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Research Article

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Melatonin influences the biological characteristics of keloid fibroblasts through the Erk and Smad signalling pathways

Shaobin Huang[1,2,3,†], Wuguo Deng[3,†], Yunxian Dong[1,†], Zhicheng Hu[1], Yi Zhang[4], Peng Wang[1], Xiaoling Cao[1], Miao Chen[3], Pu Cheng[1], Hailin Xu[1], Wenkai Zhu[5], Bing Tang[1,*] and Jiayuan Zhu[1,*]

[1]Department of Burn, First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China, [2]Department of Cosmetic and Plastic Surgery, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, China, [3]Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, China, [4]Department of Burn and Plastic Surgery, Affiliated Hospital of Nantong University, Nantong 226001, China and [5]Department of Obstetrics and Gynecology, School of Medicine, Stanford University, Stanford, CA 94305, United States

*Correspondence. Bing Tang, Email: tangbing@mail.sysu.edu.cn; Jiayuan Zhu, Email: zhujiay@mail.sysu.edu.cn

[†]These authors contributed equally to this work.

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Abstract

Background: Keloids are abnormal fibrous hyperplasias that are difficult to treat. Melatonin can be used to inhibit the development of certain fibrotic diseases but has never been used to treat keloids. We aimed to discover the effects and mechanisms of melatonin in keloid fibroblasts (KFs).

Methods: Flow cytometry, CCK-8 assays, western blotting, wound-healing assays, transwell assays, collagen gel contraction assays and immunofluorescence assays were applied to demonstrate the effects and mechanisms of melatonin in fibroblasts derived from normal skin, hypertrophic scars and keloids. The therapeutic potential of the combination of melatonin and 5-fluorouracil (5-FU) was investigated in KFs.

Results: Melatonin significantly promoted cell apoptosis and inhibited cell proliferation, migration and invasion, contractile capability and collagen production in KFs. Further mechanistic studies demonstrated that melatonin could inhibit the cAMP/PKA/Erk and Smad pathways through the membrane receptor MT2 to alter the biological characteristics of KFs. Moreover, the combination of melatonin and 5-FU remarkably promoted cell apoptosis and inhibited cell migration and invasion, contractile capability and collagen production in KFs. Furthermore, 5-FU suppressed the phosphorylation of Akt, mTOR, Smad3 and Erk, and melatonin in combination with 5-FU markedly suppressed the activation of the Akt, Erk and Smad pathways.

Conclusions: Collectively, melatonin may inhibit the Erk and Smad pathways through the membrane receptor MT2 to alter the cell functions of KFs, while combination with 5-FU could exert even more inhibitory effects in KFs through simultaneous suppression of multiple signalling pathways.

Key words: Keloid; Fibroblast, Melatonin, 5-fluorouracil, Erk, Smad; Signalling pathways; Hyperplasias; Phosphorylation

Highlights

- Melatoninpromoted cell apoptosis and inhibited cell proliferation, migration andinvasion, contractile capability and collagen production in KFs.
- Melatonininhibited the cAMP/PKA/Erk and Smad pathways through the membrane receptor MT2.
- Thecombination of melatonin and 5-FU remarkably promoted cell apoptosis andinhibited cell migration and invasion, contractile capability and collagenproduction in KFs.
- Melatoninin combination with 5-FU markedly suppressed the activation of the Akt, Erk andSmad pathways.

Background

Keloids are abnormal scar tissue that is caused by skin damage. The aesthetic problems caused by keloids, their accompanying clinical symptoms and scar contractures cause joint and limb dysfunction, which greatly affects patient quality of life. The most common treatments for keloids include corticosteroid injections, cryotherapy, surgical resection (because keloids recur in 45–100% of cases, postoperative adjuvant treatment is usually required), radiotherapy, laser therapy and local injection of imiquimod, botulinum toxin and 5-fluorouracil (5-FU). Multiple courses of treatment are typically necessary for the treatment of keloids, which places a tremendous financial and emotional load on sufferers [1–6].

Compared with normal wound healing, keloid fibroblasts (KFs) have enhanced proliferative ability, enhanced invasive ability and a lower apoptosis rate. Transforming growth factor-β1 (TGF-β1) is an indispensable cytokine for wound healing that can activate downstream Smad signalling, leading to fibroblast activation. Increased fibroblast activity leads to elevated production of extracellular matrix, especially collagen I and collagen III, and abnormal scar tissue formation. Antibodies against TGF-β1 can reduce scar tissue formation [7–13]. Studies have shown that the activation of the phosphoinositide 3-kinase/RAC-α serine/threonine-protein kinase (PI3K/Akt/mTOR) pathway can enhance KF cell proliferation, migration and invasion and promote the expression of collagen I and III [14–16]. Studies have also found that the phosphorylation of p44/42 MAPK (Erk1/2) in human keloid tissue is enhanced; therefore, inhibition of Erk phosphorylation may become a potential pharmacological target for the clinical treatment of keloids [17].

Melatonin is a tryptophan-derived indoleamine that stimulates antioxidant enzymes in tissues and has the ability to scavenge free radicals [18]. In mammals there are two types of melatonin receptors, MT1 and MT2. They are heterologous Gi/Go and Gq/11 protein-coupled receptors that can interact with downstream messengers such as adenylate cyclase (cAMP), guanylate cyclase (cGMP), phospholipase A2, phospholipase C and calcium and potassium channels. Studies have shown that melatonin can inhibit liver fibrosis and renal fibrosis [19,20], indicating that melatonin has antifibrotic effects. Currently, there is no research on the application of melatonin in keloids.

The malignant growth pattern of keloids is similar to that of tumours, and the antitumour chemotherapeutic drug 5-FU has also been reported to have been used for the treatment of keloids. Studies have shown that TGF-induced type I collagen gene 2 (COL1A2) expression in human fibroblasts is blocked by 5-FU. Moreover, 5-FU does inhibit the production of type I collagen [21–23]. The effect of 5-FU on collagen expression in fibroblasts needs to be investigated.

In this study, we attempt to investigate the combined inhibitory effects of 5-FU and melatonin in human KFs.

Methods

Fibroblast isolation and culture

Patients who had not been previously treated for keloids prior to surgical excision were included in the study. Using clinical criteria, i.e. a scar with a history of earlier local trauma and growth that has gone beyond the scar's border, the surgeon was able to identify keloids. Pathologists then confirmed the histology by examining tissue slices using the clinical criteria for a normal scar—light-coloured and flat [24–26]. The skin tissues of the normal control group were taken from circumcision patients without underlying diseases. Six keloid specimens, six normal scar specimens and six foreskin specimens from urology patients were collected (Table S1, see online supplementary material). Because margins for surgical removal of keloids are relatively narrow, the amount of tissue that can be used to extract normal fibroblasts (NFs) is very small. To ensure that sufficient NFs could be extracted, we used foreskin tissues as the source of NFs. To minimize the impact of cell heterogeneity due to differences in keloid biopsy sites and cell generations, intralesional keloid samples were used to extract KFs, and healthy cells at passages three to five were used in the experiments. The tissues were cut into small pieces $\sim$1 mm^3 in size, placed in 20 ml of 0.2% type I collagenase and digested at 37°C for 4–6 h. The digested mixture was filtered through a 100-mesh cell strainer, centrifuged and rinsed twice with serum-containing culture solution. Isolated cells were inoculated into a 25 cm^2 disposable culture flask, cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 g/ml streptomycin at a cell density of 10^6/ml and placed in a 37°C, 5% CO_2 incubator. This study was approved by the Medical Ethics

Committee of the Medical Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). Written informed consent was obtained from all participants.

Preparation of melatonin solution

Melatonin was purchased from MedChemExpress, Monmouth Junction, NJ, USA (HY-B0075, 10 mM/mL in Dimethyl sulfoxide (DMSO)).

Preparation of 5-FU solution

5-FU was purchased from MedChemExpress, Monmouth Junction, NJ, USA (HY-90006, 10 mM/mL in DMSO).

CCK-8 assay

Fibroblasts in a good growth state were plated in 96-well plates at a density of 4000 cells/200 μl/well, and six replicates were made for each group. Melatonin was added at final concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 2, 4 and 8 mmol/l in each group. 5-FU was added at final concentrations of 0, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2 and 4 g/l in each group. After 24, 48, 72 and 96 h, 10 μl of CCK-8 solution was added to each well and incubated for 1 h. Using a microplate reader, the optical density (OD) value at 450 nm was measured. Based on the OD value, the cell proliferation inhibition rate and the IC_{50} of the inhibitory concentration of melatonin or 5-FU on fibroblasts were calculated. The cell viability was calculated using the following formula: cell survival rate (%) = $[(As-Ab)/(Ac-Ab)] \times 100$. *A*s = absorbance of experimental wells (absorbance of wells containing cells, culture medium, CCK-8 and melatonin or 0.02% DMSO). *A*b = Absorbance of blank wells (absorbance of wells containing medium and CCK-8). *A*c = absorbance of control wells (absorbance of wells containing cells, culture medium and CCK-8).

Cell apoptosis

Fibroblasts in logarithmic growth phase were plated in six-well plates and placed in an incubator overnight. Melatonin was added to final concentrations of 0, 0.5, 1 and 2 mmol/l in each well, and the cells were incubated in a 37°C in a 5% CO_2 incubator for 72 h. After 72 h, both the cells in suspension and those attached to the plate were collected. Adherent cells were digested with 0.25% trypsin without ethylene diamine tetraacetie acid (EDTA) and centrifuged at 800 rpm for 5 min. The cells were then washed three times with phosphate buffered saline (PBS), centrifuged at 800 rpm for 5 min and resuspended at 1×10^5 cells/ml. Next, 100 μl of 1× binding buffer was added to the cell pellet, transferred to a flow cytometry tube, and 10 μl propidium iodide (PI) was added and incubated at room temperature in the dark for 10 min. Subsequently, 5 μl of Annexin V-FITC was added, mixed well and incubated at room temperature in the dark for 10 min. Then, 400 μl of PBS was added before flow cytometry to detect apoptosis. Three strains of fibroblasts were examined. Each experiment was repeated three times.

Western blot analysis

Western blotting was performed using antibodies (all obtained from Cell Signaling Technology unless otherwise indicated) directed against poly ADP-ribose polymerase (PARP) (5625; 1 : 1000), cleaved-PARP (9542; 1 : 1000), caspase-3 (9665; 1 : 1000), cleaved-caspase-3 (9662; 1 : 1000), caspase-9 (9508; 1 : 1000), cleaved-caspase-9 (2876; 1 : 1000), E-cadherin (3195; 1 : 1000), N-cadherin (13 116; 1 : 1000), vimentin (5741; 1 : 1000), Zonula occludens (ZO-1) (13 663; 1 : 1000), collagen I (39 952; 1 : 1000), collagen III (Proteintech; 22 734–1-AP; 1 : 1000); BRAF (9433; 1 : 1000), p-BRAF (2696; 1 : 1000), p-ERK (4370 T; 1 : 1000), ERK (4695 T; 1 : 1000), Akt (9272; 1 : 1000), p-Akt (9271; 1 : 1000), mTOR (2972; 1 : 1000), p-mTOR (2971; 1 : 1000), Smad2 (5339; 1 : 1000), p-Smad2 (3108; 1 : 1000), Smad3 (9523; 1 : 1000), p-Smad3 (9520; 1 : 1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma–Aldrich; SAB1405848; 1 : 6000). It is preferable to use GAPDH as an internal control for comparing the expression levels of multitissue and multicell samples since it is a metabolic protein with generally consistent expression in live tissues. GAPDH served as an internal control as reported [27,28]. For western blotting, cells were lysed with radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology) containing Phenylmethanesulfonyl fluoride (PMSF) (100:1, v/v) (Cell Signaling Technology) for 30 min. A bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific) was used to measure the total protein concentrations. Aliquots (40 μg) of total cellular protein were resolved using sodiumdodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (5–12%), electrotransferred to PVDF membranes and blocked with 5% skim milk (w/v) at room temperature for 1 h. The membranes were then incubated with primary antibodies on an orbital shaker at 4°C overnight, and secondary antibodies (Horseradish peroxidase (HRP)-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit) were added and incubated for 1 h at room temperature. Protein–antibody complexes were then detected using chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo, USA). Three strains of fibroblasts were examined. Each experiment was repeated three times.

Wound-healing assay

Fibroblasts in good growth conditions were inoculated into six-well plates and placed in an incubator until the cell density reached ~100%. The cells were scratched using a sterile 200 μl pipette tip and were washed three times with sterile PBS. The floating cells were washed away, pictures were taken under an inverted microscope to document the scratches at 0h and the site was recorded. DMEM with 1% BSA was used

to culture the cells to prevent the proliferation of fibroblasts. Melatonin was added to the wells at final concentrations of 0, 0.5, 1 and 2 mmol/l, the culture plates were placed in a 37°C in a 5% CO_2 incubator for 72 h, and wound pictures were taken under an inverted microscope at 0, 24, 48 and 72 h. ImageJ software was used to calculate the changes in the wound area. Three strains of fibroblasts were examined. Each experiment was repeated three times.

Transwell assay

The fibroblasts were digested and centrifuged and the culture medium was discarded. Then, 600 μl of medium containing 20% FBS was added to the lower chamber of the 24-well plate. Then, 200 μl of cell suspension was added to the Transwell chamber (the chamber was precoated with gel). The high nutritional content of the medium in the lower chamber should promote cell invasion. The number of cells entering the lower chamber reflects the invasion ability of cells. A total of 40,000 cells were seeded in each chamber. Melatonin was added to final concentrations of 0, 0.5, 1 and 2 mmol/l and the plates were incubated in an incubator for 48 h. After 48 h, the Transwell chamber was removed and the culture solution was discarded. After the upper layer of nonmigrated cells was wiped off with a cotton swab, the insert was fixed with paraformaldehyde for 30 min. The cells were stained with 0.1% crystal violet for 30 min and washed three times with PBS to remove crystal violet. A cotton swab was used to gently wipe off moisture from the chamber. The number of cells in five visual fields was counted immediately under 10X magnification. Three strains of fibroblasts were examined. Each experiment was repeated three times.

Collagen gel contraction assay

The cells were digested and fibroblasts were collected at a density of 5×10^5 cells/ml. Then, 900 μl of 5 mg/ml rat tail collagen was added to 54 μl of 0.1 mol/l NaOH and mixed. Immediately, 100 μl of 10 × DMEM culture medium was added and mixed again. Then, 2 ml of cell suspension was added and mixed. The mixture was dispensed into three sterile centrifuge tubes and melatonin was added to final concentrations of 0, 0.5, 1 and 2 mmol/l. Each sample was seeded into a 24-well plate at 300 μl per well. The 24-well plate was placed in a 37°C incubator for 30 min, at which point the collagen gel became visible. The gel was separated from the well wall along the edge with a sterile syringe needle, 200 μl of complete medium was added and the gel was transferred to a 37°C, 5% CO_2 incubator. Images of gel contraction were taken after 24 and 48 h and the rate of change in the gel area in each well was measured using ImageJ software. Three strains of fibroblasts were examined. Each experiment was repeated three times.

Immunofluorescence staining and imaging

Cells were digested and collected and KFs were resuspended at 2×10^5 cells/ml. One millilitre of the cell suspension was seeded in a Petri dish containing glass discs for confocal microscopy and incubated with 100 μM L-ascorbic acid (MedChemExpress, Monmouth Junction, NJ, USA, HY-B0166, 10 mM/mL in DMSO) overnight. Melatonin was added to final concentrations of 0, 0.5, 1 and 2 mmol/l in each group and placed in an incubator for 48 h. The cells were then fixed with 4% paraformaldehyde for 20 min. Triton X-100 (0.5% in PBS) was added to the Petri dish to permeabilize the cells at room temperature for 20 min. After three washes with PBS, 5% goat serum was dropped onto the glass discs in the Petri dish to block nonspecific binding at room temperature for 40 min. The blocking solution was poured off and blotted with absorbent paper and 100 μl of primary antibody diluted in 5% goat serum was added dropwise to the glass disc in the Petri dish and incubated at 4°C overnight. The next day, the primary antibody was removed and 100 μl of fluorescent secondary antibody diluted with 1% goat serum was added onto the glass disc and incubated at room temperature for 1 h in the dark. Starting with the addition of a fluorescent secondary antibody, all subsequent steps were performed in the dark. After 4',6-diamidino2-phenylindole (DAPI) was added dropwise and incubated in the dark for 5 min, the dish was washed four times with PBST to wash away excess DAPI. The remaining liquid on the dish was absorbed with absorbent paper, an anti-fluorescence quencher was added dropwise and the images were captured under a fluorescence microscope. Three strains of fibroblasts were examined. Each experiment was repeated three times.

Enzyme-linked immunosorbent assay

TGF-β1 in the cell culture medium was detected using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Abcam; ab108912) according to the manufacturer's instructions. Six replicates were made for each group.

cAMP formation assay

The cAMP levels in the cell lysates were assayed according to the manufacturer's protocol (Abcam; ab65355). Six replicates were made for each group. The concentration of cAMP in the samples was then determined by comparing the OD value of the samples to the standard curve.

cGMP formation assay

The cGMP levels in the cell lysates were assayed according to the manufacturer's protocol (Abcam; ab65356). Six replicates were made for each group. The concentration of cGMP in the samples was then determined by comparing the OD value of the samples to the standard curve.

Protein kinase A (PKA) activity assay

PKA activity in the cell lysates was assayed according to the manufacturer's protocol (Abcam; ab139435). Six replicates were made for each group. The activity of PKA in the samples

was then determined by comparing the OD value of the samples to the standard curve.

Lentivirus and transfection

To generate cell lines with stable MT1 and MT2 knockdown, lentivirus-mediated short hairpin RNA (shRNA) (OBiO Technology, Shanghai, China) was used according to the manufacturer's instructions. Stable clones were selected after 2 weeks using 1 μg/ml puromycin, and the expression levels of MT1 and MT2 were determined using western blotting.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The data were evaluated with a two-tailed unpaired Student's t-test and the results are expressed as means ± SDs. The significant differences among multiple groups were compared using one-way analysis of variance (ANOVA) and the Newman–Keuls *post hoc* test. The combined effects of two factors were analysed with two-way ANOVA and Tukey's post test. $P < 0.05$ was considered statistically significant.

Results

Melatonin significantly inhibits the cell survival of KFs

Melatonin inhibited KF proliferation at $IC_{50} = 1.22 \pm 0.1$ mmol/l (72 h). Melatonin concentrations as low as 0.5 mmol/l were sufficient to inhibit cell survival and this effect became significantly prominent at 1–2 mmol/l. Furthermore, the effect of melatonin was observed in a time-dependent manner and the inhibitory effect on KFs became more prominent after 48 h (Figure 1a). The inhibitory effect of melatonin on normal scar fibroblast (NSF) survival was weaker (Figure S1a, see online supplementary material). For NFs, even when the melatonin concentration was increased or the duration of treatment was extended, melatonin hardly affected cell viability (Figure 1b). As previous research [29] and our CCK-8 research has suggested that DMSO did not affect cell growth, the melatonin solvent control (DMSO) was not used in subsequent experiments. When KFs and NFs were cultured in six-well plates, we could visually observe under the microscope that the cell density of KFs decreased with an increase in the melatonin concentration; at 1 and 2 mmol/l, the cell density of KFs decreased significantly, the cell morphology changed, the cells became slenderer and light transmission increased (Figure 1c). The cell density of NSFs decreased but cell morphology remained the same (Figure S1b, see online supplementary material). However, melatonin did not affect the cell density or cell morphology of NFs (Figure 1d).

Melatonin promotes apoptosis of KFs

When KFs were treated with melatonin at 0, 0.5, 1 and 2 mmol/l, the percentages of apoptotic cells were 1.77, 3.33, 29.12 and 32.45%, respectively. Clearly, the apoptosis of KFs was promoted significantly by melatonin at concentrations ≥1 mmol/l (Figure 1e). Moreover, melatonin also promoted the apoptosis of NSFs (Figure S1c, see online supplementary material). On the other hand, melatonin did not promote cell apoptosis in NFs (Figure 1f). Western blot analysis showed that after melatonin treatment, the expression of the apoptosis-related proteins c-PARP, c-caspase3 and c-caspase9 in KFs increased significantly in a dose-dependent manner (Figure 1g). However, for NSFs, the expression of apoptosis-related proteins was not significantly different (Figure S1d).

Melatonin inhibits KF migration and invasion

The wound-healing experiment results showed that melatonin significantly inhibited the migration of KF cells (Figure 2a). On the other hand, melatonin had no effect on the cell migration of NSFs and NFs, and NSFs and NFs were slower to fill in the scratch than KFs (Figure S2a, b, see online supplementary material). In the Transwell experiment, a significantly smaller number of KFs passed through the chamber in the presence of >0.5 mmol/l melatonin after 48 h of incubation compared to the control group (Figure 2b), while NFs could not invade (Figure S2c, see online supplementary material). Western blot results suggested that in KFs treated with melatonin, the expression of vimentin and N-cadherin decreased, while the expression of E-cadherin and ZO-1 increased, indicating that melatonin can inhibit the invasion and migration of KFs (Figure 2c). However, melatonin had no effect on the NSFs (Figure S2d, see online supplementary material) and NFs (Figure 2d).

Melatonin inhibits contractile capability and collagen production of KFs

The contractile capability of KFs was significantly weakened when the cells were incubated with >1 mmol/l melatonin for 24 and 48 h (Figure 2e). On the other hand, melatonin did not affect the contractile ability of NSFs (Figure S2e, see online supplementary material) and NFs (Figure 2f). Western blot results indicated that in the presence of melatonin the expression of type I collagen and type III collagen in KFs was significantly reduced compared to that in the control group (Figure 2g).

Melatonin inhibits the activation of the Erk pathway in KFs

Melatonin significantly inhibited the production of cAMP in KFs at ≥1 mmol/l (Figure 3a) but did not affect the production of cGMP (Figure 3b). In the presence of ≥1 mmol/l melatonin, the PKA activity of keloid cells also decreased significantly (Figure 3c). Western blots indicated that in the presence of melatonin, cAMP/PKA downstream kinase activity was also inhibited in KFs, and the expression of p-BRAF and p-Erk was reduced (Figure 3d). 6-Bnz-cAMP is a selective activator of PKA. 6-Bnz-cAMP significantly increased the activity of PKA in KFs even in the presence of melatonin (Figure 3e). The western blot results showed that regardless of whether

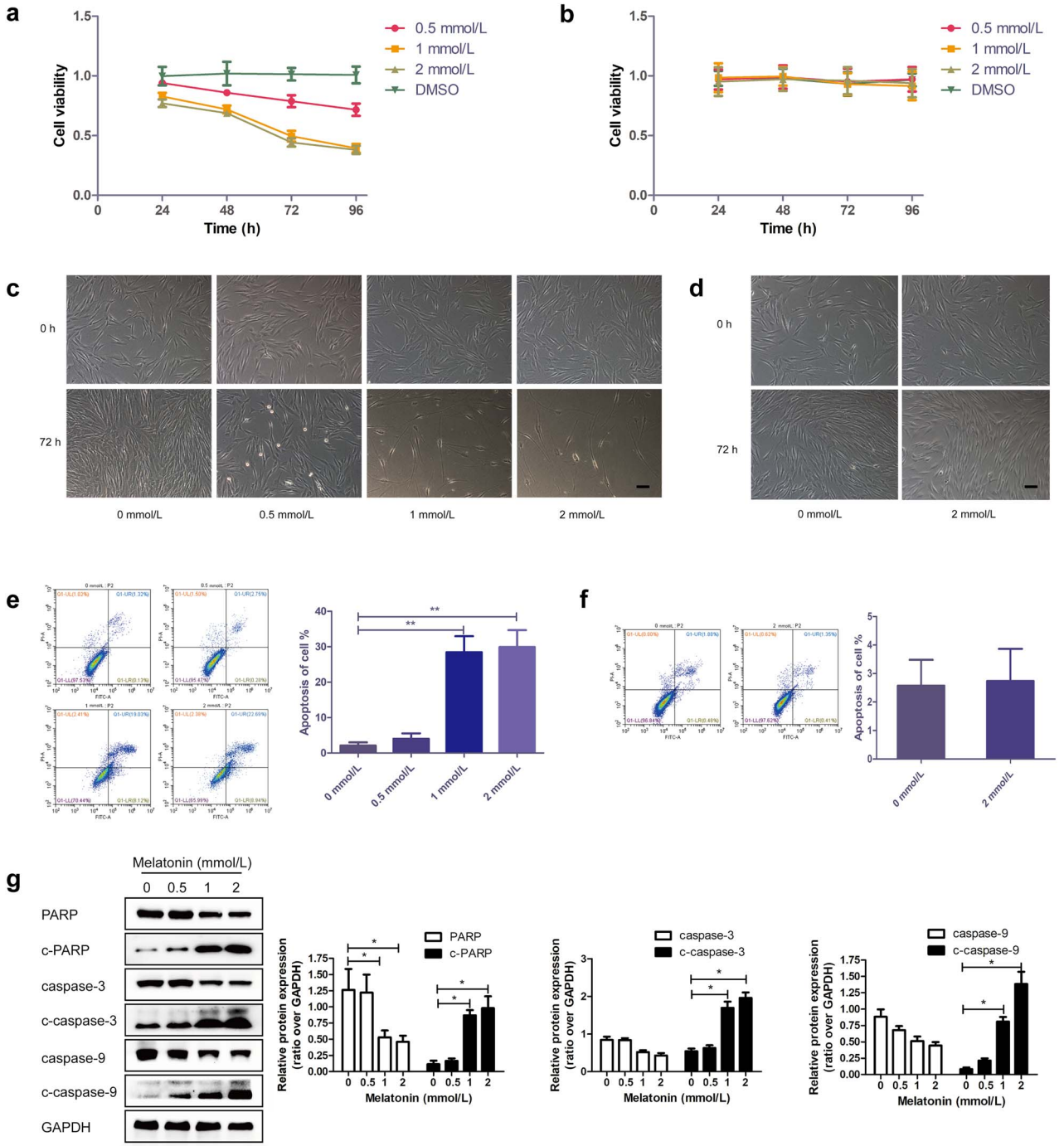

Figure 1. Melatonin specifically induced morphological changes, reduced the viability and promoted the apoptosis of keloid fibroblasts (KFs) but not normal fibroblasts (NFs). (**a**) KFs and (**b**) NFs were treated with melatonin at the indicated doses. At 24, 48, 72 and 96 h after treatment, the cell viability was determined with a CCK-8 assay ($n = 6$). Changes in the cell morphology of KFs (**c**) and NFs (**d**) at 72 h after melatonin treatment were imaged under light microscopy. Apoptosis of KFs (**e**) and NFs (**f**) was determined using flow cytometry on Annexin V and propidium iodide (PI) double-positive cells after 72 h of treatment with melatonin ($n = 3$). (**g**) After 72 h of treatment with melatonin the levels of cleaved PARP (c-PARP), cleaved caspase 3 (c-caspase-3) and cleaved caspase 9 (c-caspase-9) in KFs were examined using western blotting ($n = 3$). Scale bar: 100 μm. Data are the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$

melatonin was present the expression of p-BRAF and p-Erk increased upon the addition of 6-Bnz-cAMP. Overall, we conclude that melatonin can influence the cAMP/PKA/BRAF/Erk pathway in KFs (Figure 3f).

Melatonin inhibits activation of the Smad pathway in KFs

First, melatonin had no effect on TGF-β1 secretion (Figure 4a). TGF-β1 showed no effects on the production of

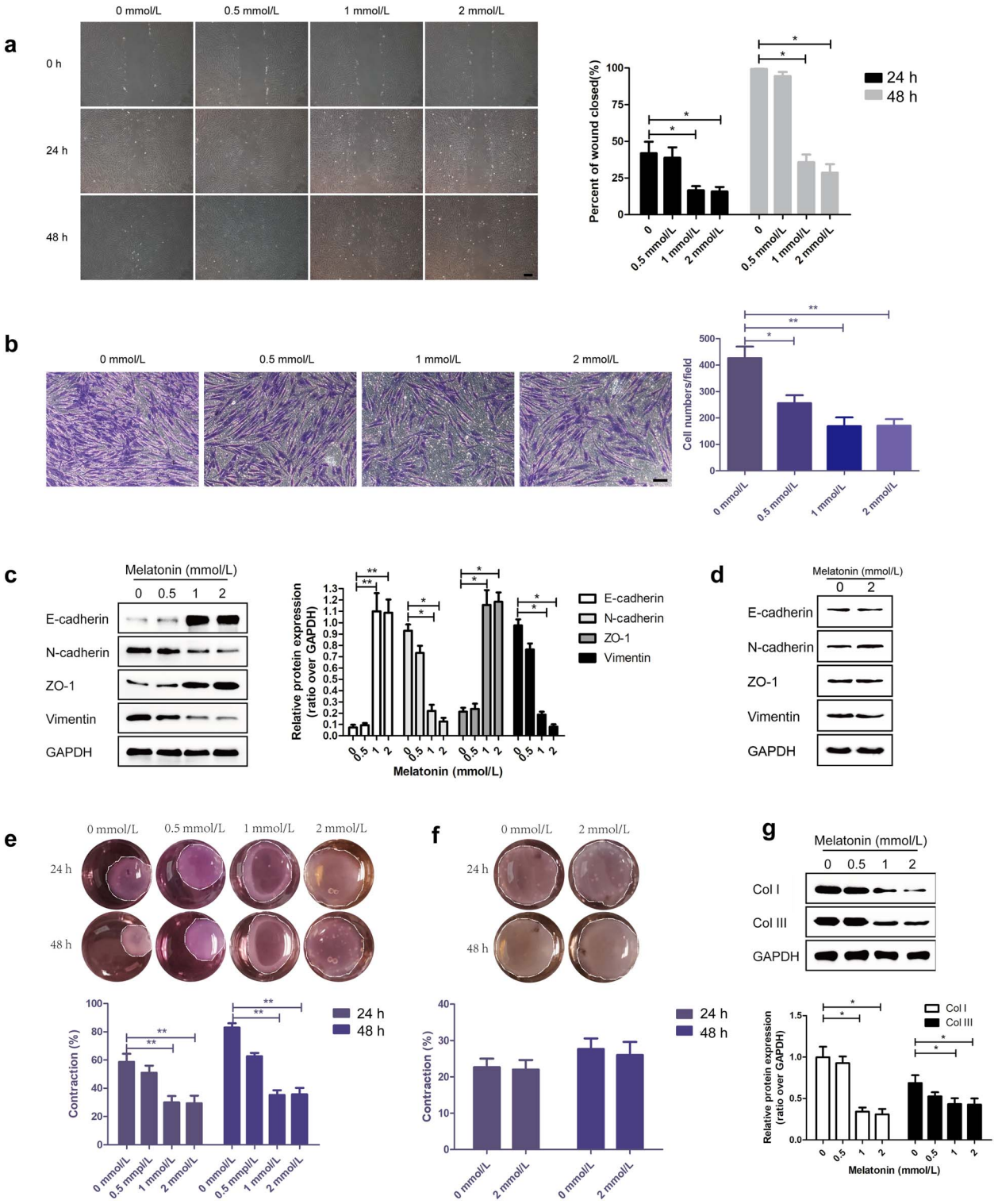

Figure 2. Melatonin reduced the migratory, invasive and contractile capability of KFs. (**a**) After a scratch was made in confluent KF monolayers (0 h), cells were treated with melatonin at the indicated concentrations. Migration is shown at 24 and 48 h. (**b**) In the transwell assay, KFs were treated with melatonin at the indicated concentrations for 48 h and the number of cells passing through the chamber was counted. After 72 h of treatment with melatonin, the levels of vimentin, N-cadherin, E-cadherin and ZO-1 in (**c**) KFs and (**d**) NFs were examined using western blotting. (**e**) KFs and (**f**) NFs were mixed with neutralized collagen in the presence of melatonin at the indicated concentrations and the collagen gel was imaged at various time points up to 48 h. (**g**) KFs were treated with melatonin at the indicated concentrations for 72 h. The expression levels of collagen I and III were detected using western blotting. Scale bar : 100 μm. Data are the mean ± SD, $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$. *KFs* keloid fibroblasts, *NFs* normal fibroblasts

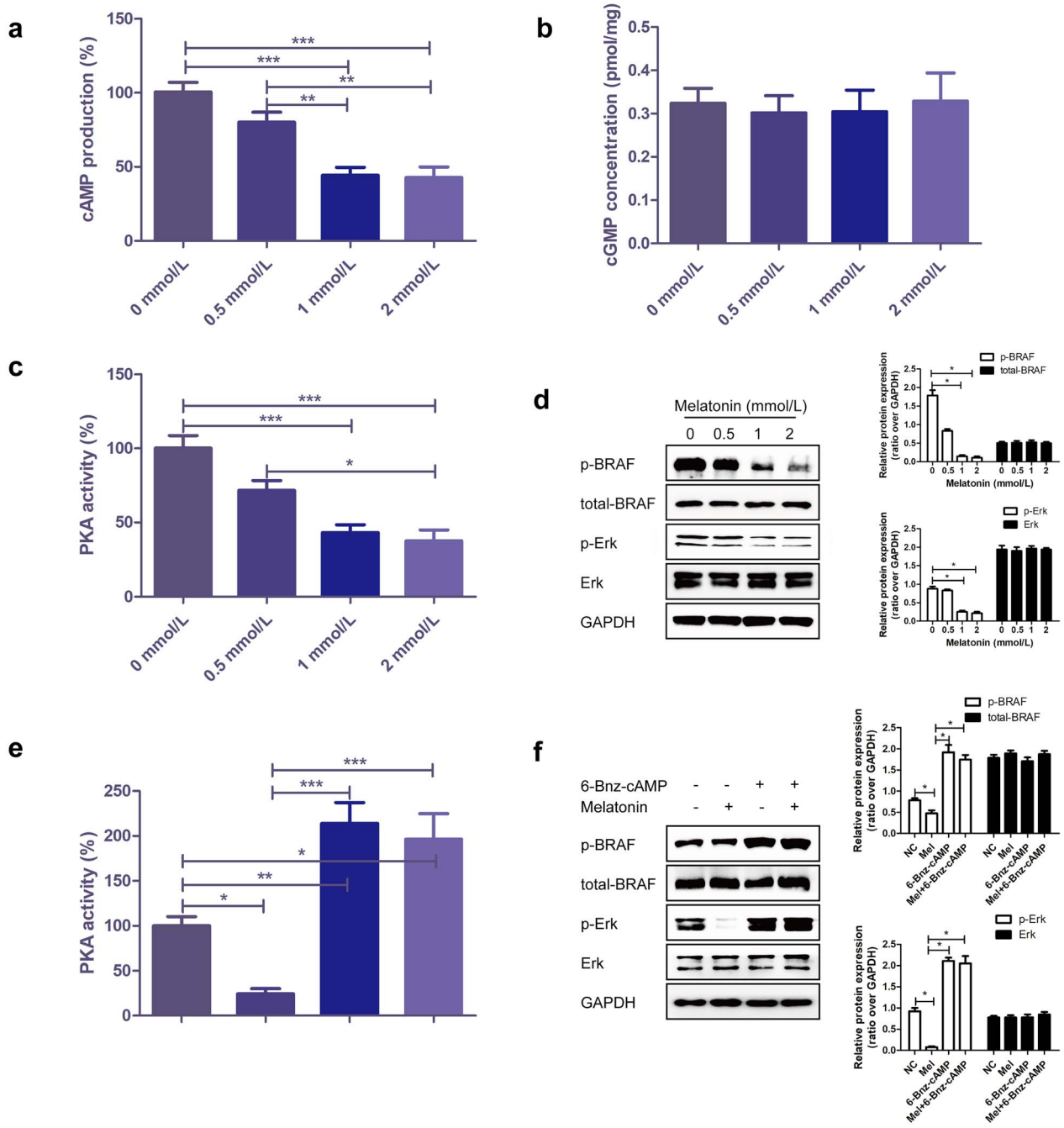

Figure 3. Melatonin inhibits activation of the Erk pathway in KFs. (**a**) cAMP and (**b**) cGMP production of KFs in the presence of melatonin at the indicated concentrations for 72 h ($n = 6$). (**c**) PKA activity of KFs in the presence of melatonin at the indicated concentrations for 72 h ($n = 6$). (**d**) After 72 h of treatment with melatonin, the levels of p-BRAF and p-Erk were examined using western blotting ($n = 3$). (**e**) PKA activity of KFs after cells were treated with melatonin or 6-Bnz-cAMP alone or their combination at the indicated doses for 72 h ($n = 6$). (**f**) After 72 h of treatment with melatonin or 6-Bnz-cAMP alone or their combination, the levels of p-BRAF and p-Erk were examined using western blotting (n = 3). Data are the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *KFs* keloid fibroblasts, *PKA* protein kinase A

cAMP in KFs (Figure 4b) and the activity of its downstream PKA was also not affected (Figure 4c). Upon TGF-β1 treatment, the expression of p-BRAF and p-Erk increased, and melatonin inhibited the increase in p-BRAF and p-Erk expression induced by TGF-β1 (Figure 4d). Western blot results indicated that TGF-β1 upregulated the expression of p-Smad2 and p-Smad3 as well as p-Akt and p-mTOR, and melatonin inhibited the expression of p-Smad2 and p-Smad3 induced by TGF-β1 but had no effect on the expression of p-Akt or p-mTOR (Figure 4e). Next, we explored the relationship between cAMP/PKA and the Smad pathway. Western blot results indicate that melatonin inhibits the

expression of p-Smad2 and p-Smad3 in KFs. Treatment with the PKA activator 6-Bnz-cAMP reversed the downregulation of p-Smad2 and p-Smad3 induced by melatonin treatment (Figure 4f). B-Raf IN 1 is a selective B-Raf inhibitor with an IC_{50} for KFs of 0.83 ± 0.1 μmol/l (72 h). When 1 μmol/l B-Raf IN 1 was administered to KFs, western blot results showed that B-Raf IN 1 did not inhibit the upregulation of p-Smad2 and p-Smad3 induced by 6-Bnz-cAMP (Figure 4g). The above results indicate that TGF-β1 can activate the Erk, Smad and Akt pathways in KFs, while melatonin can inhibit cAMP/PKA/Erk and cAMP/PKA/Smad pathway activation in KFs. Moreover, KFs could express the myofibroblast marker α-smooth muscle actin (α-SMA) after induction with TGF-β1, and melatonin treatment suppressed the expression of α-SMA (Figure S3, see online supplementary material), which suggested that melatonin suppressed TGF-β1-induced fibroblast–myofibroblast transdifferentiation.

Melatonin inhibits the activation of the Erk and Smad pathways in KFs through the MT2 receptor

Western blot results showed that the expression of MT2 was significantly higher in KFs than in NSFs and NFs, but there was no significant difference in the expression of MT1 (Figure 5a). Next, we used lentiviruses to knock down the expression of MT1 and MT2 in KFs. We selected MT1 shRNA-2 and MT1 shRNA-3 to knock down MT1 for subsequent experiments and selected MT2 shRNA-1 and MT2 shRNA-2 for knockdown of MT2 (Figure 5b, c, and Figure S4, see online supplementary material). After MT1 knockdown, melatonin still inhibited cAMP production (Figure 5d) and its downstream PKA activity (Figure 5e). Western blot analysis showed that after the knockdown of MT1, melatonin still inhibited the expression of p-BRAF and p-Erk as well as the expression of p-Smad2 and p-Smad3 (Figure 5f). After the knockdown of MT2, however, melatonin no longer inhibited the production of cAMP in KFs (Figure 5g), and its downstream PKA activity was intact in the presence of melatonin (Figure 5h). Western blot analysis showed that after knocking down MT2, the expression of p-BRAF and p-Erk was not inhibited by melatonin, and similarly, the expression of p-Smad2 and p-Smad3 was no longer inhibited by melatonin (Figure 5i). From these observations, we conclude that melatonin can inhibit the cAMP/PKA/Erk pathway and Smad pathway through the MT2 receptor in KFs. Moreover, after knocking down the MT2 receptor, in addition to cell apoptosis, the ability of melatonin to inhibit KF migration, invasion, collagen contraction and collagen expression was significantly reduced (Figure S5, see online supplementary material).

Inhibitory effects of the combination of melatonin and 5-FU on keloid fibroblasts

The IC_{50} of 5-FU in KFs was 0.87 ± 0.1 g/l, and the IC_{50} of 5-FU and melatonin for KFs was 0.44 ± 0.1 g/l (72 h). A 5-FU concentration as low as 0.25 g/l was sufficient to inhibit cell survival and this effect became significantly prominent at 0.75–1 g/l. Furthermore, the effect of 5-FU was observed in a time-dependent manner and the inhibitory effect on KFs became more prominent after 48 h (Figure S6, see online supplementary material). Western blot results indicated that 5-FU inhibited the expression of p-Akt, p-mTOR, p-Smad3 and p-Erk. When administered in combination with melatonin, 5-FU further inhibited the expression of p-Smad3 and p-Erk (Figure 6a). Flow cytometry detection showed that the average percentages of apoptotic cells in the control, melatonin, 5-FU and combination groups were 2.44, 28.04, 13.61 and 42.44%, respectively. The percentage of apoptotic KFs in the combination group was significantly higher than that in the melatonin monotherapy or 5-FU monotherapy group (both $p < 0.05$) (Figure 6b). The wound-healing experiment results showed that after 24 h of treatment with melatonin and 5-FU, the closed scratch area of KFs was significantly smaller than that of the melatonin monotherapy or the 5-FU monotherapy group (both $p < 0.05$) (Figure 6c). The Transwell experiment results indicated that after 48 h of treatment with melatonin and 5-FU, the number of KFs passing through the chamber was significantly smaller than that in the melatonin monotherapy or the 5-FU monotherapy group (both $p < 0.05$). Treatment with a combination of melatonin and 5-FU significantly inhibited the invasion of KFs (Figure 6d). The collagen contraction experiment results indicated that after 24 and 48 h of treatment with melatonin and 5-FU, the collagen contractility of KFs was significantly weaker than that of the melatonin monotherapy or 5-FU monotherapy group (both $p < 0.01$) (Figure 6e). Western blot results indicated that in the presence of melatonin and 5-FU, the expression of type I collagen and type III collagen in KFs was significantly lower than that in the single drug groups (Figure 6f).

Discussion

The molecular mechanism of keloids is still poorly understood, and effective prevention and treatment are lacking. Fibroblasts are the key effector cells in the formation of keloids. The dysfunction of KFs during the formation of keloids is common, including abnormal proliferation, aggressive invasion and excessive deposition of extracellular matrix. Therefore, targeted regulation of KFs is a treatment strategy for keloids [30,31].

Initially, we found that melatonin can specifically act on KFs, promote cell apoptosis, and inhibit cell proliferation, migration and invasion, collagen contraction and collagen synthesis. Next, we further explored the differences in the phenotypes of KFs and NFs and studied the specific mechanism of melatonin-specific responses in KFs.

The effect of melatonin depends on the target cells and corresponding intracellular melatonin signalling pathways [31]. In mammals, cAMP functions through direct intracellular targets such as cAMP-dependent PKA. PKA is directly activated by cAMP [32–36]. In our experiment, we found that

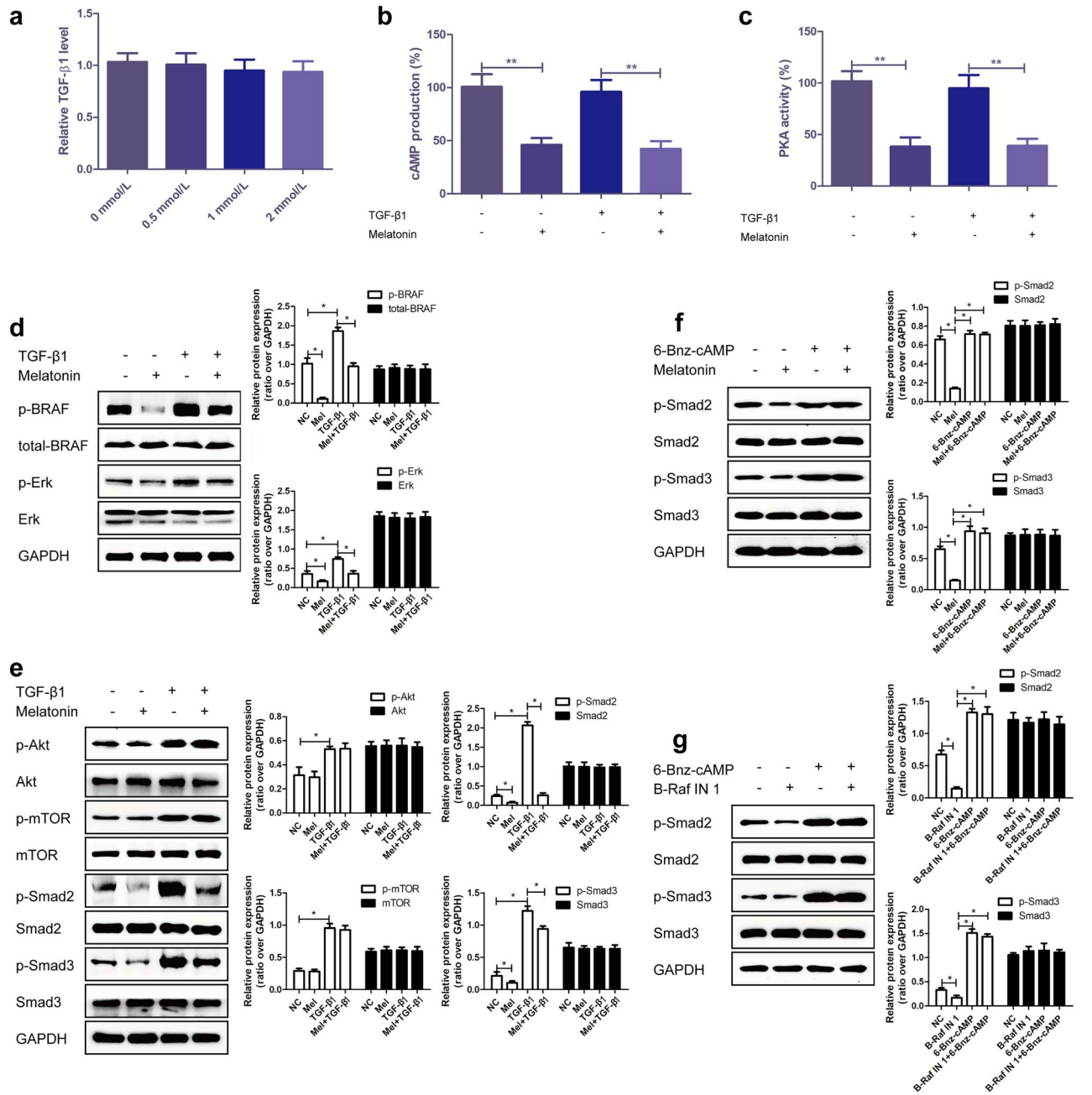

Figure 4. Melatonin does not affect Akt signalling but suppresses the Smad signalling pathway in KFs. (**a**) The effects of melatonin on TGF-β1 secretion ($n = 3$). (**b**) cAMP production and (**c**) PKA activity of KFs in the presence of melatonin or TGF-β1 alone or their combination for 72 h ($n = 6$). (**d**) After 72 h of treatment with melatonin or TGF-β1 alone or their combination, the levels of p-BRAF and p-Erk were examined using western blotting ($n = 3$). (**e**) After 72 h of treatment with melatonin or TGF-β1 alone or their combination, the levels of p-Akt, p-mTOR, p-Smad2 and p-Smad3 were examined using western blotting ($n = 3$). (**f**) After 72 h of treatment with melatonin or 6-Bnz-cAMP alone or in combination, the levels of p-Smad2 and p-Smad3 were examined using western blotting ($n = 3$). (**g**) After 72 h of treatment with B-Raf IN 1 or 6-Bnz-cAMP alone or in combination, the levels of p-Smad2 and p-Smad3 were examined using western blotting ($n = 3$). Data are the mean $\pm$ SD. $^{*}P < 0.05$; $^{**}p < 0.01$. *KFs* keloid fibroblasts, *TGF-β1* transforming growth factor-β1, *PKA* protein kinase A, *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase

melatonin inhibited cAMP production in KFs, thereby inhibiting the activation of PKA. In addition, melatonin inhibited the phosphorylation of BRAF and Erk. After treatment with a PKA agonist, melatonin reversed the downregulation of BRAF and Erk phosphorylation. Therefore, our experiments prove that melatonin affects the biological characteristics of KFs by inhibiting the activation of the cAMP/PKA/Erk pathway.

TGF-β1 is considered an important inducer of keloid formation [37,38]. KFs show significant sensitivity to TGF-β1 stimulation [39,40]. The signalling pathways involved in TGF-β1 include Smad-dependent and non-Smad-dependent

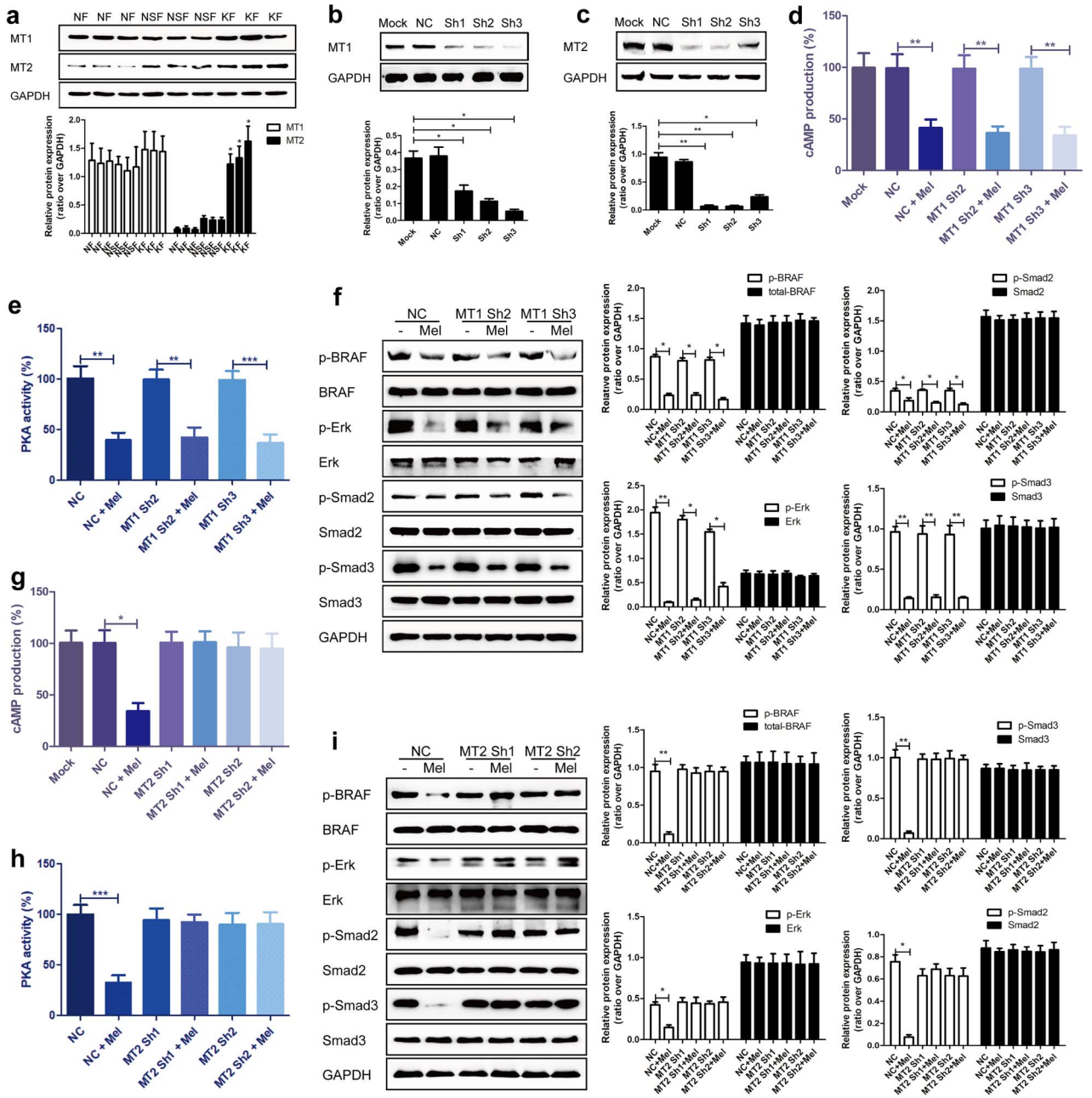

Figure 5. Melatonin acts on KFs through MT2. (**a**) The protein levels of MT1 and MT2 were detected using western blotting in NFs and KFs ($n = 3$). (**b**) KFs were transfected with negative control RNA (NC) or MT1-targeted shRNAs, and the levels of MT1 were examined using western blotting ($n = 3$). (**c**) KFs were transfected with negative control RNA (NC) or MT2-targeted shRNAs, and the levels of MT2 were examined using western blotting ($n = 3$). (**d**) cAMP production of KFs in different groups ($n = 6$). (**e**) PKA activity of KFs after transfection with negative control RNA (NC) or MT1-targeted shRNAs ($n = 6$). (**f**) After transfection with negative control RNA (NC) or MT1-targeted shRNAs, the levels of p-BRAF, p-Erk, p-Smad2 and p-Smad3 were examined using western blotting ($n = 3$). (**g**) cAMP production of KFs in different groups ($n = 6$). (**h**) PKA activity of KFs after transfection with negative control RNA (NC) or MT2-targeted shRNAs ($n = 6$). (**i**) After transfection with negative control RNA (NC) or MT2-targeted shRNAs, the levels of p-BRAF, p-Erk, p-Smad2 and p-Smad3 were examined using western blotting ($n = 3$). Data are the mean ± SD. $^{*}P < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *KFs* keloid fibroblasts, *PKA* protein kinase A

signalling pathways. Smad-dependent signalling pathways are considered to be the main pathway of TGF-β1 signalling. TGF-β receptor kinase phosphorylates Smad2 and Smad3, stimulates the formation of Smad2/3/4 complex and induces the translocation of the complex into the nucleus. According to reports in the literature, the expression of phosphorylated Smad2 and Smad3 in KFs is increased compared to that in NFs. The nonclassical (independent of Smad) pathway of TGF-β1 also affects various biological functions of cells. Studies have shown that many signalling pathways, including the MAPK/Erk, p38, JNK, NF-κb and PI3K/Akt signalling pathways, can be affected by TGF-β1, and their activation

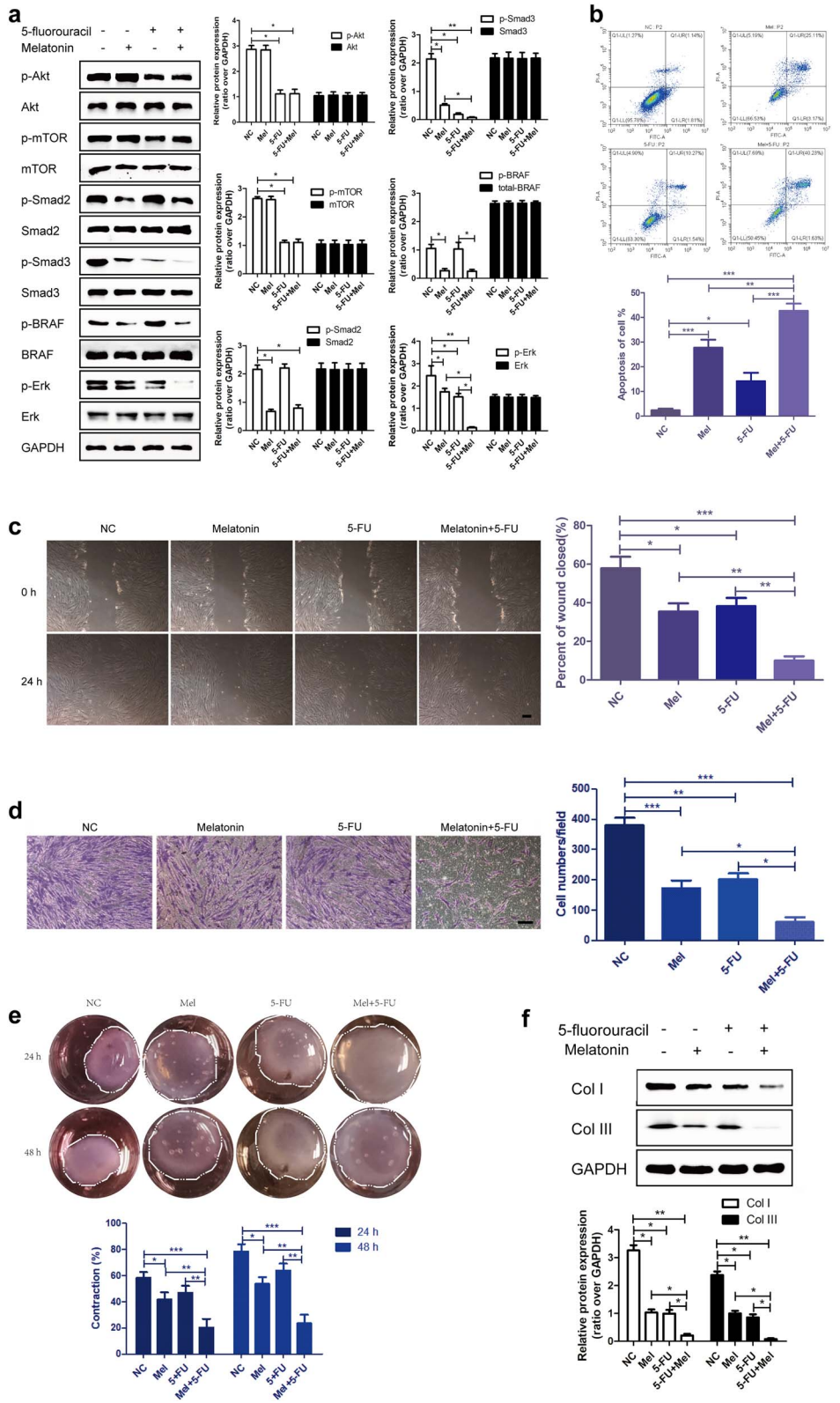

Figure 6. Inhibitory effect of combined melatonin and 5-FU on KFs. (a) After 72 h of treatment with melatonin or 5-FU alone or in combination, the levels of p-Akt, p-mTOR, p-BRAF, p-Erk, p-Smad2 and p-Smad3 were examined using western blotting. (b) The apoptosis of KFs was determined using flow cytometry on Annexin V and propidium iodide (PI) double-positive cells after 72 h of treatment with melatonin or 5-FU alone or in combination. (c) After a scratch was made in the confluent KF monolayer (0 h), cells were treated with melatonin or 5-FU alone or in combination. Migration is shown at 24 h. (d) In the transwell assay, KFs were treated with melatonin or 5-FU alone or their combination for 48 h, and the number of cells passing through the chamber was counted. (e) KFs were mixed with neutralized collagen in the presence of melatonin or 5-FU alone or their combination, and the collagen gel was imaged at various time points up to 48 h. (f) KFs were treated with melatonin or 5-FU alone or their combination for 72 h. The expression levels of collagen I and III were detected using western blot. Scale bar: 100 μm. Data are the mean ± SD, $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *KFs* keloid fibroblasts, *5-FU* 5-Fluorouracil

depends on the cell type and microenvironment [41–45]. In our study, we found that TGF-β1 upregulated the expression of p-BRAF and p-Erk, p-Smad2 and p-Smad3, and p-Akt and p-mTOR in KFs. Melatonin inhibited the TGF-β1-induced increase in p-BRAF, p-Erk, p-Smad2 and p-Smad3 expression but showed no effect on the expression of p-Akt or p-mTOR. Therefore, the experimental results show that TGF-β1 can activate the Erk, Smad and Akt pathways in KFs, while melatonin can inhibit the cAMP/PKA/Erk and Smad pathways.

Melatonin works through different molecular pathways. The most classic approach is the activation of membrane-specific receptors, where activation of G protein-coupled receptors (including MT1 and MT2) can inhibit cAMP in target cells [46]. In our present research, we found that the expression of MT2 in KFs was significantly higher than that in NFs, but there was no significant difference in the expression of MT1. After knocking down MT2, the production of cAMP in KFs was no longer inhibited by melatonin, and its downstream PKA activity was not inhibited by melatonin nor p-BRAF, p-Erk, p-Smad2 or p-Smad3. Therefore, we believe that melatonin can specifically act on the MT2 receptor and inhibit the cAMP/PKA/Erk pathway and the Smad pathway in KFs. Certain studies have proven that melatonin receptors are widely expressed in scar fibroblasts but are not expressed in fibroblasts of normal skin. Furthermore, melatonin might inhibit inflammation in scars by suppressing the PI3K/Akt and ERK signalling pathways [47]. Our research is consistent with previous studies and further explores the changes in specific pathways. Research has also found that melatonin can prevent renal interstitial fibroblasts from transdifferentiating into myofibroblasts by inhibiting reactive oxygen species (ROS)-mediated mechanisms in a receptor-independent manner [48]. Our transcriptome sequencing results suggest that the effect of melatonin on KFs may also occur in a receptor-independent manner. We will further verify the possible receptor-independent mechanism in future experiments. The effect of melatonin on the body has diurnal variations. However, there are no ideal animal models to study keloids [25,49]. We tried to build keloid animal models by implanting keloid scar tissue into subcutaneous pockets in immunodeficient mice, but the survival of keloid tissue was unstable. With the development of tissue engineering, animal models with features of human keloid scars might be built to study keloids.

As a common antitumour drug, there are many studies on the mechanism of action of 5-FU in the treatment of tumours. In colorectal cancer, studies have shown that 5-FU inhibits the expression of p-Akt and promotes the apoptosis of cancer cells. Additional research showed that melatonin significantly enhanced the inhibitory effect of 5-FU on the proliferation, colony formation, migration and invasion of colon cancer cells. Mechanistically, melatonin enhanced the antitumour effect of 5-FU by inhibiting the PI3K/Akt and NF-κB/iNOS signalling pathways [50,51]. 5-FU inhibited the expression of p-Erk in the pancreatic cancer cell line PANC-1, promoted apoptosis and inhibited cell migration [52]. Treatment of the melanoma cell line SK-MEL-2 with 5-FU promoted apoptosis, and the expression of p-Smad2 and p-Smad3 was downregulated [53]. In our experiment, we found that 5-FU inhibited the expression of p-Akt, p-mTOR, p-Smad3 and p-Erk in KFs, and in combination with melatonin, it further inhibited the expression of p-Smad3 and p-Erk. Moreover, compared with the melatonin monotherapy and 5-FU monotherapy groups, melatonin combined with 5-FU promoted KF apoptosis and inhibited cell migration, collagen contraction and collagen production much more significantly.

Conclusions

In summary, melatonin can inhibit the proliferation of KFs, promote cell apoptosis, inhibit cell migration and invasion and collagen contraction and inhibit the expression of collagen. Mechanistically, melatonin can inhibit the cAMP/PKA/Erk and Smad pathways through the membrane receptor MT2 of KFs. When melatonin and 5-FU were combined to treat KFs, 5-FU was found to inhibit p-Akt and p-mTOR, and compared with 5-FU monotherapy and melatonin monotherapy, 5-FU combined with melatonin further inhibited p-Erk and p-Smad3. For cell biological characteristics, compared with the single drug treatment, the combination drug treatment promoted apoptosis and inhibited cell migration and invasion, collagen contraction and collagen expression more robustly. Our study provides new insights into the molecular mechanisms by which melatonin affects keloids and suggests that melatonin and 5-FU combination treatment might potentially become a more effective keloid therapy.

Abbreviations

5-FU: 5-Fluorouracil; KF: Keloid fibroblasts; NF: Normal fibroblasts; TGF-β1: Transforming growth factor-β1; DAPI,4',6-diamidino2-phenylindole; PBS, Phosphate buffered saline; shRNA, short hairpin RNA; ANOVA, Analysis of variance; DMEM, Dulbecco's modified eagle medium; OD, Optical density; Half maximal inhibitory concentration (IC50), Half maximal inhibitory concentration; EDTA, Ethylene diamine tetraacetie acid; Fluorescein isothiocyanate (FITC), Fluorescein isothiocyanate; PARP, Poly ADP-ribose polymerase; PKA, Protein kinase A; DMSO, Dimethyl sulfoxide; ZO-1, Zonula occludens 1; α-SMA, α-smooth muscle actin; ROS, reactive oxygen species; Inducible nitric oxide synthase (iNOS), Inducible nitric oxide synthase; PMSF, Phenylmethanesulfonyl fluoride; BCA, Bicinchoninic acid; SDS-PAGE, Sodiumdodecyl sulphate-polyacrylamide gel electrophoresis; HRP, Horseradish peroxidase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

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Authors' contributions

JZ, WD and BT designed the research and acquired funding; SH and ZH isolated and cultured the KFs and NFs; SH, WD, ZH, PW, YZ, XC and MC carried out most of the experiments and drafted the manuscript; YD participated in data analysis; PC and HX performed the western blot analysis; and WZ participated in reviewing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). Written informed consent was obtained from all participants.

Data availability

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

Conflicts of interest

None declared.

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