Targeting the Akt/PI3K/mTOR signaling pathway for complete eradication of keloid disease by sunitinib

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Abstract
Keloid disease is a nodular lesion, tumor-like but not cancerous, and characterized of excessive proliferation of fibroblasts and deposition of extracellular matrix (ECM) components. This condition often causes itching, pain and cosmetic disfigurement, significantly reducing patient quality of life. To date, no universally effective therapies are available, possibly due to inadequate understanding of keloid pathogenesis. As an oral small-molecule inhibitor of certain tyrosine kinase receptors, sunitinib has shown significant therapeutic effects in renal cell carcinoma (RCC) and gastrointestinal stromal tumor (GIST). However, it has never been tested if keloid therapy can be effective for the management of keloids. This study thus aims to explore the potential of sunitinib for keloid treatment. Keloid-derived fibroblasts (KFs) were successfully isolated and demonstrated proliferative advantage to normal skin-derived fibroblasts (NFs). Additionally, sunitinib showed specific cytotoxicity and inhibition of invasion, and induced cell cycle arrest and significant apoptosis in KFs. These effects were accompanied by complete suppression of ECM component expression, including collagen types 1 and 3, upregulation of autophagy-associated LC3B and significant suppression of the Akt/PI3K/mTOR pathway. Moreover, a keloid explant culture model was successfully established and used to test the therapeutic efficacy of sunitinib on keloid formation in nude mice. Sunitinib was found to induce complete regression of keloid explant fragments in nude mice, showing significantly higher therapeutic efficacy than the most commonly used intralesional drug triamcinolone acetonide (TAC). These data suggest that sunitinib effectively inhibits keloid development through suppression of the Akt/PI3K/mTOR pathway and thus can be potentially developed as a monotherapy or combination therapy for the effective treatment of keloid disease.

Keywords Keloid · Keloid fibroblasts · Sunitinib · Akt/PI3K/mTOR pathway · Keloid explant model

Introduction
Currently regarded as benign skin lesions, keloid diseases (KDs) are commonly caused during disrupted dermal wound healing processes [1, 2]. This condition is characterized by dysregulated proliferation of dermal fibroblasts and excessive deposition of extracellular matrix (ECM) components and has a high recurrence rate after surgery. Keloids grow and develop like tumors beyond the boundaries of the original wound and frequently cause severe pain and itching [3, 4]. The current treatments include surgery, cryotherapy, radiotherapy and intradermal injections of chemotherapeutic agents, such as bleomycin and 5-fluorouracil [5]. However, it is very difficult to cure KDs with any of these available
therapies due to their limited efficacy and a high frequency of disease recurrence. Therefore, improvements to KD treatment are needed.

It has been shown that the pathogenesis of keloids is closely linked to fibroblast dysfunction [6]. Keloid fibroblasts (KF) are the specific fibroblasts in keloid conditions and are thought to play a key role in the formation of keloids. In addition, various cytokines, such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), are thought to promote keloid formation [7–9, 14].

Gene alterations, protein overexpression or activation of the phosphatidylinositol-3-kinase (PI3K), v-Akt murine thymoma viral oncogene homolog 1 (Akt) and mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) pathway components are frequent manifestations in many types of cancer, such as prostate cancer [10, 11], breast cancer, ovarian cancer, lung cancer, acute myeloid leukemia, and glioblastoma [15]. These changes often result in dysregulated cell growth, proliferation, mobility, survival, apoptosis and angiogenesis and contribute to tumorigenesis and progression and drug resistance in cancers. Therefore, this pathway may be targeted in tumor therapy and actually extensive investigations are undergoing to develop effective cancer treatments through targeting these pathway elements. Blockade or suppression of this pathway has become an attractive strategy for controlling of tumor development and progression.

Sunitinib (SU11248) is an oral small-molecule inhibitor targeting over 80 kinases [12], such as vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGF-Rs), Fms-like tyrosine kinase 3 (FLT3), stem cell factor receptor kinase (c-KIT) and glial cell-derived neurotrophic factor receptor kinase (RET) [13]. Sunitinib has been shown to target the JNK and MEK/ERK signaling pathways, leading to reduced tumor angiogenesis, cell proliferation, and apoptosis and tumor shrinkage in thyroid cancers [16]. Sunitinib has been approved for treating refractory gastrointestinal stromal tumor (GIST) and advanced renal cell carcinoma (RCC) due to observed robust clinical responses of these two tumors [17]. As a promising antitumor candidate drug, sunitinib is now being tested for treating an extensive range of solid tumors [18]. Considering the great potential of this drug for both kinase inhibition and tumor treatment, we hypothesize that sunitinib may suppress the PI3K/AKT/mTOR pathway in KDs and is possibly a candidate therapeutic agent for the management of the refractory skin lesions.

We sought to investigate the effects of sunitinib on KF proliferation, apoptosis and 3D migration in vitro and also its ex vivo therapeutic efficacy in an organ culture model of keloid in mice in this study. Essential signaling molecules were examined for changes of expression levels or phosphorylation in the PI3K/AKT/mTOR pathway to elucidate the action mechanisms by which sunitinib attenuates the development of keloids.

**Materials and methods**

**Collection of keloid samples**

Eight freshly excised keloid samples were collected from six patients (three males and three females ranging from 12 to 52 years old) who had not received any preoperative treatment and gave informed consent. These keloid tissues were taken from the chest or ear of the patients.

**Isolation and culturing of fibroblasts from normal skin and keloid biopsy samples**

The keloid tissues were aseptically excised from the chest and ears of patients who gave informed consent and were treated with localized anesthesia and collected in 50-mL centrifuge tubes to isolate KFs, followed by washing three times in phosphate-buffered saline (PBS). After removal of epidermal tissues from the samples, the remaining dermal tissues were cut into small pieces with a scalpel and incubated in 0.3% collagenase in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C with shaking for 6 h. Subsequently, the treated tissues were squashed to release detached cells and filtered with 100 µm cell strainers. The obtained cell suspension was centrifuged at 1200 rpm for 5 min, and then, the cell pellets were resuspended for use in cell culture with DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. The isolated cells were grown until reaching 80% confluence, then washed, trypsinized in 0.25% trypsin–EDTA, collected by centrifugation and designated as passage 0 (P0), and subsequently frozen and stored in liquid nitrogen. Some of the P0 cells were plated for subsequent cell culture and experiments, and cells grown from the P0 population were regarded as P1. Cells in the third to fifth passages (P3–P5) were used in cellular experiments. Normal human skin fibroblasts (NFs) were isolated from human foreskin tissue, which was obtained with informed consent following the above-described KF isolation and culturing procedures.

**Cell lines and cell cultures**

All cell culture agents were obtained from Gibco (Life Technologies, Gaithersburg, MD, USA) unless otherwise stated. Human KFs and NFs were isolated separately, and 293 T cells, human adipose tissue-derived mesenchymal stem cells (ADSCs), and bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from Fuheng Biology.
(Shanghai, China). All cell lines were grown and cultured in DMEM supplemented with 10% FBS in a humidified atmosphere containing 5% CO₂ at 37°C.

**Chemical reagents**

Sunitinib (SU11248) was purchased from MedChemExpress (China), dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution and stored at -20°C under light-proof condition until use. In the experiments described below, the final concentration of DMSO did not exceed 0.1% (v/v), which is not toxic to KFs. Triamcinolone acetonide (TAC) was obtained from APExBIO (China), dissolved in DMSO at 14.3 mg/mL to produce a stock solution, and used as the positive control for keloid treatment.

**Cell proliferation assay**

In vitro cellular viability and proliferation assays were carried out with Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) to examine the growth and proliferative potential of KFs and NFs or to test the cytotoxicity of sunitinib on KFs and normal cell lines, including NFs, 293 T cells, ADSCs and BMSCs. Passages 3–5 of NFs and KFs were tested. Cells were seeded in 96-well plates (2 × 10³/well in 100 µl of complete culture medium) and allowed to settle down for overnight. Subsequently, cells were subjected to the CCK-8 test or starved in serum-free medium for 24 h and then treated with vehicle or sunitinib at the concentrations of 2.0–6.0 µM, or at 0.0–100.0 µM for 24 h, respectively, followed by CCK-8 assay.

Assays were carried out in triplicate and repeated for at least 3 times. The optical density (OD) values were measured at 450 nm with a microplate reader (model 2300, PerkinElmer, Boston, MA, USA), and the survival cell percentage is calculated using the equation: cellular viability (%) = (ODsample - ODblank)/(ODcontrol - ODblank) × 100. The data are presented as the means ± SD (n = 3).

**Cellular 3D migration/invasion assay**

The migration/invasion assay was performed to examine the 3D cell migration potential/invasiveness using Matrigel (BD Biosciences, UK) and Transwell inserts with permeable polycarbonate membranes (8.0-µm pore size) (Corning Inc., NY) based on a previous description [19] but with some modifications. Briefly, 40 µl Matrigel was added into each Transwell insert, and 2 × 10⁴ cells were seeded on the top of Matrigel reconstructed matrix, followed by supplementation of serum-free DMEM with or without drugs above Matrigel and 10% FBS containing DMEM in the lower chamber. The cells were allowed to migrate through the Matrigel matrix and invade to the bottom of the porous membrane for 3 days. Subsequently, the Matrigel matrix was removed, and cells that had migrated through the matrix and attached to the membrane were regarded as invading cells, which were fixed with 4% paraformaldehyde, stained with crystalline violet, imaged and quantitated. Each assay was carried out in triplicate.

**Cell cycle analysis**

Passage 3 (P3) of KFs were cultured and treated with vehicle or 6 µM sunitinib for 24 h, harvested by trypsinization, washed with cold PBS, and fixed overnight with ice-cold 70% ethanol at 4 °C. Subsequently, the cells were pelleted by centrifugation at 2000×g for 8 min, washed twice with PBS, resuspended in cell cycle assay staining buffer containing 50 µg/mL propidium iodide (PI) and 100 µg/mL RNase A (Solarbio Life Sciences, Beijing, China), and incubated for 1 h at 37°C. The stained cells were pelleted by centrifugation at 2000×g for 10 min, washed with PBS, resuspended in PBS, and detected by flow cytometry (FCM). The fluorescence staining signal intensity was measured at a 480 nm excitation wavelength through an FL-2 filter. Finally, the FCM data were analyzed with FlowJo 7.6 software (Becton Dickinson, Ashland, OR, USA).

**Apoptosis assay**

KFs were seeded in 12-well plates at a density of 1 × 10⁵ cells/well, allowed to settle down for overnight, and then treated with vehicle or 6 µM sunitinib for 24 h. Then, the treated cells were harvested, washed and stained with FITC-Annexin V and PI (BestBio, Shanghai, China) for 30 min, and then the number of apoptotic cells was assessed by FCM (FACSCalibur; Becton Dickinson). Annexin V+/PI- and Annexin V+/PI+ cells were considered to have undergone early and late apoptosis, respectively, and Annexin V-/PI-cells were regarded as live cells.

**Real-time relative quantification PCR**

Real-time relative quantification PCR (RQ-PCR) was performed to study the effect of sunitinib treatment on cellular collagen I and III gene expression at the mRNA level in KFs. Total RNA was extracted with TRIzol reagent (Invitrogen, China) from cultured cells with or without sunitinib treatment. Then, 1 µg of total RNA was used to prepare cDNA for each sample using a Revert Aid First-Strand cDNA Synthesis Kit (Fermentas, Life Sciences, Canada). Subsequently, RQ-PCR was carried out with SYBR Premix ExTaq TM II reagent, 2 µl of cDNA per sample (0.1 µg) and collagen I- and III-specific primer sets (Supplementary Table-1). Reactions were carried out on an ABI PRISM® 7900HT system (Takara Biotechnology, Japan) following the manufacturer’s
instructions. A GAPDH primer set (Supplementary Table-1) was used as the endogenous control, and all PCRs were carried out in triplicate. The RQ-PCR data were analyzed using the $2^{-\Delta\Delta C_{T}}$ method [20], and relative mRNA expression levels were compared between the vehicle and sunitinib treatment KF groups.

**Immunoblotting assay**

Western blotting was carried out to detect the expression of the proteins of interest. To this end, cultured or treated cells were harvested, and cellular proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (MedChemExpress, China). Protein quantitation was performed by BCA assay. Then, 10 µg of cellular protein was separated by 7.5% SDS–PAGE for both mTOR and p-mTOR or by 12% SDS–PAGE for all other examined proteins. After electrophoresis, the proteins in the gel were transferred onto a PVDF membrane, which was then blocked with TBST buffer containing 5% skim milk. Then, primary antibody (Ab) incubation was performed overnight at 4 °C with antibodies against the following proteins: GAPDH (dilution 1:1000, AF0911-100, Affinity Biosciences), collagen I (dilution 1:1000, AF0134-50, Affinity Biosciences), collagen III (dilution 1:1000, AF0136-50, Affinity Biosciences), Akt (dilution 1:2000, 4691S, Cell Signaling Technology, Danvers, USA), p-Akt (dilution 1:2000, 4060S, Cell Signaling Technology, Danvers, USA), PI3K (dilution 1:2000, AB86714, Abcam, Cambridge, UK), mTOR (dilution 1:1000, 2972S, Cell Signaling Technology, Danvers, USA), and p-mTOR (dilution 1:1000, 5536S, Cell Signaling Technology, Danvers, USA), LC3B (dilution 1:1000, 43,566, Cell Signaling Technology, Danvers, USA). Subsequently, the membrane was washed in TBST 3 times and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (dilution 1:2500, ProteinTech, Chicago, IL, USA) for 1 h at room temperature. Each membrane was then washed in TBST again 3 times, developed with an enhanced chemiluminescence (ECL) detection kit (Merck Millipore), and imaged and photographed with BioImage Lab (Bio–Rad, Hercules, CA, USA). The immunoblots results were analyzed for expression quantification of the target proteins with ImageJ software (National Institutes of Health, Bethesda, MA, USA) and the values were normalized using GAPDH as the protein loading internal control. The expression level of each target protein is presented relative to the Ctrl level, for which the value was set to 1.0.

**Immunofluorescence assay**

Approximately $1 \times 10^{4}$ KFs and NFs per well were seeded in chamber slides and allowed to grow for 3 days. Then, the medium was removed from the wells, and the cells were washed twice with PBS and fixed overnight in 4% paraformaldehyde, stained for ki67 with a rabbit anti-human ki67 primary polyclonal Ab (AF0198, Affinity Bioscience) and a second AF594-conjugated goat anti-rabbit polyclonal IgG (H+L) Ab (Affinity Bioscience). The stained cells were examined and imaged with a confocal microscope (LSM800, Zeiss, Jena, Germany).

**In vivo study**

An in vivo study was performed using female Balb/c nude mice (4–6 weeks old) to evaluate the therapeutic efficacy of sunitinib on keloids. These mice were purchased from the SPF Biotechnology Company (Beijing, China). Animals were kept in pathogen-filtered conditions with filtered air, and sterilized water and food were provided all the time. To establish a keloid explant culture model, freshly excised keloids were cut into small fragments (~10 mm˟8 mm˟8 mm), and implanted under forelimb and hindlimb skin in mice, which were first anesthetized by intraperitoneal injection of 5% chloral hydrate. The keloid fragments were implanted approximately 1 cm from skin cut sites, and then, the cut was closed with a 5.0 suture. Three limb sites were implanted with keloid fragments in each mouse, and 9 mice were tested in total, producing a test sample number of 9 for each group.

One-week post-implantation, the mice were allocated to three treatment groups: the vehicle control, sunitinib and TAC groups. As a positive therapeutic control, TAC was intralesionally injected at a dose of 2.0 mg/0.1 mL/kg, as described in a previous report. Sunitinib was administered by intralesional injection at 100.0 mg/0.1 mL/kg. Injection therapy was performed once weekly for four weeks.

Keloid explants were measured every 3 days starting from 6 days post-implantation until day 33 with a Vernier caliper, and the keloid volume (KV) was calculated as KV (mm$^3$) = $d^2 \times D/2$, where $d$ and $D$ represent the shortest and the longest diameter, respectively. One animal from each group was culled 24 h post the third administration of reagents, and the mouse lesions, skins and organs were collected for immunohistochemistry (IHC) analysis. All remained mice were sacrificed on day 33, and animal skins, remained lesions and organs were collected, fixed, embedded in wax and stored until use.
**Immunohistochemistry analyses**

For immunohistochemistry staining, paraffin-embedded keloid explants, mouse skin or organs were sectioned (~3-mm thick), deparaffinized using xylene, hydrated in a series of graded alcohol, and subsequently immersed in sodium citrate solution to retrieve tissue antigens at 100 °C for 20 min. Then, the sections were treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The tissue slides were then incubated with a 3% bovine serum albumin (BSA) solution (Servicebio, Beijing China) at room temperature for 15 min to reduce the staining background. Specific primary antibodies, including collagen I Ab (ab138492; Abcam), collagen III Ab (22,734–1-AP; Proteintech, Rosemont, IL, USA) and ki67 (1:600 dilution, AF0198, Affinity Biosciences), were first incubated with the sections, and then, the appropriate secondary antibodies were added and incubated for 1 h incubation at room temperature. Nuclei were stained with hematoxylin. The stained tissues were developed to identify the labeled targeted proteins with an UltraVisionQuanto Detection System HRP (Thermo Fisher Scientific Inc., Waltham, MA, USA) and DAB (Liquid DABþ Substrate Chromogen System, Dako, Copenhagen, Denmark). Finally, the stained sections were imaged with an Eclipse E600 microscope (Nikon, Tokyo, Japan).

**Statistical analysis**

All data were analyzed by GraphPad Prism 9.0 Software (GraphPad Software Inc., La Jolla, CA, USA) and are presented as the means ± SD. Differences between two groups were analyzed by Student’s t test, and multiple group comparisons were performed with one-way ANOVA/Bonferroni post hoc correction. Probability values of significant differences are denoted as *p<0.05, **p<0.01, and ***p<0.001.

**Results**

**Isolation of normal skin fibroblasts (NFs) and keloid fibroblasts (KFs) and examination of their cellular proliferation potential**

Keloid fibroblasts (KFs) were isolated and cultured from fresh keloid tissues through an explant culturing method described previously [21] and briefly described in the Materials and methods section. Normal skin fibroblasts (NFs) were similarly isolated from foreskin tissues. The proliferative potential of the NFs and KFs was assessed with viability and proliferation assays and compared. As shown in Fig. 1A, KFs proliferated significantly faster than NFs in the in vitro monolayer culturing system. Detection of the proliferation marker Ki67 confirmed the proliferative advantage of the KFs over that of NFs (Fig. 1B-C). This indicates that KFs may be more active for cell division in keloid lesions than NFs in normal skin.

**Sunitinib is specifically cytotoxic and significant apoptosis in KFs**

KFs and normal cell lines were incubated with a serial concentration of sunitinib to test the potential efficacy of this inhibitor as a keloid treatment. As shown in Fig. 2A, sunitinib significantly inhibited KF proliferation in a dose-dependent manner but induced no cytotoxicity in the normal cell lines, including 293 T cells, NFs, ADSCs and BMSCs. The inhibitory effect of sunitinib on KFs was evident at 6.0 µM showing 62.3 ± 5.2% viability inhibition rate, and at this concentration, it was safe and nontoxic to normal cell lines.

A broader range of sunitinib concentrations (0.0–100.0 µM) were further examined for the cytotoxicity on KFs. As shown in Fig. 2B, along with the increase of sunitinib concentration, further increased cell toxicities were observed. Notably, over 95% cells were killed by treatment with 25.0–100.0 µM of the drug.

Next, cell cycle was examined for KFs with or without sunitinib treatment. The obtained results revealed that the inhibitor significantly reduced G1/G0 cell percentage whilst inducing significant increase of S phase (P<0.05, Fig. 2C-D), indicating cell cycle arrest in the S phase induced by sunitinib treatment. KFs were further investigated to investigate their apoptosis induction by sunitinib treatment. As shown in Fig. 2E, treatment with sunitinib at 2.0–6.0 µM induced more than 60% of apoptosis in KFs. The treated KFs were stained with FITC-phalloidin for filamentous actin (F-actin) labelling, and cytoskeletal disruption was clearly observed in the cells treated by sunitinib (2.0–6.0 µM) but not in the cells treated with vehicle (Fig. 2F), confirming apoptosis induction by sunitinib. Collectively, these data demonstrate that sunitinib is effective for the suppression of cell proliferation and inducing apoptosis in KFs and thus can be a potential candidate drug for keloid therapy.

**Sunitinib inhibits the 3D migration/invasion of KFs**

One of the typical features of keloids is their extended growth, which extends beyond the boundary of the antecedent trauma forming a tongue-like extending edge in the dermis [22]. These characteristics suggest that keloid cells such as KFs may be invasive. To test the invasiveness, KFs were examined for their 3D migration/invasion potential in a Transwell/Matrigel set. As shown in Fig. 3, KFs showed high invasiveness in the Matrigel matrix. Interestingly, sunitinib treatment at 2.0–6.0 µM demonstrated potent inhibition
on the invasion of KFs (Fig. 3). This observation further supports using sunitinib as a therapeutic candidate drug for KDs.

The sunitinib treatment significantly reduced collagen expression and induced an autophagy flux in KFs

Upregulated expression of both collagen I and III has been frequently observed in keloids [23, 24] and is thus a biomarker of keloids. Therefore, it would be interesting to know whether sunitinib can suppress the expression of collagen I and III in keloids. To this end, KFs and NFs were treated with sunitinib and then examined for collagen I and III expression at both the mRNA and protein levels. As shown in Fig. 4A, sunitinib treatment at 2.0–6.0 μM significantly downregulated the transcription of both collagen I and III genes. Consistent with this finding, the protein expression of collagen I and III was abolished by sunitinib treatment at 6.0 μM in KFs (Fig. 4B), while NFs also showed reduced expression of both collagen I and III but apparently with lesser downregulation extent.

Additionally, the autophagy related factors LC3B was examined by immunoblotting in KFs treated by sunitinib. As shown in Fig. 4C, the drug treatment upregulated the expression of LC3B-II (14 kDa), whilst suppressing the expression of LC3B-I (16 kDa), indicating the induction of an autophagy flux by sunitinib treatment in KFs.

Collectively, these data indicate that sunitinib may exert anti-fibrotic and pro-autophagy effects in keloids.
Sunitinib effectively suppressed the expression of key components of the PI3K/Akt/mTOR pathway in KFs

Both KFs and NFs were cultured and treated with sunitinib at 6 µM to assess the effects of drug treatment on the expression of the PI3K/Akt/mTOR pathway components, including Akt, PI3K and mTOR. As shown in Fig. 5, both NFs and KFs expressed abundant amounts of Akt, PI3K and mTOR. Notably, both Akt and mTOR expression was significantly reduced by sunitinib treatment in both NFs and KFs. However, the downregulation extent was greater in KFs than in NFs. In KFs, the expression of mTOR was decreased to undetectable levels after sunitinib treatment. Moreover, the expression of PI3K was significantly suppressed in KFs but not in NFs. The expressions of both phosphorylated Akt and mTOR (p-Akt and p-mTOR) were also found to be significantly reduced by sunitinib treatment. In line with the complete suppression of mTOR expression, the p-Akt expression was not detected in the KFs treated with the drug. These observations showed that sunitinib potentially suppressed the Akt/PI3K/mTOR signaling pathway in both NFs and KFs.
and KFs, but KFs appeared more susceptible to the drug treatment.

**Sunitinib treatment suppressed cellular proliferation and collagen production in keloid explants**

An explant culture model of keloids was established by subcutaneous implantation of freshly excised human keloid tissue fragments into female Balb/c nude mice. As scheduled in Fig. 6A, this model was then used to test the therapeutic efficacy of sunitinib for keloids. Intrallesional injection of sunitinib at a dose of 100 mg/kg was performed once a week for 4 continuous weeks. As the most commonly used intrallesional injection corticosteroid for keloid treatment, TAC was used as the positive treatment control at the reported dose of 2 mg/kg in mice [25]. As shown in Fig. 6B, sunitinib therapy ultimately induced complete regression of the keloid explants after 4 weeks of treatment, while TAC treatment caused a significant decrease in keloid tissue volumes compared with vehicle control. The IHC detection of ki67 expression revealed that both TAC and sunitinib treatments significantly suppressed cell proliferation in keloids; however, the latter seemed to be more effective (Fig. 6C-D). In addition, both drug treatments reduced collagen I production in the keloid explants, and sunitinib therapy resulted in a reduction in collagen III secretion as well. These data suggest that sunitinib is more efficient than TAC for the in vivo suppression of both cellular proliferation and ECM deposition in keloids.

**Fig. 3** Invasion/3D migration assays. Transwell inserts were covered in Matrigel, and then, the KFs were seeded into the top chamber for 3D migration/invasion evaluation with vehicle (Ctrl) or sunitinib treatment (2.0–6.0 µM) for 2 days. The cells that invaded through the Matrigel layer and attached to the bottom membrane were fixed with 4% paraformaldehyde and stained with crystalline violet imaged and quantitated as invading cells. **A** Invading cells. **B** Invasion/3D migration rates were calculated and compared. All values are presented as the means ± SD (n = 4). ****p < 0.001, versus Ctrl by Student’s t test.
Discussion

Keloid disease (KD) has been recognized as a tumor-like but not cancerous skin condition with rarely spontaneous regression [26, 27]. In contrast, KD generally continues to develop over time. Although not deadly, the KD causes cosmetic disfigurement, leading to a significant reduction in quality of life for KD patients. In addition, the condition can result in pain and itching and impairs emotional well-being [28–30]. Therefore, it is essential for KD patients to have an effective therapy. Unfortunately, neither a thorough understanding of the mechanisms behind keloid formation nor reliable therapies have been developed to date. It is still a challenge to manage skin disorders to a satisfactory level.

Surgical excision is a common practice for the management of keloid patients, but this condition frequently recurs at rates of 55–100%. A worsened skin condition may even follow and necessitate surgical interventions again [29, 31]. The most common chemotherapy involves the intralesional injection of corticosteroid-like drugs, such as triamcinolone acetonide (TAC), alone or in combination with other therapeutics. The TAC injection can lead to 50–100% regression rates, but high recurrence rates up to 50% can be observed within 5 years of this treatment. Therefore, more effective therapies are needed. In this study, we revealed that the upregulated activation of the Akt/PI3K/mTOR pathway is an important mechanism in keloid. And the interrupted autophagy was observed in...
In this study, which may contribute to the pathogenesis of KD. The effective suppression of the Akt/PI3K/mTOR pathway by sunitinib led to a complete regression response in keloid explant culture. This may be closely correlated with the increase of autophagy by sunitinib treatment in KFs, since the enhanced autophagy by epidrugs led to increased autophagy cell death previously [33].

More importantly, sunitinib showed significantly higher efficiency than TAC in attenuating keloid development and fibrosis. These findings can further advance the understanding of keloid pathogenesis and facilitate the development of more effective treatment approaches. Next, it is necessary to test the efficacy of sunitinib in combination with other therapeutic modalities, such as surgical excision and intraliesional TAC administration.

Animal models of keloid have not been successfully established to date [34, 35]. This has hampered research efforts on understanding keloid pathogenesis. However, our data in this study suggest that explant culture of human keloid fragments in mice can be a true-to-life alternative to animal model of keloid. This study thus may provide an example for future in vivo keloid investigations and the development of novel therapeutic approaches.

In conclusion, we successfully isolated KFs and showed their greater proliferation potential compared to that of NFs in monolayer culture. Sunitinib, a kinase inhibitor, was shown to inhibit cell proliferation, arrest the cell cycle in the S phase, block cell invasion and induce significant apoptosis in KFs but not in NFs. In addition, sunitinib treatment drastically reduced collagen I and III expression at both the mRNA and protein levels and may induce an autophagy flux.

**Fig. 5** Effects of sunitinib on the expression of the PI3K/Akt/mTOR pathway components. NFs and KFs were cultured and treated with sunitinib at 6.0 µM for 48 h, followed by cell harvest, protein extraction and immunoblot analysis of the expression of Akt, p-Akt, PI3K, mTOR, p-mTOR and GAPDH. The expression is shown relative to NFs with vehicle treatment (NF) for KFs with vehicle treatment (KF) or for NFs and KFs with sunitinib treatment at 6.0 µM for 48 h (NF+S and KF+S); set to 1.0. All values are presented the means ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA/Bonferroni multiple-comparison post hoc correction.
Fig. 6 In vivo determination of sunitinib therapy efficacy on human keloid disease. A Schedule of animal study of keloid explant culture. B Development curve of the keloid explants in mice. C Immunohistochemistry (IHC) examination of the cell proliferation marker Ki67 and extracellular matrix (ECM) components collagen I and III in implanted keloid fragments in nude mice. Keloid lesions were excised and examined 24 h after the final treatment. D Quantification and comparison of the IHC analysis results. IHC quantification was carried out by determining the histochemistry score (H-SCORE) of 8 histologically similar microscopic fields for each individual sample with Image-Pro Plus 6.0 software. All values are presented as the means ± SD (n = 9 or 8), *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA/Bonferroni multiple-comparison post hoc correction test.
These effects were accompanied with evident suppression of the Akt/PI3K/mTOR signaling pathway in KFs. Importantly, an in vivo test demonstrated complete regression induction of keloid explants in mice after sunitinib treatment. Therefore, sunitinib can be a promising candidate drug for the treatment of the notorious skin keloid disease.

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Data availability The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interests The authors declare no conflict of interest.

Ethical approval This study was approved by the Ethics Committee of Guangdong Second Provincial General Hospital, and the Guideline for the Care and Use of Laboratory Animals was followed.

Consent for publication Not applicable.

References

1. Zhu HY, Bai WD, Li C, Zheng Z, Guan H, Liu JQ et al (2016) Knockdown of lncRNA-ATB suppresses autocrine secretion of TGF-β2 by targeting ZNF217 via miR-200c in keloid fibroblasts. Sci Rep 6:24728
2. Lee WJ, Ahn HM, Na YJ, Wadhwa R, Hong JW, Yun CO (2017) Mortaline deficiency suppresses fibrosis and induces apoptosis in keloid spheroids. Sci Rep 7:12957
3. English RS, Shenefelt PD (1999) Keloids and hypertrophic scars. Dermatol Surg 25:631–638
4. Yan L, Cao R, Liu YB, Wang LZ, Pan B, Lv XY et al (2016) MiR-21-5p Links epithelial-mesenchymal transition phenotype with stem-like cell signatures via AKT signaling in keloid keratinocytes. Sci Rep 6:28281
5. Mari W, Alsabri SG, Tahal N, Younes S, Sherif A, Simman R (2015) Novel insights on understanding of keloid scar: article review. J Am Coll Clin Wound Spec 7(1–3):1–7
6. Wang W, Qu M, Xu L, Wu X, Gao Z, Gu T et al (2016) Sorafenib exerts an anti-keloid activity by antagonizing TGF-beta/Smad and MAPK/ERK signaling pathways. J Mol Med (Berl) 94:1181–1194
7. Shih B, Garside E, McGrourther DA, Bayat A (2010) Molecular dissection of abnormal wound healing processes resulting in keloid disease. Wound Repair Regen 18:139–153
8. Yu C, Xu ZX, Hao YH, Gao YB, Yao BW, Zhang J et al (2019) A novel microcurrent dressing for wound healing in a rat skin defect model. Mil Med Res 6(1):22
9. Yang F, Chen Y, Shen T, Guo D, Dakhova O, Ittmann MM et al (2014) Stromal TGF-beta signaling induces AR activation in prostate cancer. Oncotarget 5:10858–10869
10. Xue G, Hemmings BA (2013) PKB/Akt-dependent regulation of cell motility. J Natl Cancer Inst 105:393–404
11. Syed F, Sherris D, Paus R, Varmeh S, Singh S, Pandolfi PP et al (2012) Keloid disease can be inhibited by antagonizing excessive mTOR signaling with a novel dual TORC1/2 inhibitor. Am J Pathol 181:1642–1658
12. Choueiri TK, Heng DYC, Lee JL, Cancel M, Verheijen RB, Mellemgaard A et al (2020) Efficacy of sunitinib vs sunitinib in patients With MET-driven papillary renal cell carcinoma: The SAVOIR phase 3 randomized clinical trial. JAMA Oncol 6:1247–1255
13. Westerdijk K, Desar IM, Steeghs N, van der Graaf WTA, van Erp NP (2020) Imatinib, sunitinib and pazopanib: from flat-fixed dosing towards a pharmacochemically guided personalized dosed. Br J Clin Pharmacol 86:258–273
14. Klingberg F, Hinz B, White ES (2013) The myofibroblast matrix: implications for tissue repair and fibrosis. J Pathol 229:298–309
15. Chaker D, Mouawad A, Azar A, Quilliot D, Achkar I, Fajloun Z et al (2018) Inhibition of the RhoGTPase Cdc42 by ML141 enhances hepatocyte differentiation from human adipose-derived mesenchymal stem cells via the Wnt5a/PI3K/miR-122 pathway: impact of the age of the donor. Stem Cell Res Ther 9:167
16. Rini Brian I (2007) Sunitinib. Expert Opin Pharmacother 8:2359–2369
17. Heng Daniel YC, Christian K (2010) Sunitinib. Recent Results Cancer Res 184:71–82
18. Amal I, Heng Daniel YC, Christian K (2014) Sunitinib in the treatment of advanced solid tumors. Recent Results Cancer Res 201:165–184
19. ZhengQiang Y, Gobeil Phille Philame AM, Saveria CM et al (2010) Equine sarcoid fibroblasts over-express matrix metalloproteinases and are invasive. Virology 396:143–151
20. Liviak KJ, Schmittgen TD (2001) Analysis of relative gene expression using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408
21. Yuan ZQ, Nicholson L, Marchetti B et al (2008) Transcriptional changes induced by bovine papillomavirus type 1 in equine fibroblasts. J Virol 82:6481–6491
22. Limandjaja Grace C, Niessen Frank B, Scheper Rik J et al (2020) The keloid disorder: heterogeneity, histopathology, mechanisms and models. Front Cell Dev Biol 8:2360
23. Naitoh M, Hosokawa N, Kubota H et al (2001) Upregulation of HSP47 and collagen type III in the dermal fibrotic disease, keloid. Biochem Biophys Res Commun 280:1316–1322
24. Syed F, Ahmadi E, Iqbal SA et al (2011) Fibroblasts from the growing margin of keloid scars produce higher levels of collagen I and III compared with intralesional and extraleional sites: clinical implications for lesional site-directed therapy. Br J Dermatol 164:83–96
25. Chen Austin D, Rong-Fu C, Yun-Ting Li et al (2019) Triamcinolone acetonide suppresses keloid formation through enhancing apoptosis in a nude mouse model. Ann Plast Surg 83:S50–S54
26. Siong-See L, Gil Y, Yong-Huak C et al (2004) Pruritus, pain, and small nerve fiber function in keloids: a controlled study. J Am Acad Dermatol 51:1002–1006

27. Didar BD, Tacettin I, Asena DC et al (2009) DLQI scores in patients with keloids and hypertrophic scars: a prospective case control study. J Dtsch Dermatol Ges 7:688–692

28. Eveline B, Kouwenberg Casimir AE, Reinier T et al (2017) Burden of Keloid Disease: a cross-sectional health-related quality of life assessment. Acta Derm Venereol 97:225–229

29. Barbara S, Eloise G, Angus MD et al (2010) Molecular dissection of abnormal wound healing processes resulting in keloid disease. Wound Repair Regen 18:139–153

30. Peixoto P, Grandvallet C, Feugeas JP, Guittaut M, Hervouet E (2019) Epigenetic control of autophagy in cancer cells: a key process for cancer-Related phenotypes. Cells 8(12):1656

31. van den Broek LJ, Limandjaja GC, Niessen FB, Gibbs S (2014) Human hypertrophic and keloid scar models: principles, limitations and future challenges from a tissue engineering perspective. Exp Dermatol 23:382–386

32. Wang Q, Wang P, Qin Z, Yang X, Pan B, Nie F, Bi H (2021) Altered glucose metabolism and cell function in keloid fibroblasts under hypoxia. Redox Biol 38:101815

33. Lv W, Ren Y, Hou K, Hu W, Yi Y, Xiong M, Wu M, Wu Y, Zhang Q (2020) Epigenetic modification mechanisms involved in keloid: current status and prospect. Clin Epigenet 12(1):183

34. Deng CC, Hu YF, Zhu DH, Cheng Q, Gu JJ, Feng QL, Zhang LX, Xu YP, Wang D, Rong Z, Yang B (2021) Single-cell RNA-seq reveals fibroblast heterogeneity and increased mesenchymal fibroblasts in human fibrotic skin diseases. Nat Commun 12(1):3709

35. Griffin Michelle F, Borrelli Mimi R, Garcia Julia T et al (2021) JUN promotes hypertrophic skin scarring via CD36 in preclinical in vitro and in vivo models. Sci Transl Med 13(609):3312

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