is a gelatin-based coating material.
6. Change the medium 24 h after the initial plating to remove unattached cells, and change the medium daily until the cells reach the desired confluency prior to the experiment.

## 3. *In Vitro* Differentiation of KCs by High Calcium

1. To induce terminal differentiation, add $CaCl_2$ to 0.2 mM in the culture medium.
2. Alternatively, starve the cultured KCs overnight without added growth supplements in low calcium KC basal medium prior to the addition of high calcium.
   NOTE: Growth factor starvation will enhance cell commitment to early differentiation and the induction of early differentiation markers, such as K10[5].

## 4. UVB Irradiation Mediated Cell Death and TNFα Release from KCs

1. Culture the mouse KCs in KC growth medium (in humidified incubator with 5% $CO_2$ at 37 °C) with daily medium change until the cells reach confluency. Immediately before the UVB irradiation, remove the growth medium and replace with 500 µL of PBS. Then subject the cells to 25 mJ/$cm^2$ UVB or mock control (no UVB).
   NOTE: UVB irradiation is performed using handheld UVB lamps with two 8 W bulbs (312 nm) as previously described[10]. After UVB irradiation, PBS is aspirated and fresh medium is added.
2. Measure and quantify the cell viability by the CCK-8 cell viability assay at 6, 8, and 24 h post-UVB treatment following the manufacturer's instruction[11].
3. Collect the conditioned medium from the KCs treated with UVB at desired time points and measure the TNFα released in the conditioned medium by the Mouse TNF (Mono/Mono) ELISA kit following the manufacturer's instruction[7,12].

## Representative Results

**High calcium induced terminal differentiation of neonatal and adult KCs.** The primary mouse epidermal KCs plated and maintained at 0.06 mM $CaCl_2$ grew as a monolayer, and individual cells had a polygonal shape with distinct intercellular space, showing a cobblestone appearance when confluent (**Figure 1A** and **Figure 2A**). Elevating the $CaCl_2$ to 0.2 mM induced a rapid morphology change of the cells. Within 8 h after the high calcium switch, the cells became flattened and the distinct intercellular space became less apparent, and by 24 h the cell-cell adhesion with tight junctions became obvious (**Figure 1A** and **Figure 2B**). The formation of the cornified cell envelope and vertical cell stratification started around 48-72 h after the high calcium switch (**Figure 2B**). To analyze the actin remodeling and cell-cell tight junction formation during the high calcium switch, KCs treated with 0.2 mM $CaCl_2$ were stained with phalloidin (**Figure 1B**) to measure the actin remodeling during KC differentiation[13]. The formation of the actin fiber-rich filopodial projections between the adjacent cells were detected as early as 3 h post calcium switch, and the actin fiber remodeling further progressed between 6-24 h, and by 48 h the cell stratification became prominent (**Figure 1B**).

**Susceptibility of mouse KCs to UVB-triggered cell death and TNFα release**. The cultured adult mouse KCs were exposed to 25 mJ/$cm^2$ UVB irradiation, and the cell viability and TNFα released in the culture medium were analyzed. As shown by the phase contrast images (**Figure 3A**), as well as the cell viability assay (**Figure 3B**), the UVB triggered a time dependent death of the KCs. By 24 h, the majority of the cells were rounded and were detached from the dish (**Figure 3A**). As quantified by the cell viability assay, ~90% of cells were dead within 24 h post UVB treatment (**Figure 3B**; $p < 0.001$ as calculated by one-way ANOVA multiple comparison test). Finally, we showed that TNFα, an important cytokine that is induced by UVB and drives KC apoptosis following UVB irradiation[14], was abundantly secreted (~250 pg/mL within 24 h post UVB-irradiation; $p < 0.001$) in the culture medium of UVB-treated KCs in a time dependent manner (**Figure 3C**).

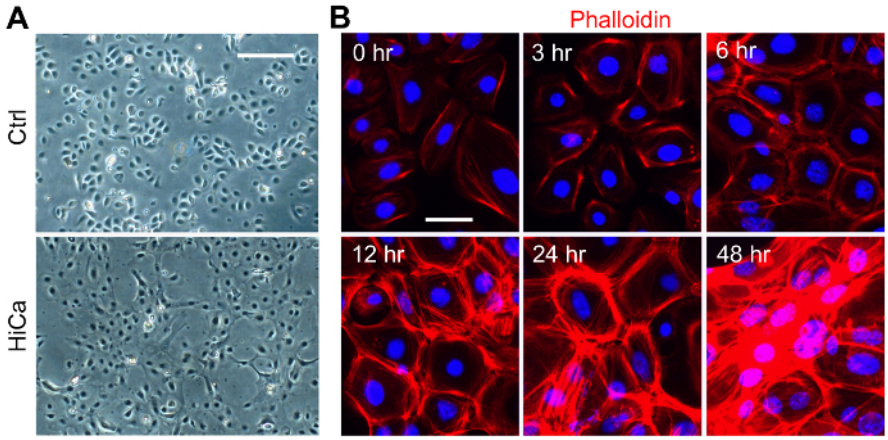


**Figure 1: Primary Culture of Neonatal Mouse KCs, and High Calcium Induced Terminal Differentiation and Tight Cell-cell Junction Formation.** (**A**). Primary neonatal mouse KCs were treated with high calcium (0.2 mM $CaCl_2$), and phase contrast images at 4X magnification were taken at 0 hour (ctrl) and at 8 h after treatment. Scale bar = 100 µm. (**B**). Neonatal KCs were differentiated in the presence of 0.2 mM $CaCl_2$, and the cells were stained with phalloidin (red) to visualize the formation of actin fiber-rich filopodial projections between the adjacent cells during differentiation. Nuclei were counterstained with DAPI, and actin fibers were stained with rhodamine phalloidin. Scale bar = 25 µm. Images were taken at 20X magnification using a fluorescence microscope set to the DAPI channel (for nuclei staining) and RFP channel (for actin staining). Please click here to view a larger version of this figure.

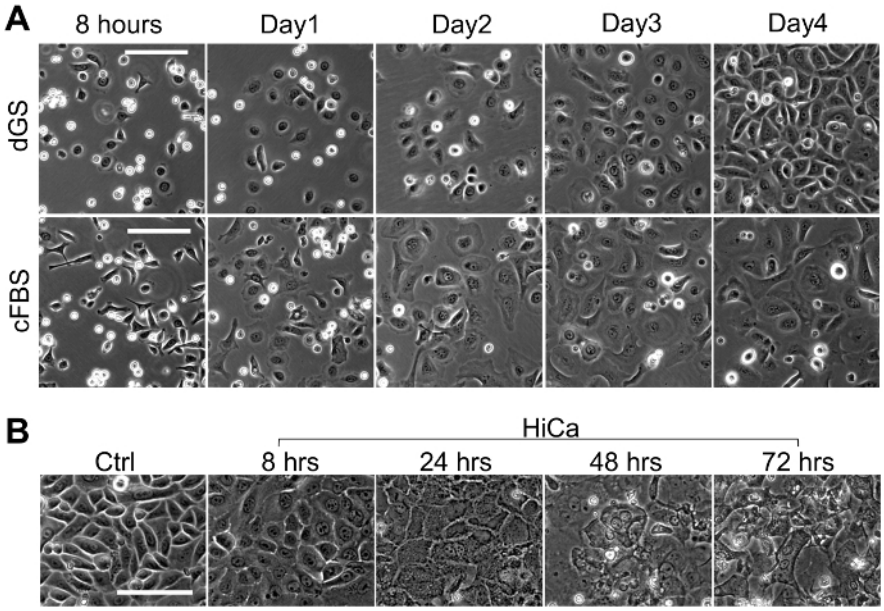


**Figure 2: Primary Culture and High Calcium Induced Terminal Differentiation of Adult mouse KCs.** (**A**). Phase contrast images at 10x magnification of primary adult mouse KCs at 8 h, 1 day, 2 days and 3 days after the initial plating. The upper panel represents cells grown in dGS, and the lower panel represents cells grown in 10% chelexed FBS (cFBS) with 8 ng/mL recombinant mouse EGF. Scale bar = 100 µm. (**B**). Confluent adult mouse KCs were differentiated in the presence of 0.2 mM $CaCl_2$, and phase contrast images at 10X magnification were taken at 0 (ctrl), 8, 24, 48, and 72 h post high calcium switch. Scale bar = 100 µm. Please click here to view a larger version of this figure.

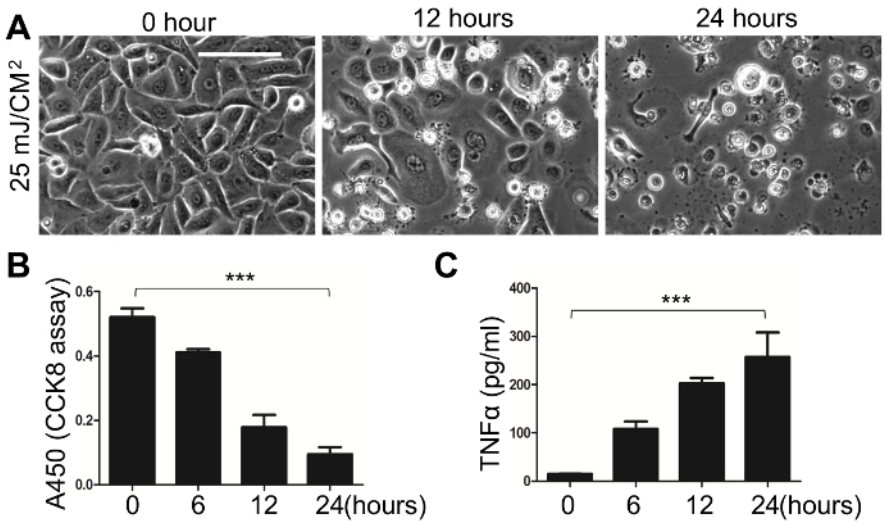


**Figure 3: Primary Culture of Adult mouse KCs, and Susceptibility of Adult KCs to UVB-induced Cell Death and Release of TNFα.** Primary adult mouse KCs were cultured to confluency, then exposed to 25 mJ/cm$^2$ UVB irradiation. (**A**) Phase contrast images at 10X magnification taken at 12 and 24 hours post-UVB exposure showed a time dependent UVB-induced cell death. Scale bar = 100 µm. (**B**) The cell viability was quantified by the CCK-8 cell viability assay at 6, 8, and 24 h post-UVB treatment. (**C**) UVB-induced release of TNFα to the media at indicated time points was measured by ELISA. All error bars indicate mean ± SEM. The p-values were calculated by one-way ANOVA multiple comparison test (***, p <0.001) Please click here to view a larger version of this figure.

## Discussion

The skin epidermis functions as a critical barrier to separate and protect the body from the outside environment and damage from water loss, pathogens, heat and UV irradiation. The KCs are the predominant cell lineage of the epidermis, and primary culture of epidermal KCs is a useful tool to study and understand the biological processes of barrier formation and the response of KCs to environmental insults *in vitro*.

Here we describe methods to isolate and culture primary epidermal KCs from neonatal and adult mouse skin. While primary mouse KCs can be conveniently isolated from neonatal mouse skin, the isolation and successful culture of adult KCs are considered difficult due to the thinning of the epidermis and the high hair follicle density of the adult mouse dorsal skin. The method described here uses adult mouse tail skin as a convenient and abundant source of basal KCs. Because of the presence of a thick epidermis and low hair follicle density in the tail skin, the critical step of isolating KCs by separating the epidermis from the dermis becomes feasible after overnight dispase digestion of the tail skin. In contrast, our attempt to isolate KCs from adult dorsal skin using this protocol was not successful, resulting in low cell yield after isolation and low cell attachment after plating. However, successful isolation and culture of KCs from adult dorsal skin has been reported after overnight trypsin digestion of the fur-free skin[15], suggesting that dispase alone may not be sufficient to extract follicular KCs from the dermis of the adult dorsal skin.

In this protocol, the primary KCs were grown in low calcium medium supplemented with dGS instead of the calcium-depleted chelexed-FBS, which was widely used in several previously published mouse KC culture protocols[4,15,16,17,18]. In this study, we observed that dGS is superior to chelexed FBS to culture adult mouse KCs and to maintain KC's basal cell morphology (**Figure 2A**). However, it is likely that the effectiveness of chelexed-FBS to maintain KC's basal morphology may depend on the FBS lot used in each study.

Using the methods presented here, we demonstrated that both the neonatal and adult mouse epidermal KCs responded to high calcium triggered terminal differentiation, tight cell junction formation and stratification (**Figure 1** and **Figure 2**). In general, elevating the extracellular calcium level to greater than 0.1 mM triggers terminal differentiation of basal KCs[4,5,17,19]. It has been reported that an intermediate level of calcium (0.1- 0.16 mM) substantially induced the expression of early differentiation markers, such as K1 and K10, during the first 24 h upon calcium switch, whereas K1 and K10 was only induced in a small fraction of cells when treated with higher calcium medium (1 mM)[19,20]. However, the higher calcium medium ( ≥1 mM) has been widely used in many other studies to rapidly and robustly induce KC stratification and the expression of late differentiation genes, such as INV and LOR[4,21,22,23]. Based on our experience, an intermediate level of calcium, such as 0.2 mM $CaCl_2$, leads to a gradual and slower onset of terminal differentiation, whereas a higher calcium levels (≥1 mM) drastically speed up the onset of KC terminal differentiation, leading to a shorter time window to study the cellular changes during differentiation. Therefore, we used 0.2 mM $CaCl_2$ to induce KC terminal differentiation, and we have shown that this intermediate calcium level led to a gradual change of the basal cell morphology to a stratified corneocyte morphology within 72 h after the calcium switch (**Figure 2B**).

Our group has previously demonstrated that epidermal KCs also play an important role to initiate inflammatory responses to pathogens or DAMPs released during injury and/or UVB-irradiation[6,7,8,9,10]. In consistent with those *in vivo* observations, here we showed that cultured adult mouse KCs were highly susceptible to UVB-triggered cell death and UVB-treated KCs released a large amount of TNFα, which is a key inflammatory cytokine that activates the immune system.

In summary, the primary mouse epidermal KC cell culture provides a useful *in vitro* model to study the epidermal barrier formation and innate immune response of KCs, and the methods described here will be of interest to researchers pursuing studies in cutaneous biology in the neonatal mouse as well as in the adult mouse.

## Disclosures

The authors have nothing to disclose.

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