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Original article

Halofuginone regulates keloid fibroblast fibrotic response to TGF-β induction

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ABSTRACT

Keloids are characterized by increased deposition of fibrous tissue in the skin and subcutaneous tissue following an abnormal wound healing process. Although keloid etiology is yet to be fully understood, fibroblasts are known to be key players in its development. Here we analyze the antifibrotic mechanisms of Halofuginone (HF), a drug reportedly able to inhibit the TGF-β1-Smad3 pathway and to attenuate collagen synthesis, in an *in-vitro* keloid model using patient-derived Keloid Fibroblasts (KFs) isolated from fibrotic tissue collected during the "Scar Wars" clinical study (NCT NCT03312166). TGF-β1 was used as a pro-fibrotic agent to stimulate fibroblasts response under HF treatment. The fibrotic related properties of KFs, including survival, migration, proliferation, myofibroblasts conversion, ECM synthesis and remodeling, were investigated in 2D and 3D cultures. HF at 50 nM concentration impaired KFs proliferation, and decreased TGF-β1-induced expression of α-SMA and type I pro-collagen production. HF treatment also reduced KFs migration, prevented matrix contraction and increased the metallo-proteases/inhibitors (MMP/TIMP) ratio. Overall, HF elicits an anti-fibrotic contrasting the TGF-β1 stimulation of KFs, thus supporting its therapeutic use for keloid prevention and management.

1. Introduction

Since 2018, World Health Organization defines keloids as a member of a group of disorders *"characterized by increased deposition of fibrous tissue in the skin and subcutaneous tissue"* [1]. Accordingly, keloids are considered not just pathological scars, rather the outcome of an abnormal wound healing process with features similar to that of chronic inflammatory diseases and cancer [2]. Clinically, keloids present as a fibrotic tissue that proliferates beyond the primary injured area and persist over time without natural regression. Keloids may cause pain and pruritus, and seriously affect patient quality of life when they are located in visible areas [3]. Keloids may result from different skin injuries, including surgery, burns, trauma, piercing, and folliculitis, though their location appears mainly confined to chest, shoulders, neck and ears [4].

Despite the ample variety of treatments available, which include occlusive dressing, compressive therapy, cryotherapy, radiation, laser, and pharmacotherapies with steroids, mitomycin C, 5-FU, bleomycin, no effective therapeutic protocols or standardized guidelines have been published yet [5]. Surgery is commonly indicated in order to reduce keloid volume, despite the high recurrence rate after excision and the development of a fibrosis worse than the initial one [6]. The lack of effective treatments clearly reflects our poor understanding of keloid

Abbreviations: TGF-β1, transforming growth factor β1; ECM, extracellular matrix; SMADs, Mothers against decapentaplegic homolog; α-SMA, alpha-smooth muscle actin; MMP, matrix metalloproteinases; TIMP, tissue inhibitors of metalloproteinases; HF, Halofuginone; KF, keloid fibroblast; AUC, Area Under the Curve.

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pathogenesis, though keloid histopathology has been extensively described in the past decades [7]. The papillary-reticular distribution of dermal compartment is lost in keloid tissue, where the Extracellular Matrix (ECM) is mainly composed of randomly distributed thick collagen fibers. Compared to normal skin, the keloid fibrotic tissue shows an increased collagen type-I to type–III ratio along with other ECM-related components such as fibronectin, glycosaminoglycans and chondroitin sulfate [8]. However, keloid etiology and pathogenesis remain largely unknown. Recently, Limandjaja et al. [8] proposed that keloid could be triggered by a combination of three risk factors, the "keloid triad", involving genetics, wound healing process and external factors [9], and in this scenario the fibroblast is a key player.

Keloid fibroblasts (KFs) are the central effectors of the "keloid triad" leading to the abnormal deposition of fibrous tissue. Quantitatively speaking, fibroblasts and myofibroblasts are more numerous in keloids than in non-pathological scars [10,11]. Compared to normal skin fibroblasts, KFs have an increased proliferation rate [12,13] and a reduced apoptosis rate [14,15]. Moreover, KFs are more responsive to growth factors activation (*i.e* by TGF-β1 and PDGF) than their normal counterparts [16–18], and this leads to higher production of type I and III collagens (ratio I/III in keloid is 17:1 *vs* 5:1 in normal skin) and ECM accumulation [19]. During wound healing, ECM degradation and remodeling is controlled by a family of enzymes called matrix metalloproteinases (MMPs). MMPs are synthesized by fibroblasts and their activity is regulated by the tissue inhibitor of matrix metalloproteinase (TIMPs). Dysregulation of such MMP/TIMP equilibrium may underlie ECM over-accumulation. In keloid, the levels of MMP-1 and -13 (collagenase), MMP-2 (gelatinase) and TIMP-1 and -2 increase, whereas the level of MMP3 (stromelysin) decreases, compared to the respective levels in normal scars [19–21]. The TGF-β1/Smad signaling pathway is pivotal in the process of keloid fibrosis. TGF-β1 regulates fibroblast-to-myofibroblast conversion, α-SMA expression and collagen synthesis during the development of fibrosis. TGF-β1 is found in higher quantity in keloid than in normal scar [22–24]. Accordingly, a variety of therapeutic strategies (drugs, siRNA, shRNA, miRNA, *etc*) have been designed to target the TGF-β1/Smad signaling pathway in order to interfere with fibroblast-mediated keloid progression [25].

In the past years, Halofuginone [7-Bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3 H)-quinazolinone] has been proposed as a new potential agent capable of contrasting tissue fibrogenesis [26]. Halofuginone (HF) is a chemical derivative of febrifugine originally isolated from the chinese plant *Dichroa febrifuga*. HF is widely used for its anti-coccidiosis and anti-parasitic properties in the poultry industry [27]. Additionally, HF has been reported to inhibit Smad3 phosphorylation downstream of the TGF-β1 signaling pathway, impairing the fibroblasts-to-myofibroblasts phenoconversion and reduction of collagen synthesis and ECM accumulation [28]. In 2007, Lista and Emanuele [29] proposed HF as a new therapeutic option for keloid management, yet no mechanistic data in support of this hypothesis have been published to date.

The present study aims to fill this gap of knowledge by investigating *in vitro* the effects and mechanism of action of HF on keloid fibroblasts cultured in a pro-fibrotic environment. To this end, we analyzed the key events and factors involved in the abnormal functioning of keloid fibroblasts within the profibrotic environment, including survival, migration, proliferation, myofibroblasts conversion, ECM synthesis and remodeling. We show that HF greatly attenuates the response of keloid fibroblasts to TGF-β1 stimulation. Our results support the clinical use of HF as a new therapeutic tool for keloid management.

2. Material and method

2.1. Reagents

Phosphate Buffer Saline (P04-36500), Dulbecco Modified Eagle's Medium (P04-05540), Penicillin-Streptomycin (P06-07100), Trypsin-EDTA (P10-023100), NaHCO3 (P04-49100) and Fetal Calf Serum (S1810-500) were purchased from Dutscher (Brumath, France); MMP1 Human ELISA Kit (EHMMP1) and MMP3 Human ELISA Kit (BMS2014-3) from Thermo Fisher Scientific (Villebon-sur-Yvette, France); Collagenase (Collagen degradation/zymography) assay kit (ab234624) and Human alpha SMA ELISA Kit (ab240678) from Abcam (Paris, France); Pierce solution (C2284 and B9643), Triton x100 (T8787), Goat serum (S26), Glycine (G7403), BSA (A3294), DAPI (D9542), anti- α-smooth actin mAb (A2547), anti-Mouse IgG (Fab specific) F(ab′)2 fragment-FITC antibody (F8521), Halofuginone (HF) hydrobromide (VETRANAL™, 32481), cell proliferation ELISA kit (11647229001), Protease inhibitor cocktail (P1860), MTT (M5655), DMSO (D4556), NaOH (1091372500), and p-Aminophenylmercuric Acetate (164610) from Sigma-aldrich (Saint Quentin Fallavier, France); Type I collagen (207050257) from Jacques Boy (Reims, France); Recombinant Human TGF-beta 1 Protein (ref 240-B-002) from R&D Systems Europe Ltd (Lille, France). Quanti-Blue™ kit and HEK-Blue™ TGF-β cells were purchased from Invivogen (Toulouse, France).

2.2. Clinical study approval

Keloid tissues were obtained from patients undergoing reductive plastic surgery (Table 1) performed at Maxillo-Facial Surgery Department of the University Hospital of Besançon (France). All included-patients were informed and provided consent. The clinical study was conducted in compliance with the ethical standards, namely the Declaration of Helsinki. This work was ethically approved by the French Regulatory Agency (ANSM), Ethic committee (CPP Sud-Ouest et Outre-Mer I) and was registered on clinicaltrial.gov as "SCAR WARS" (NCT03312166). "SCAR WARS" is a monocentric and single group assignment clinical trial which is carried out on 27 patients with earlobe keloid.

2.3. Human primary keloid fibroblasts collection

Keloid tissues were processed for cell extraction immediately after surgery. Under laminar flow, biopsies (5 mm diameter) were harvested from the center of each keloid tissue. Then, biopsies were incubated with antibiotics (PS, 10 min, RT), washed three times in PBS (5 min, RT) and cut into smaller pieces (2 mm x 2 mm). Samples were then plated in 25 mm^2 tissue culture flasks (dermis side on plastic) and cultured in complete medium [DMEM supplemented with 10 % FCS, penicillin (100 U/mL) and streptomycin (0.1 mg/mL)] in cell culture incubator (37 C, 5 % CO_2). Culture medium was changed every 3 days. After 7–10 days of culture, spindle-shaped fibroblasts began to proliferate from explants edges. When cells reached local confluence, fibroblasts were detached from culture flask with trypsin (0.05 %) - EDTA (0.02 %) solution and sub-cultured for cell expansion. Primary keloid fibroblasts (KF) used in this experimental study was extracted from seven patients (Table 1). For further experiments, cells were used between the third and eighth passage.

2.4. Human keloid fibroblasts culture and treatment

During routine cell culture and cell seeding prior to treatment, KFs

Table 1
Details of patient sample processed for cell extraction.

Inclusion number	Gender	Patient age	Keloid site
003	Female	59	Earlobe
005	Female	38	Earlobe
007	Male	21	Earlobe
008	Female	44	Earlobe
010	Male	37	Earlobe
016	Female	19	Earlobe
017	Female	54	Earlobe

were cultured in complete medium [DMEM supplemented with 10 % FCS, penicillin (100 U/mL) and streptomycin (0.1 mg/mL)]. During treatment, low serum concentrated medium (5 %) was used as drug vehicle. In further experimentation, FK were treated with HF (50 nM), TGF-β1 (2 ng/mL), a combination of HF and TGF-β1 (respectively 50 nM and 2 ng/mL) or vehicle alone (Ctrl). If necessary, other ways of treatment will be specified. Bioactivity of TGF-β1 (non-latent form) was verified using HEK-Blue™ TGF-β cells prior to investigation (data not shown). HEK-Blue™ TGF-β cells allow the detection of bioactive TGF-β by monitoring the activation of the TGF-β/Smad pathway.

2.5. Determination of suitable Halofuginone working concentration

2.5.1. Cytotoxicity

Viability of KFs was determined by MTT assay. KFs were seeded into 96 wells culture plate ($7.5.10^3$ cells per well) and treated during 24 h with a range of HF concentrations (1−5-10−25-50−100-200−500-1000−10000 nM) *vs* vehicle. After treatment, MTT solution (final concentration: 2 mg/mL) was added to each well and incubated 4 h. Then, supernatant was discarded, cells were washed two times with PBS and formazan crystals were dissolved by adding 100 µl of DMSO. Absorbance at 490 nm was measured by a microplate reader (Multiskan FC, Thermo Scientific). Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 10 per condition).

2.5.2. Cell cycle

Keloid fibroblasts were seeded into 12-well culture plate ($2\ 10^5$ cells per well) and treated during 24 h with a panel of HF concentration (10, 25, 50 nM) *vs* vehicle. To analyze cell-cycle progression, cells were harvested and immobilized in 70 % ethanol at 4 °C for 12 h followed by washing with phosphate buffer solution (PBS). Then, cells were incubated in PBS 1X supplemented with RNase (0.32 µg/mL), propidium iodide (0.16 µg/mL). Cells were stored in a dark room until flux cytometry measurement (FC500 Beckman Coulter). Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 2 per condition).

2.5.3. Adhesion test

Keloid fibroblasts were seeded into 24-well culture plate ($2.5\ 10^4$ cells per well) into increasing halofuginone concentration (50−100-1000 nM) *vs* vehicle and incubated 6 h. Then, culture medium and detached cells were discarded, the adherent cells were washed with PBS and subsequently trypsinized and counted. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition).

2.5.4. Cell growth

Keloid fibroblasts were seeded into 12-well culture plate (5.10^4 cells per well) and treated for increasing time with 50 nM HF or vehicle to determine the time-dependent effect of HF on KF growth. At day 3 post treatment, part of cells cultured with HF were then cultured into vehicle and *vice versa*. The other part was cultured into the same medium as at the beginning. Medium was changed every 3 days and cells were counted at day 3, 6 and 9. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition). The effect of HF combined with TGF-β1 on KFs growth was also determined by cell counting. To this end, KF were seeded into 12-well culture plate (5.10^4 cells per well) and treated as described in 2.4 section. After 3 days, cells were collected by trypsination and viable cells were counted excluding the trypan blue-positive. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition).

2.6. Proliferation assay

Cell proliferation was assessed by BrDU incorporation during DNA synthesis. Keloid fibroblasts were seeded into 96 wells culture plate (5.10^3 cells per well) and treated as described in 2.4 section. Cell proliferation ELISA kit was used according to the manufacturer's instruction. Absorbance at 450 nm was measured by a microplate reader (Multiskan FC, Thermo Scientific). Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 10 per condition).

2.7. Scratch wound assay

Keloid fibroblasts were seeded into IncuCyte® ImageLock 96-well culture plate (2.10^4 cells per well) in complete medium. After overnight adhesion, both confluence status and homogenous cell covering were checked prior to wounding. WoundMaker™ device (Essen BioScience) was used to create standardized scratches into 96-well plate. After wounding, medium was aspirated; each well was washed twice with PBS and refilled with treatment or vehicle (100 μl). Plates were incubated in IncuCyte S3™ and automatically photographed in bright field every 2 h for 72 h. After a preliminary visualization of the resulting images, three typical wells were chosen for setting automatic analysis parameters as proposed by the manufacturer. Relative wound density at each time point was analyzed using the IncuCyte™ Scratch Wound Cell Migration Software Module (Essen BioScience). Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 10 per condition).

2.8. ECM synthesis and remodeling protein

2.8.1. ELISA assay

Keloid fibroblasts were seeded into 6 wells culture plate ($0.5\ 10^6$ cells per well) and treated as described in *2.4* section during 48 h. Supernatants were collected on ice in the presence of anti-proteinases (10 %) and frozen at −80 °C until use. The protein quantity of Type-I Procollagen, MMP1, TIMP1 and MMP3 was measured in the supernatant by ELISA assays according to manufacturer instruction. Data obtained from ELISA assays were normalized to total cell protein. In brief, after supernatant collection, cells were washed twice with PBS and NaOH (0.1 N, 500 µl per well) was added. Cell lysate were collected on ice and stocked at −80 °C until total protein assay. Pierce BCA reaction assay was used to quantify total protein concentration. Briefly, 20 µl of each cell lysate was mixed to 200 µL Pierce solution (Copper II sulfate solution/bicinchonic acid, 1:50) and incubated 30 min at 37 °C. Absorbance at 571 nm was measured by a microplate reader (Multiskan FC, Thermo Scientific). BSA standard was used to determine protein quantity in cell lysate. Results were expressed as a ratio of control condition. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition).

2.8.2. Collagenase activity – zymography assay

Keloid fibroblasts were seeded into 6 wells culture plate ($0.5\ 10^6$ cells per well) and treated as described in *2.4* section during 48 h. After treatment, cell lysates were collected, inactive zymogen forms of collagenase were activated with 1 mM p-aminophenylmercuric acetate (AMPA) and collagenase activity was determined using a collagenase zymography assay kit (ab234624) in accordance with manufacturer instruction. Collagenase activity was normalized by total protein concentration in cell lysate as described in 2.8.1. Results were expressed in U/mg and raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition).

2.8.3. Collagen gel retraction

ECM remodeling was assessed using free retracted collagen gel as previously described [12]. First, a specific medium was prepared [DMEM medium (0.8 X), FCS (9 % v/v), NaOH (0.005 N), NaHCO3 (0.3 %), Penicillin (200 U/mL) and Streptomycin (0.2 mg/mL)] and kept chilled on ice. Then, acid-extracted type I collagen (0.6 mg/mL) and

keloid fibroblast suspension (8.10^4 cell/mL) were added. 2.5 ml of the liquid gel mixture was dispensed into Petri dish (30 mm diameter) and incubated at 37 °C for at least 1 h to allow for gel polymerization. Immediately after polymerization, gels were smoothly detached from plastic and treated as previously described. Petri dish were scanned daily and gel diameter were determined using Image J software [30]. Results about collagen gel retraction were expressed as a percentage of initial area. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 4 per condition).

2.8.4. Myofibroblasts phenoconversion

Alpha-smooth muscle actin (α-SMA) was used as the main phenoconversion marker of fibroblast into myofibroblast. α-SMA was quantified by ELISA and ratio of α-SMA positive cells were determined after immunofluorescence staining. For ELISA assay, keloid fibroblasts were seeded into 6 wells culture plate (0.3 10^6 cells per well) and treated as described in *2.4* section during 96 h. After treatment, cell lysate were collected and α-SMA was quantified using α-SMA *in vitro* SimpleStep ELISA® kit (Abcam) according to manufacturer instruction. Data were normalized to total cell protein, as described in 2.8.1. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition).

For immunostaining, KFs (0.3 106) were seeded into Petri dish (60 mm) containing 3 sterile glass coverslip and treated as described in 2.4 section during 96 h. After treatment, cells were washed with PBS and fixed in 4 % PFA. Cell membranes were permeabilized with Triton x100 (0.1 % in PBS) and subsequently washed with PBS. Cells were then incubated in 1 % Glycine (10 min, RT), afterwards a blocking solution (2.7 % BSA; sheep serum 10 %; 1 % Triton x100) was used to saturate nonspecific binding site. After saturation step, cells were incubated overnight in 1:200 mouse anti- α-sm actin mAb (A2547) at 4 °C. The next day, following several subsequent washes, cells were incubated for 1 h at room temperature with Anti-Mouse IgG (Fab specific) F(ab′)2 fragment-FITC antibody (F88521). Nuclei were counterstained with DAPI for 15 min before microscopy. A negative control was obtained by omitting the primary antibody. Images were obtained using a confocal microscope (Zeiss LSM800). On each slide, pictures from five areas were randomly taken excluding the edges of the coverslip due to cells homogeneity issues. Total cells and α-SMA positive cells were counted from these pictures. Raw data were obtained from independent experimentation performed with three different primary cell lines (n = 3 per condition).

2.9. Statistics

Results are expressed as mean ± SD. Statistical analyses were performed using one-way or two-way analysis of variance followed by Tukey test for multiple comparisons. All analyses were performed using GraphPad Prism 7 software. Differences were considered as statistically significant * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

3. Results

3.1. HF [50 nM] is a suitable concentration to impair keloid fibroblasts proliferation

The effect of HF on cell morphology was also followed during treatment (Fig. 1A). Below 10^2nM, keloid fibroblasts demonstrated a classical fibroblast-like morphology. Above 10^2nM, increased cell granularity and cellular shrinkage were observed as well as detachment from culture surface. As shown in Fig. 1B, HF concentration from 0 to 10 nM did not trigger any modification in cell viability while HF at 25 nM and 50 nm has a moderate effect compared to control (respectively 86.3 % and 75.1 %). Highest concentrations (from 10^2nM to 10^5) induced a significant and critically reduction of cell viability. 70 % of viability was used as a threshold to choose the maximum dose tolerated by cells. Therefore, 50 nM was selected as the treatment dose for subsequent experiments. In addition, we examined the cell-cycle profile of keloid fibroblasts treated 24 h with HF at 0, 10, 25 and 50 nM (Fig. 1C). HF treated fibroblasts showed the same cell-cycle profile as control. Moreover, during a short-term adhesion test, HF did not show any influence on KF adhesion properties (Fig. 1D).

3.2. HF impairs KF proliferation in a persistent way

At day 3, the addition of HF [50 nM] in culture medium led to the decrease of KF proliferation rate in comparison to control, for which basal medium was used for the 6 next days (Fig. 1D). KF proliferation slowed down but was not entirely suppressed, as shown by the positive slope of the proliferation curve. Conversely, after 3 days of treatment with HF [50 nM], drug replacement by control medium was not able to fully restore the proliferation level as in control condition, even if cell quantity slightly increased compared to HF treated cells over time.

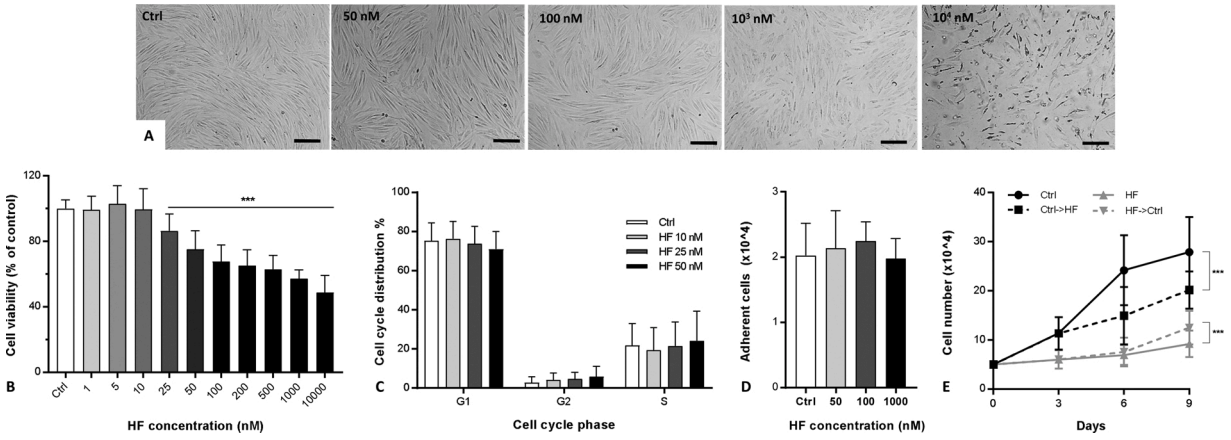

Fig. 1. Halofuginone concentration screening and determination HF suitable working concentration (50 nM). (A) HF do not affect KF morphology at low concentration (scale bar = 200 μm). (B) HF at 25 nM and 50 nm has a moderate effect compared to control (*** $p < 0001$). (C, D) At low concentration HF do not modify neither fibroblast cell cycle nor adhesion properties. (E) HF blocks KF proliferation (dot black line) compared to control (black line). This effect is maintained and persistent over time even after HF withdrawing (dot gray line), (*** $p < 0001$). Results are expressed as mean ± SD. Statistical analyses were performed using one-way (B, C, D) or two-way analysis (E) of variance followed by Tukey test for multiple comparisons.

3.3. HF retains anti-proliferative properties in profibrotic culture condition

After 24 h of treatment, BrdU incorporation assay showed that HF significantly blocked proliferation capacities of KF even in the presence of TGF-β1 (Fig. 2A). In terms of cell quantity, similar results were observed 3 days after treatment (Fig. 2B). Cell number were significantly lower after HF treatment with or without TGF-β1, compared to the respective controls. In both BrdU assay and cell counting experiments, TGF-β1 showed no significant effects in short term incubation. However, while in control cultures fibroblasts showed a clear spindle-like shape and were parallel-oriented, in the presence of TGF-β1 KF morphology and orientation were affected as shown in Fig. 2C.

3.4. HF limits FK migration over time

Real-time monitoring of KF wounded monolayers was used to investigate HF effect on KF migration. Fig. 3A shows representative images obtained with Incucyte system over time according culture conditions. Software analysis led to the measurement of initial wound mask (blue area) and time-dependent wound area (yellow), both used for Relative Wound Density (RWD) calculation. Representative results of RWD evolution over time are presented in Fig. 3B. Apparently, TGF-β1 did not influence KF migration rate compared to control, yet HF shows a synergistic effect during the first 36 h when combined with the pro-fibrotic growth factor. Overall, KF migration is reduced in the presence of HF in culture medium with and without TGF-β1, compared to control. This global effect is shown in Fig. 3C and is also evidenced by the lower area under the curve (AUC) in HF treated cells (statistically significant).

3.5. HF prevents TGF-β1-induced myofibroblast phenoconversion and ECM remodeling

α-SMA was used as a phenoconversion marker of fibroblast into myofibroblast, both in immunostaining investigation (Fig. 4A and B) and ELISA assay (Fig. 4C). While TGF-β1 treatment led to a 2-to-3-times higher number of α-SMA positive cells, HF could significantly bring back α-SMA expression to the basal level, in terms of either positive cells or protein quantity. During tridimensional collagen gel retraction assay (Fig. 4D), HF reduced the mechanical contraction ability of KF. Over time, the retraction ratio (expressed as the percentage of initial gel area) of HF treated gel increased slowly compared to control. The presence of TGF-β1 did not influence the retracting capacity of gel embedded KF.

3.6. HF downregulates procollagen type I synthesis without impacting overall collagenase activity

In TGF-β1-stimulated keloid fibroblasts, procollagen type-I quantity significantly increased by 1.5 to 2-fold (Fig. 5A), and this effect was completely nullified by HF. To be noted, HF *per se* did not modify the expression of procollagen type-I. MMP1 expression level (Fig. 5B) was not modified either by HF or TGF-β1 when drugs were added individually in KF culture. When HF was combined with TGF-β1, MMP1 was significantly lower compared to control condition. MMP3 expression level (Fig. 5C) significantly increased in TGF-β1-stimulated KF culture, while it was not modified by HF. HF significantly reduced MMP3 protein synthesis in pro-fibrotic culture condition. TIMP1 expression level (Fig. 5D) was not modified by HF compared to control. The addition of TGF-β1 to KF led to an increase of TIMP1 protein, and HF significantly blocked this induction and actually further reduced its expression at level lower than control. The effect of HF on collagenase activity is shown in Fig. 5E. Pro-fibrotic treatment with TGF-β1 led to a slight but significant reduction of collagenase activity mediated by KFs, and the addition of HF had no impact on this activity nor it prevented the TGF-β1 effect.

4. Discussion

Keloid fibroblasts (KF) are the key effectors involved in fibrotic tissue production. In keloid tissue, TGF-β1 is overexpressed and regulates fibroblast-to-myofibroblast conversion, α-SMA expression and ECM synthesis during wound healing and fibrosis progression [22–24]. Halofuginone (HF) has been shown to be a potent inhibitor of tissue fibrosis [25,26], and its effect have been already described on different type of pathological fibroblasts in cardiac fibrosis [31], rheumatoid arthritis [32], corneal fibrosis [33], Interstitial fibrosis [34], and sclerodermia [35].

The present *in vitro* study aimed to investigate for the first time the anti-fibrotic effect and the underlying mechanisms of HF in patient-derived keloid fibroblast. First, we have determined that a low HF concentration [50 nM] was efficient to limit KF proliferation without any major cytotoxicity effect or cell cycle disruption. These results are in accordance with the literature where comparable effects were observed with low HF concentration from 10^{-7} to 10^{-10} M [35–37]. Our results also proved that HF effect was maintained in a persistent way. This latter observation has interesting clinical implication, since suggests the possibility to get a maximum drug effect while limiting the amount or frequency of drug application. Then, we focused on the effect of HF on KF

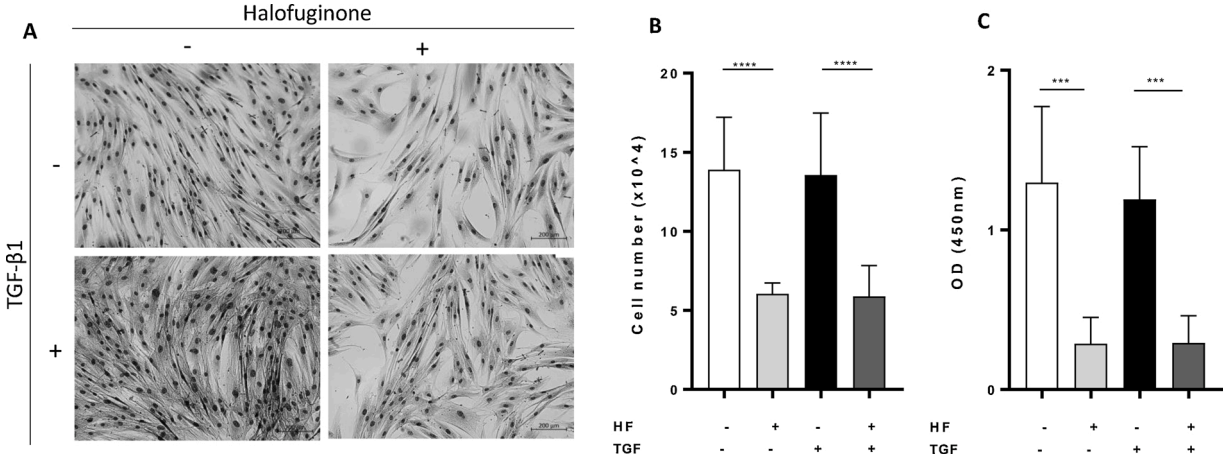

Fig. 2. Effect of Halofuginone on keloid fibroblast proliferation in pro-fibrotic culture condition. (A, B) Scale bar = 200 μm. TGF-β1 treatment do not enhance short term cell proliferation. Cell number is statistically lower when HF is used in control or pro-fribrotic medium (**** $p < 0.0001$). (C) BrdU incorporation assay shows that HF significantly blocked proliferation capacities of KF with or with combination with TGF-β1 (*** $p < 0.001$). Results are expressed as mean ± SD. Statistical analyses were performed using one-way analysis of variance followed by Tukey test for multiple comparisons.

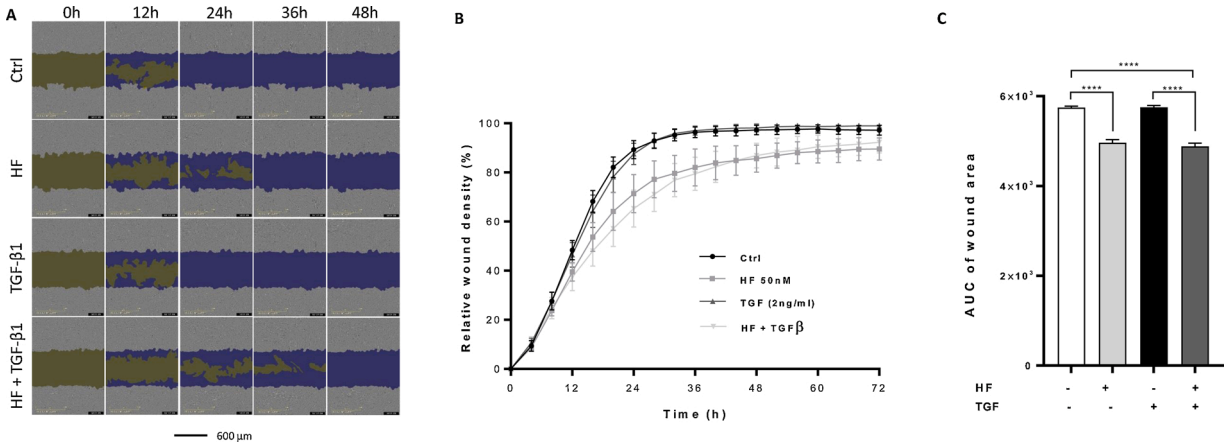

Fig. 3. (A) Representative images of wounded monolayer over time. Initial wound (blue area) and wound at different time (yellow) are represented for each culture condition, Scale bar =600 µm). (B) Relative Wound Density (RWD) was calculated from picture using Incucyte 2019B rev 2 software. The graph is representative of result obtain with one of the three keloid fibroblast strain. (C) Area under the curve was determined from RWD for each cell strain. HF significantly limits KF migration compared to control and pro-fribrotic medium (**** $p < 0.0001$). Results are expressed as mean ± SD. Statistical analyses were performed using one-way analysis of variance followed by Tukey test for multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

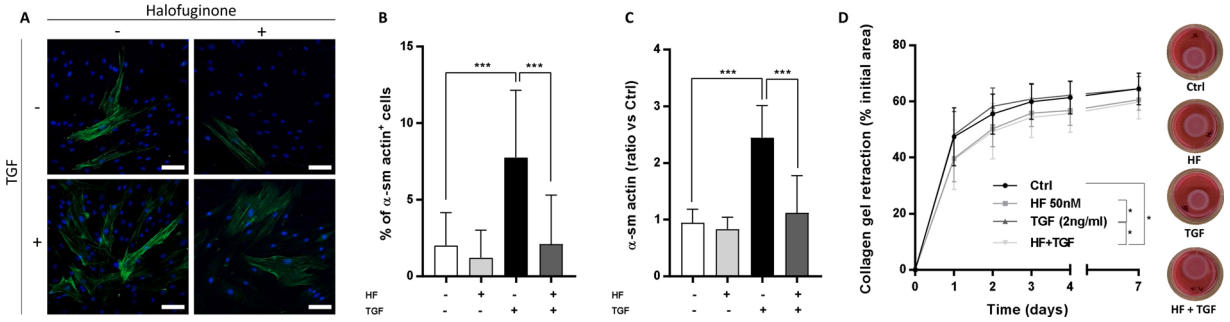

Fig. 4. HF prevents myofibroblast phenoconversion from TGF-β1 induction as well as ECM remodeling. (A, B) α-sm actin was stained in green and cell nuclei were counterstained with DAPI (blue). Scale Bar = 100 µm). TGF-β1 treatment led to a 2-to-3-time higher number of α-sm actin positive cells (*** $p < 0.001$). HF was able to significantly bring cells back to a basal level of α-sm actin expression (*** $p < 0.001$). (C) α-sm actin protein quantification confirmed that HF is able to reverse myofibroblasts activation induced by TGF-β1 (***$p < 0.001$). (D, E) Collagen gel retraction assay was used to investigate the effect of HF on ECM mechanical remodeling over time. HF was also able to slow down mechanical contraction ability of KF. The retraction ratio of HF treated gel increased slowly compared to control or pro-fribrotic condition (* $p < 0.05$). Results are expressed as mean ± SD. Statistical analyses were performed using one-way (B, C) or two-way (D) analysis of variance followed by Tukey test for multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

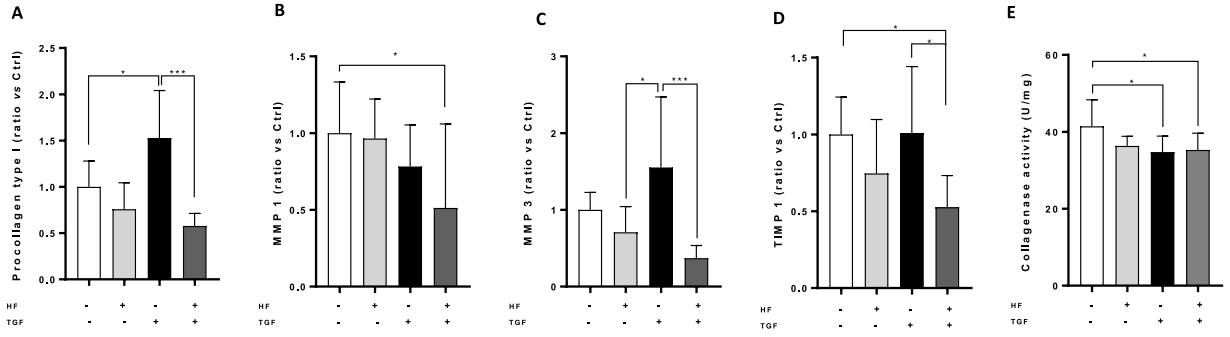

Fig. 5. Effect of HF on ECM deposition and degradation. (A) When HF was combined to TGF-β1, Halofuginone was able to restore the basal level of synthesized procollagen type I, while HF only did not modify the protein expression level compared to control (* $p < 0.05$ and ***$p < 0.001$). (B) HF decrease MMP1 protein expression in KF treated with TGF-β1 (* $p < 0.05$). (C) MMP3 expression level significantly increased in TGF-β1 induced KF culture. HF significantly reduced MMP3 protein synthesis in pro-fibrotic culture condition (* $p < 0.05$ and ***$p < 0.001$). (D) TGF-β1 induction increases TIMP1 protein synthesis by KF. HF significantly downregulates TIMP1 synthesis l (* $p < 0.05$). (E) TGF-β1 lightly reduces collagenase activity in treated keloid fibroblasts whereas HF has no impact whatever the culture conditions. Results are expressed as mean ± SD. Statistical analyses were performed using one-way analysis of variance followed by Tukey test for multiple comparisons.

properties involved in pathological wound healing and fibrosis (*i.e* proliferation and remodeling phases). Fibroblast migration and proliferation play a crucial role in pathological healing process by initiating the proliferative step of tissue repair. Fibroblast recruitment is regulated by a range of molecules involving numerous growth factors, cytokines and chemokines released by immune cells and fibroblasts during the inflammation step [38]. In a time-lapse monolayer scratch assay, we showed that HF limits KF migration over time even in a pro-fibrotic environment of culture (mimicked by the presence of TGF-β1), suggesting that HF could contribute *in vivo* to limit fibroblasts recruitment to the wound site and their over proliferation during keloid progression. Our results are consistent with those obtained by Zeng et al. [32] in the context of rheumatoid arthritis, where HF decreased F-actin expression and migration of synoviocyte fibroblast. Myofibroblasts are the ultimate key cell for physiological tissue repair as well as in generating fibrotic tissue in pathological situation [39]. Here, we provide evidence that myofibroblasts phenoconversion by TGF-β1 is prevented by HF. Same properties were previously described in corneal fibroblast by Nelson et al. [33] and Sheffer et al. [40]. During remodeling, myofibroblasts play an important role in tissue contraction [41]. We observed that HF did not totally blocked fibroblasts-mediated ECM contraction, but it slightly decreased the intensity of this phenomenon. Our findings are in agreement with those published by Tacheau et al. [42] who showed that HF concentration below 100 nM could decrease the capacity of fibroblasts to contract collagen gel without affecting fibroblast cell cycle. This suggests that HF potentially contributes *in vivo* to reduce the high number of contractile myofibroblasts observed in keloid tissue [10,11]. As a result, from fibroblasts hyper-proliferation and myofibroblasts differentiation, ECM deposition is the tip of the "fibrosis" iceberg. Two major approaches could be considered to revert the pathological ECM deposition: either by reducing ECM proteins synthesis or by triggering their degradation. As expected, TGFβ-1 promoted the synthesis of procollagen type I by keloid fibroblasts and the matrix accumulation. In agreement with previously published data [35–37], HF was able to reverse such TGFβ-1 stimulation by bringing back type I procollagen synthesis to control level. In keloid, the ratio between MMPs (matrix metalloproteinases) and TIMPs (tissue inhibitors of metalloproteinases) is critically disrupted [19–21]. As much as MMPs/TIMPs are regulated by TGFβ-1 [43], they could be targeted to restore the right degradation/synthesis balance [44–46]. While TGFβ-treated KF produced less MMP1 and HF treatment decreased the production of MMP3 and TIMP1, the zymography assay did not detect any changes in the overall collagenase activity.

In summary, our findings point that HF could contributes to keloid fibrosis resolution by restoring basal level of ECM deposition as well as by decreasing proliferation, migration and ECM protein synthesis of TGFβ-activated myofibroblasts.

In the future, we wish to further decipher the molecular mechanisms underlying the anti-fibrotic potential of HF in keloid formation. Indeed, at the molecular level, numerous studies showed that the action of HF on TGF-β signaling is mediated by the inhibition of TGFβ-dependent Smad3 phosphorylation, the elevation of the expression of the inhibitory Smad7 and by decreasing of TGFBII protein [47]. Besides, at the pharmacological level, HF is a competitive inhibitor of the glutamyl-prolyl-tRNA-synthetase [48], that subsequently inhibits production of high proline content (*i.e* collagen) and triggers the AAR (Amino Acid starvation Response). Beyond AAR, HF is also proposed as a time-dependently inducers of autophagy [49], and this is another major pathway that likely plays a role in keloid fibroblast regulation. Finally, HF is also able to prevent the development of Th17 cells by the mechanism of AAR activation [50], leading to reduction of IL-17 production, a cytokine which promotes fibrosis by inducing inflammatory response and regulating the activation of fibroblasts. The mutual contribution to fibrosis by a 'vicious circle' between Th17 cells and keloid fibroblasts has been recently documented by Lee et al. [51]. Thus, the contribution of HF to counteract IL-17 effect on KF definitely should also be investigated. Finally, and because no strongly relevant animal models for keloid are available yet [52], further investigation should be done on 3D complex models such as spheroids [53]. In this way, we will be able to consider keloid *in vitro* as a chronic inflammatory disease sharing close similarity to cancer [2]. This approach will take into account the effect of cytokines and growth factors other than TGFβ-1 only (*i.e* TNF-α, IL-1, -6, -10, -17…) [54,55], as well as the cell-cell interplay during fibrosis initiation (*i.e* KFs and macrophages).

5. Conclusion

Taken together, the present data indicate that Halofuginone has a strong potential to contribute to solve keloid disease issues. As an anti-fibrotic molecule, HF was able to regulate the fibrotic response induced by TGF-β1 in keloid fibroblasts by reverting their myofibroblast phenotype from the dark side of the wound healing process. Our study can be considered as a first milestone leading to move Halofuginone forward from bench to bedside as a new tool for keloid disease management.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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