Original Paper

Epidermal CFTR Suppresses MAPK/NF- κ B to Promote Cutaneous Wound Healing

Jing Chen^a Yu Chen^a Yajie Chen^a Zicheng Yang^a Bo You^a Ye Chun Ruan^b
Yizhi Peng^a

^aState Key Laboratory of Trauma, Burns and Combined Injury, Chongqing Key Laboratory for Proteomics Disease, Institute of Burn Research, Southwest Hospital, the Third Military Medical University, Chongqing, China, ^bEpithelial Cell Biology Research Centre, School of Biomedical Sciences, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong

Key Words

CFTR • Cutaneous wound healing • MAPK • NF- κ B

Abstract

Background: CFTR is implicated in cutaneous wound healing although the underlying mechanisms are not fully understood. In other cell types, CFTR is reported to regulate MAPK/NF- κ B signaling. We undertook the present study to explore a possible role of CFTR in regulating MAPK/NF- κ B during cutaneous wound healing. **Methods& Results:** The splint-excisional and incisional wound healing models were used in CFTR mutant (DF508) mice. The cell-scratch model was used in a human keratinocyte line, HaCaT, in conjunction with CFTR knockdown or overexpression. The epidermal inflammation, keratinocyte proliferation and differentiation, as well as MAPK/NF- κ B signaling were examined. Inhibitors of MAPK/NF- κ B were also used. **Results:** Both DF508 mice and HaCaT cells with CFTR knockdown exhibited delayed cutaneous wound healing with exuberant inflammation, increased proliferation and aberrant differentiation. Knockdown of CFTR in HaCaT cells resulted in phosphorylation of ERK, p38 and I κ B α . The disturbance of inflammation, proliferation and differentiation in HaCaT cells were reversed by CFTR overexpression or inhibition of MAPK or NF- κ B. **Conclusion:** CFTR plays a role in suppressing MAPK/NF- κ B to relieve inflammation, reduce proliferation and promote differentiation of keratinocytes, and thus promotes cutaneous wound healing.

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Introduction

Cutaneous wound healing is a highly orchestrated and complex process involving multiple types of cells in the epidermis and dermis [1, 2]. Among them, the keratinocyte, a major cellular component of the epidermis, plays an important role in inflammation, proliferation and differentiation during epidermis repair after injuries [1]. A broad variety of molecules and signaling pathways are identified to be important to cutaneous wound healing

J. Chen and Y. Chen contributed equally to this work.

Yizhi Peng
and Ye Chun Ruan

State Key Laboratory of Trauma, Burns and Combined Injury, Chongqing Key Laboratory for Proteomics Disease, Institute of Burn Research, Southwest Hospital, the Third Military Medical University, Chongqing (China) and Epithelial Cell Biology Research Centre, School of Biomedical Sciences, Faculty of Medicine, the Chinese University of Hong Kong, (Hong Kong), E-Mail yizhipen@sina.com / ruanyechun@cuhk.edu.hk

[3-5]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinase (MAPK), two key signaling pathways involved in inflammation [6, 7], cell proliferation [8, 9] and differentiation [8-14] in many cell types and tissues, have also been implicated in cutaneous wound healing [3, 4]. However, the molecular mechanisms underlying cutaneous wound healing remain unclear.

Cystic fibrosis transmembrane conductance regulator (CFTR), is a cAMP-dependent anion channel, mutations in which cause cystic fibrosis (CF), an autosomal recessive disease [15, 16]. Of the identified 1,900 mutations of CFTR in humans, deletion of the phenylalanine at position 508 (DF508) is the most frequent mutation found in CF patients, which results in a folding defect and endoplasmic reticulum associated degradation of CFTR [17]. It is being recently recognized that apart from transporting anions, CFTR is involved in other cellular functions, including inflammation [6, 18], proliferation [19-21], and differentiation [22-24] in bronchial epithelia, granulosa cells, enterocytes, and osteoblasts. Several signaling molecules associated with CFTR have been reported, including MAPK and NF- κ B [6, 18, 25-28]. We have previously demonstrated that in the airway, CFTR plays a role in suppressing MAPK/NF- κ B signaling in pulmonary inflammation induced by thermal inhalation injury [29].

Interestingly, the expression of CFTR in the epidermis in both humans [30] and mice [31] have been reported. In a recent study, CFTR has been demonstrated to promote the formation of cellular-junctions in human keratinocytes and thus be beneficial for wound repair [31]. Using an excisional wound healing model, the same study has also shown that mice carrying DF508 mutation had delayed wound healing as compared to the wildtypes [31]. However, whether and how CFTR is involved in inflammatory or cell proliferation signaling(s) during wound healing remain elusive. Given the observed role of CFTR in regulation of MAPK/NF- κ B-mediated inflammation in other systems, we hypothesized that epidermal CFTR may also play a role in epidermal inflammation, proliferation and differentiation during cutaneous wound healing by regulating MAPK/NF- κ B signaling pathway. In the present study, DF508 mutant mice were also used to assess their wound repair. Different from the previous study [31], we used a splinting excisional wound healing model, in which wound contractions are restrained to better mimic wound healing in humans [32]. In addition, we also used an incision wound model in DF508 mice as well as a human epidermal keratinocyte cell line (HaCaT) in conjunction with knockdown or overexpression of CFTR.

Materials and Methods

Animals and procedures

The *cfr*^{tm1Kth} (DF508) mice were purchased from the Jackson Laboratory [31] and kept at the animal facility of the Third Military Medical University. Male adult DF508 mice were used for cutaneous wound healing models. All procedures were approved by the Animal Ethical Committee of the Third Military Medical University and were carried out in accordance with the approved guidelines. Mice were anesthetized, shaved at the back and sterilized with 70% alcohol. Excisional full-thickness skin wounds were made on the dorsal skin using a sterile disposable biopsy punch with a diameter of 5mm. The wounds were immediately covered with a biological membrane as splint to restrain wound contractions. In another set of experiments, two full thickness incisional wounds (1cm long each) were cut in parallel with equal distance (0.5cm) to the midline. Animals were individually caged to avoid possible traumatic damage to the wounds. Pictures were taken every day after the surgical injuries with a piece round paper (5mm diameter) placed next to the wound as a size reference. The size of the wounds was measured by Adobe Photoshop software to calculate wound closure rate.

Cell culture

HaCaT cells were grown in Calcium-free RPMI-1640 medium (HYCLONE, Cat#SH30809.01) with 10% fetal bovine serum in CO₂ (5%) incubator at 37 °C [33]. To induce differentiation, CaCl₂ (1mM) was supplemented into the culture medium to incubate HaCaT cells for 48 hours. Primary mouse keratinocytes were isolated and cultured as previously described [34]. In some experiments, Bay-11 (Cat#B5556, Sigma,

ST. Louis, USA), PD98059 (Cat#P215, Sigma) or SB203580 (Cat#S8307, Sigma) were added to the culture media.

Cell scratch model

HaCaT cells were plated in 24-well plates and grown till confluence. A line gap, mimicking an incisional wound *in vivo*, was made at the center of each well by scratching using a sterile 200 μ l pipette tip. Afterwards, cells were washed with PBS for three times before fresh growth medium was added. Pictures of cells were taken by phase-contrast microscopy (OLYMPUS, Japan) every hour after the scratch for 20-25 hours. NIH ImageJ image software was used for analysis.

CFTR knock-down and overexpression

CFTR was knocked down using hammerhead ribozymes (conjugated in pEF6/V5-His vector) targeting at a specific GUC or AUC site to degrade CFTR mRNA as described previously [29]. To overexpress CFTR, pEGFPC3 plasmid carrying wild-type human CFTR (a gift from Professor Tzyh-Chang Hwang, University of Missouri-Columbia, USA) was used. Empty pEF6/V5-His or pEGFPC3 vectors was used as controls for knockdown or overexpression experiments, respectively. Cells were transfected with 4 μ g plasmids using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time RT-PCR (QRT-PCR)

QRT-PCR was carried out using a cDNA Synthesis Kit (Cat#K1622, Thermo Scientific, Fremont, USA) and the SGExcelUltraSYBR Mixture (Cat#SK2956A, Sangon Biotech, Shanghai, China). $\Delta\Delta$ Ct was calculated and statistically analyzed. The sequences of primers used were as follows: CFTR: TGCCCTTCGGCGATGTTT (forward), GCGATAGAGCGTTC CTCCTTG (reverse); COX-2: TGTGTTGACATCCAGATCAC (forward), ACATCATGTTTGAGCCCTGG (reverse); β -actin: CATGTACGTTGCTATCCAGGC (forward), CTCCTTAATGTCACGCACGAT (reverse); IL-8: GGTGGAGTTTGCCAAGGAG (forward), TTCCTTGGGGTCCAGACAGA (reverse); IL-10: GACTTTAAGGGTTACCTGGGTTG (forward), TACCATGCGCCTTGATGTCTG (reverse); CALM5: GGTTGACACGGATGGAACG (forward), ACTCCTGGAAGCTGATTTTCGC (reverse); LOR: GCTCTCATGATGCTACCCGA (forward), TGGGGTTGGGAGGTAGTTGT (reverse); IVL: TCCTCCAGTCAATACCCATCAG (forward), CAGCAGTCATGTGCTTTTCCT (reverse); FLG: TGAAGCCTATGACACCACTGA (forward), TCCCCTACGC-TTCTCTGTCTCT (reverse).

Western blot

Western blot was carried out as previously described [29]. Antibodies used in this study were listed as follows: CFTR (1:200, Cat#ACL-006, Alomone labs), PCNA (1:1000, Cat#D120014, BBI life Sciences), FLG (1:1000, Cat#ab24584, Abcam Inc.), LOR (1:1000, Cat#ab24722, Abcam Inc.), IVL (1:1000, Cat#ab53112, Abcam Inc.), P-Erk1/2 (1:1000, Cat#4370, Cell Signaling Technology), Erk1/2 (1:1000, Cat#4695, Cell Signaling Technology), P-p38 MAPK (1:1000, Cat#4511, Cell Signaling Technology), p38 MAPK (1:1000, Cat#9212, Cell Signaling Technology), P-I κ B α (1:1000, Cat#2859, Cell Signaling Technology), I κ B α (1:1000, Cat#4814, Cell Signaling Technology), Tubulin (1:500, Cat#10759-1-AP, Proteintech, Chicago, USA) and β -actin (1:1000, Cat#60008-1-Ig, Proteintech).

Histological analysis

Tissues were fixed in 4% PFA, paraffin-embedded and sectioned. A hematoxylin and eosin (H&E) kit was purchased from Sangon Biotech (Cat#E607318-0200). Experiments were performed according to manufacturer's instructions. Histological wound width was quantified by measuring the distance between the leading edges of epidermis across the wound bed. Relative inflammatory infiltration in 8 animals per genotype (2 randomly chosen wound edge fields and 2 randomly chosen wound bed fields per animal) was semi-quantitatively evaluated by three blinded reviewers. Epithelial thickness was quantified by using the measure function of the Adobe Photoshop software.

Immunohistochemistry

For immunohistochemistry, after antigen retrieval, sections were stained overnight at 4°C with PCNA (1:100 diluted, BBI life Sciences). After washing, sections were stained with the secondary antibodies with peroxidase-conjugated anti-rabbit followed by rabbit peroxidase anti-peroxidase (Dako

Corporation, Carpinteria, CA) for 1 h at room temperature. After being washed, color was developed with 3,3-diaminobenzidine and counterstained with hematoxylin. Sections with the primary antibody omitted were used as a negative control.

Proliferation assay

Cells were plated in a 96-well plate at a density of 1×10^4 cells per well and a cell counting kit (SK6335-500T, BBI Life Science) was used to determine the proliferation rate of cells. Absorbance was measured at 450nm using an ELx800 Absorbance Microplate Reader (Bio-Tek Instruments, Winooski, VT).

PGE₂ and IL-8 ELISA

ELISA kits for human PGE₂ (ESK5090-96 T) and IL-8 (ESK5204-96 T) were purchased from Sangon Biotech and used according to manufacturer's instructions.

Statistical analysis

Data are the mean $\pm$ sem. Students'-test was used when comparing two groups of variables. Two Way or One-way ANOVA followed by post-tests was used for multiple-groups comparisons. All tests were two-tailed, and statistical significance was set at $P < 0.05$.

Results

Delayed cutaneous wound healing in DF508 mice

We first made excisional wounds on dorsal skin of mice, which were immediately covered by a biological membrane as a "splint" to restrain contractions during the repair as previously reported [32]. In wildtype (+/+) mice, the wounds were gradually closed up by ~90% within 7 days. With the same injury, the DF508 (-/-) mice showed delayed wound healing with significantly lower wound closure rate at day 3, 5 and 7 (Fig. 1a). For a better histological assessment of wound healing [35], we made incisional skin injury to the DF508 mice (Fig. 1b). H&E staining showed significant larger wound width (Fig. 1c and d) and lower re-epithelialization rate (Fig. 1e) in DF508 mice at day 3 and 5 post injury as compared to the wildtypes.

CFTR promotes keratinocyte wound healing in vitro

We next used an *in vitro* scratch wound healing model in HaCaT cells, an immortalized human keratinocyte line. A line gap in HaCaT cultures was made by a scratch, resembling a "wound", which was gradually filled up with migrating cells. Within 20 hours, the scratched gap was closed by 60-70%. In presence of a selective CFTR inhibitor (CFTRinh172, 10μM), the closure rate at 20 hours was significantly lower than cells treated with DMSO as a vehicle control (Fig. 2a). To confirm this, we knocked down CFTR expression in the cells by transfecting CFTR-targeting ribozymes. QRT-PCR and western blot assay confirmed ~75% knockdown of CFTR (Fig. 2b). With such knockdown of CFTR, cells showed significantly lower closure rate than control cells (Fig. 2c). To further confirm the involvement of CFTR, we transfected the cells with EGFP3 plasmid carrying human CFTR, which resulted in robust increase in CFTR expression (Fig. 2d). With such overexpression of CFTR, HaCaT cells showed significantly greater closure rate at 25 hours as compared to control cells (Fig. 2e).

CFTR deficiency enhances epidermal inflammation

The production and release of cytokines and chemokines by keratinocytes to recruit inflammatory leukocytes (i.e. neutrophils and macrophages) are the early events upon skin injuries, whereas over-inflammation may impair the healing process [36]. Since CFTR has been reported to suppress inflammation in other cell types (such as airway epithelial cells) [6, 37, 38], we tested whether CFTR is associated with inflammatory responses during skin injury and repair. We first examined leukocytes infiltration in the incisional wound in wildtype (+/+) and DF508 (-/-) mice. At 10 hours, 3 and 5 days after the incisional injury, significantly

Fig. 1. Delayed cutaneous wound healing in DF508 mice. **a)** Representative photograph of excisional wound and quantification of wound closure rate in DF508 (-/-) or wildtype (+/+) mice at day 0-7 post-surgical injury. The rate of wound closure was defined as the percentage of closed wound area size versus the initial wound area size. ***: $P < 0.001$, $n = 6$, Two-way ANOVA. **b)** Representative photograph of incisional wound of DF508 (-/-) or wildtype (+/+) mice at day 0-7 post injury. **c)** Hematoxylin and eosin staining of incisional wounds of DF508 (-/-) or wildtype (+/+) mice at day 3 and 5 post injury. The wound edges were marked by arrows. Bar = 200 μ m. **d-f)** Quantification of incisional wound width (**d**) re-epithelialization rate (**e**) in DF508 (-/-) or wildtype (+/+) mice at day 3 and 5 post injury. ***: $P < 0.001$, $n = 6$, Two-way ANOVA.

larger number of neutrophils and macrophages in the wound bed were observed in DF508 mice as compared to that in wildtypes (Fig. 3a). In HaCaT cells, we examined the production and release of inflammatory cytokines and chemokines that are associated with cutaneous wound healing. As shown in Fig. 3b-f, knockdown of CFTR led to elevations of COX-2/PGE₂ (Fig. 3b) and IL-8 (Fig. 3d). The effect was found more robust in the presence of TNF α (10 μ M), a factor increased immediately upon skin injuries [39]. Conversely, overexpression of CFTR significantly reduced COX-2 (Fig. 3c) and IL-8 (Fig. 3e) expression in HaCaT cells. Moreover, IL-10, an anti-inflammatory cytokine, was decreased by CFTR knockdown and increased by its overexpression (Fig. 3f).

Fig. 2. CFTR promotes keratinocyte wound healing *in vitro*. a) Representative bright-field images and quantification of wound closure rate in HaCaT cells in presence of CFTR inhibitor-172 (CFTRinh, 10 μ M) or DMSO as control 0-20 hours after a line scratch wound was made. b-c) QRT-PCR and western blot analysis of CFTR expression (b) and assessment of wound healing 0-20 hours after scratch (c) in HaCaT cells transfected with CFTR-knockdown ribosomes (CFTR-KD) and empty control6/V5-His vectors as negative control (Ctrl). *: $P < 0.05$. **: $P < 0.01$, $n = 4-5$, t-test. d-e) QRT-PCR and western blot analysis of CFTR expression (d) and assessment of wound healing 0-25 hours after scratch (e) in cells transfected with empty pEGFPC3 vectors (Ctrl) or pEGFPC3 vector conjugated with human CFTR to overexpress CFTR (CFTR-OE). ***: $P < 0.001$, $n = 3-4$, t-test.

CFTR deficiency increases keratinocyte proliferation

Delayed wound healing as observed both *in vivo* and *in vitro* might suggest altered keratinocyte proliferation. We therefore tested whether CFTR plays a role in keratinocytes proliferation. At day 5 post incisional wounding, significantly thicker epidermis and larger number of cells labeled with PCNA, a proliferation marker, in the epidermis near the wound were found in DF508 mice, as compared to the wildtypes (Fig. 4a). Moreover, in non-injured skin of DF508 mice, substantially thicker epidermis and more PCNA positive keratinocytes were also found, as compared to that of the wildtypes (Fig. 4b). Western blotting for PCNA in skin tissue homogenates also revealed higher expression level in DF508 as compared to the wildtypes (Fig. 4c). Consistently, knockdown of CFTR in HaCaT cells led to increased proliferation rate (Fig. 4d), whereas overexpression of CFTR resulted in decreased proliferation rate (Fig. 4e).

CFTR promotes keratinocyte differentiation

The observed delayed cutaneous wound healing with higher keratinocyte proliferation in DF508 mice or CFTR-knockdown HaCaT cells suggested that CFTR-defective keratinocytes may have differentiation defects in spite of higher proliferation. Indeed, significantly lower mRNA levels of keratinocyte differentiation markers including calmodulin 5 (CALML5),

Fig. 3. CFTR deficiency enhances epidermal inflammation. a) H&E staining and quantification of inflammatory cells in the wound bed of DF508 mice and wildtypes at 10 hours, day 3 and day 5 post injury. Bar=100 μ m. **: $P < 0.01$, ***: $P < 0.001$, $n = 3$, Two-way ANOVA. b) QRT-PCR for COX-2 and ELISA for PGE₂ in CFTR-KD or Ctrl cells with (+) or without (-) addition of TNF- α . **: $P < 0.01$, ***: $P < 0.001$, $n = 4-6$, Two-way ANOVA. c) QRT-PCR for COX-2 in CFTR-OE or Ctrl cells. *: $P < 0.05$, $n = 3$, t-test. d) QRT-PCR and ELISA for IL-8 in CFTR-KD or Ctrl cells with (+) or without (-) addition of TNF- α . ns: non-significant. ***: $P < 0.001$, $n = 3$, Two-way ANOVA. e) QRT-PCR for IL-8 in CFTR-OE or Ctrl cells. *: $P < 0.05$, $n = 3$, t-test. f) QRT-PCR for IL-10 in CFTR-KD, CFTR-OE or Ctrl cells. *: $P < 0.05$, **: $P < 0.01$, $n = 3$, t-test.

loricrin (LOR), filaggrin (FLG) and involucrin (IVL) were found in keratinocytes isolated from excisional wounds of DF508 mice as compared to those from the wildtypes (Fig. 5a). We next induced HaCaT cells differentiation by switching the cultures from Ca²⁺-free to Ca²⁺ (1mM)-containing medium. 48 hours after the Ca²⁺ switch, mRNA levels of CALML5, LOR, FLG and IVL were found significantly increased as compared to control cells, suggesting differentiation of HaCaT cells (Fig. 5b). In these Ca²⁺ treated cells, higher expression of CFTR was found as compared to control cells. Moreover, Ca²⁺ treated cells with CFTR knockdown exhibited significantly lower levels of LOR, CALML5, IVL and FLG as compared to control cells (Fig. 5c). Conversely, overexpression of CFTR increased the expression of these differentiation markers (Fig. 5d).

Fig. 4. CFTR deficiency increase keratinocyte proliferation. a) Immunohistochemistry staining for PCNA in skin tissues from DF508 (-/-) and wild-type (+/+) mice at day 5 after the incisional injury with epidermal thickness and number of PCNA positive cells quantified. Bars=100 μ m. ***: $P < 0.001$, $n = 4$, t-test. b) H&E staining (left top, Bars=200 μ m) and immunohistochemistry staining for PCNA (left bottom, Bars=100 μ m) with quantification (right) of epidermal thickness and number of PCNA-positive cells in uninjured skin of DF508 (-/-) and wild-type (+/+) mice. ***: $P < 0.001$, $n = 4$, t-test. c) Western blotting for PCNA in skin tissues from DF508 and wild-type mice. Protein extracts loaded in each lane were from skin tissues of four mice (+/+ or -/-) at equal amount of weight. $n = 4$. d-e) Cell proliferation assay in HaCaT cells treated with CFTR-KD (d) or CFTR-OE (e). ***: $P < 0.001$, $n = 5$, Two-way ANOVA.

CFTR regulates epidermal inflammation, proliferation and differentiation via MAPK/NF- κ B pathway

The above results suggested the involvement of CFTR in inflammation, proliferation and differentiation of keratinocytes during cutaneous wound healing. We next tested possible role of MAPK or NF- κ B in these processes, given their association with CFTR-deficiency reported in other cell types [6, 25, 27, 28]. Results showed that in absence or presence of TNF α (10 μ M), knockdown of CFTR in HaCaT cells induced phosphorylation of I κ B α , indicating activation of NF- κ B pathway. Also, ERK and p38, two MAPKs, were found to be phosphorylated/activated in CFTR knockdown cells with or without TNF α . We next examined the effects of inhibitors of NF- κ B (Bay-11), ERK (PD98059) and p38 (SB203580) on inflammation, proliferation and differentiation in HaCaT cells in conjunction of CFTR knockdown. Results showed that upregulation of COX-2 in CFTR-knockdown cells was significantly attenuated by PD98059 (20 μ M)-inhibition of ERK. Similarly, CFTR knockdown-induced IL-8 increase was significantly reduced by PD98059 (20 μ M) or SB203580 (20 μ M). More, the decrease in IL-10 as a result of CFTR knockdown was significantly reversed by either Bay-11 (40 μ M), PD98059 (20 μ M) or SB203580 (20 μ M) (Fig. 6b). HaCaT cells were next cultured in presence of these inhibitors and their proliferation was monitored daily after seeding. At day 4 and 5 post seeding, the enhanced proliferation in CFTR knock-down

Fig. 5. CFTR promotes keratinocyte differentiation. a) QRT-PCR analysis of CALM5, LOR, FLG and IVL in isolated epidermal cells of DF508 and wild-type mice. **: $P < 0.01$, ***: $P < 0.001$, $n = 4$, t test. b) QRT-PCR analysis of LOR, CALM5, IVL, FLG and CFTR in HaCaT cells treated with or without 1mM Ca^{2+} . **: $P < 0.01$; ***: $P < 0.001$, $n = 4$, t-test. c-d) QRT-PCR analysis of LOR, CALM5, IVL and FLG in HaCaT cells treated with CFTR-KD (c) or CFTR-OE (d) in presence of 1mM CaCl_2 to induce differentiation *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, $n = 3$, t-test.

cells was reduced by PD98059 (20 μM) (Fig. 6c). Moreover, the downregulated IVL, FLG and CALML5 in CFTR-knockdown cells were all reversed by either Bay-11 (40 μM), PD98059 (20 μM) or SB203580 (20 μM) (Fig. 6d).

Discussion

In summary, the present study has revealed a previously undefined role of CFTR in suppressing MAPK and NF- κ B activation in epidermal keratinocytes during cutaneous injury to relieve inflammation, reduce keratinocyte proliferation and promote differentiation for a better wound healing.

Results from present *in vivo* and *in vitro* models clearly suggest a beneficial role of CFTR in cutaneous wound healing. Consistent with a previous study [31] showing retarded wound healing in DF508 mutant mice, the present study using a splint-excisional wound model, which better mimics cutaneous wound healing in humans, has confirmed that defects in

Fig. 6. Involvement of MAPK/NF- κ B in CFTR-regulated keratinocyte function. a) Representative western blots and quantification for phosphorylated ERK (p-ERK), p38 (p-p38) and I κ B α (p-I κ B α) in HaCaT cells with or without CFTR-KD co-treated with or without TNF- α . *: P<0.05, **: P<0.01, n=3-5, t-test. b-d) QRT-PCR analysis of COX-2, IL-8 and IL-10 (b), cell proliferation assay (c) and QRT-PCR analysis of IVL, FLG, CALM5 and LOR (d) in HaCaT cells with or without CFTR-KD co-treated with DMSO, Bay11 (40 μ M), PD98059 (20 μ M) or SB203580 (20 μ M) respectively. *: P<0.05, **: P<0.01; ***: P<0.001, n=4, One-way ANOVA.

CFTR can result in impaired cutaneous wound healing. In addition, results from knockdown and overexpression of CFTR in HaCaT cells in conjunction of cell-scratch model have further confirmed an active role of keratinocyte CFTR in wound healing.

Multiple processes during cutaneous wound healing are suggested to be associated with CFTR as well as its regulated MAPKs and NF- κ B. First, the injury-induced inflammation, as indicated by infiltration of inflammatory cells to the wound, increases in inflammatory cytokines (COX-2/PGE₂, IL-8) in keratinocytes or downregulation of anti-inflammatory cytokine (IL-10), is overwhelmed under CFTR mutant or knock-down conditions (Fig. 3). This is consistent with reported over-inflammation in other organ systems such as the airway in CF patients [6]. Such an over-inflammatory state in keratinocytes is reversed by CFTR overexpression or inhibition of MAPKs (i.e. ERK or p38) (Fig. 6b). It thus suggests a role of CFTR in suppression of MAPK in keratinocytes upon injuries to prevent detrimental over-inflammation for a better wound healing. Different from the airway cells [6], NF- κ B in keratinocytes, though also activated by CFTR-deficiency and involved in IL-10 production (Fig. 6), does not seem to contribute to CFTR-deficiency-induced changes in COX-2/PGE₂ or IL-8, mechanisms underlying which await further investigation.

Second, keratinocytes proliferation is substantially enhanced by CFTR-deficiency. This is evident by largely thickened epidermal layer, more PCNA-positive keratinocytes observed in DF508 mice, as well as a higher proliferation rate with CFTR knockdown and a lower rate with its overexpression in HaCaT cell cultures (Fig. 4). Interestingly, CFTR knockdown-

accelerated proliferation was reversed by inhibition of ERK, but not NF κ B or p38, suggesting that activation of ERK, in addition to contributing to inflammation, also mediates the enhanced proliferation in CFTR-deficient keratinocytes (Fig 6c). However, using neonatal human keratinocytes (HEKn), Dong et.al showed comparable PCNA expression between CFTR-knockdown and control cells [31]. This might be due to different origins of HEKn and HaCaT, as HEKn cells are from neonatal human skin and HaCaT cells, from adult human skin [40]. Neonatal keratinocytes might have a very high proliferation rate that possibly compromises CFTR-induced effect. In adult DF508 mice and HaCaT cells, the present study suggests an active role of CFTR in suppressing keratinocyte proliferation.

Of note, the equilibrium between proliferating keratinocytes in the basal layer and their differentiation into spinous cells in the suprabasal layers of the epidermis is essential to the integrity and function of the epidermis. The alteration in keratinocyte proliferation induced by CFTR-deficiency may preclude keratinocyte differentiation or re-epithelialization required for the epidermis to be fully healed from injuries. Indeed, the differentiated keratinocytes markers including IVL, FLG, CAML5 and LOR are all downregulated in keratinocytes from DF508 mice or CFTR-knockdown HaCaT cells. This is consistent with previous studies that implicate CFTR in differentiation of different epithelial cell types, including keratinocytes [24, 30, 31]. Interestingly, the downregulation of these keratinocyte differentiation markers is largely reversed by inhibition of either NF- κ B, ERK or p38, suggesting NF- κ B and MAPK pathways in mediating the effect of CFTR on keratinocyte differentiation during wound healing. Notably, it was reported that p38, in particular p38-delta, suppresses ERK activity in inhibiting keratinocyte differentiation [13, 41]. However, in the present study, downregulation of differentiation markers in CFTR-knockdown cells was reversed by either PD98059, the ERK inhibitor, or SB203580, a selective inhibitor of p38-alpha and beta, suggesting inhibitory role of both p38 (alpha and beta) and ERK in keratinocytes differentiation under CFTR-deficient condition. Taken together, in addition to regulating tight-junction complex proteins required for epithelial differentiation as previously reported [24, 31], CFTR may additionally play a role in suppressing NF- κ B and MAPK pathways to promote re-epithelialization during cutaneous wound healing.

Similarly as observed presently in keratinocytes, the association of CFTR deficiency with MAPK or NF- κ B activation has been reported in other organ-systems [6, 18, 25-28, 42] with various mechanisms proposed such as increased oxidative stress [27]. However, the exact mechanisms underlying how CFTR suppresses MAPK and NF- κ B in keratinocytes during wound healing await further investigation.

Collectively, the present results have suggested a role of CFTR in keratinocytes in switching from inflammation/proliferation into differentiation/re-epithelialization to promote cutaneous wound healing. Of note, Psoriasis, an inflammatory skin disorder, is characterized by excessive inflammatory cytokines, keratinocyte hyper-proliferation as well as impaired differentiation[43], similarly as observed in CFTR-defective epidermis in the present study. Therefore, the present findings may suggest the potential of CFTR as a therapeutic target for such skin disease(s).

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Disclosure Statement

The authors of this article declare that there are no conflicts of interest.

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