Biochimie 171-172 (2020) 72–78

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Cartilage acidic protein 1 promotes increased cell viability, cell proliferation and energy metabolism in primary human dermal fibroblasts

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ARTICLE INFO

Article history:
Received 31 August 2019
Accepted 15 February 2020
Available online 18 February 2020

Keywords:
ECIS biosensor
Cell migration
Cell proliferation
hCRTAC1
In vitro scratch assay
Primary human fibroblasts

ABSTRACT

Cartilage acidic protein 1 (CRTAC1) is an extracellular matrix protein of human chondrogenic tissue that is also present in other vertebrates, non-vertebrate eukaryotes and in some prokaryotes. The function of CRTAC1 remains unknown but the protein's structure indicates a role in cell-cell or cell-matrix interactions and calcium-binding. The aim of the present study was to evaluate the *in vitro* effects of hCRTAC1-A on normal human dermal fibroblasts (NHDF). A battery of *in vitro* assays (biochemical and PCR), immunofluorescence and a biosensor approach were used to characterize the protein's biological activities on NHDF cells in a scratch assay. Gene expression analysis revealed that hCRTAC1-A protein is associated with altered levels of expression for genes involved in the processes of cell proliferation (*CXCL12* and *NOS2*), cell migration (*AQP3* and *TNC*), and extracellular matrix-ECM regeneration and remodeling (*FMOD, TIMP1, FN1*) indicating a role for hCRTAC1-A in promoting these activities in a scratch assay. In parallel, the candidate processes identified by differential gene transcription were substantiated and extended using Electric cell-substrate impedance sensing (ECIS) technology, immunofluorescence and cell viability assays. Our findings indicate that hCRTAC1-A stimulated cell proliferation, migration and ECM production in primary human fibroblasts *in vitro*.

1. Introduction

Dermal fibroblasts have a key role in maintaining and remodeling the extracellular matrix of the skin. They play a major role in the wound repair process, synthesizing collagen and elastin as well as many other so far unidentified ECM proteins and under mechanical influence can differentiate into myofibroblasts to facilitate wound contraction. Wound healing is delayed by impairment of the biosynthetic or functional response of fibroblasts [1]. Three principal phases of wound healing can be identified, inflammation, tissue formation and remodeling and it is regulated by both chemicals (e.g. growth factors) and biomechanical stimuli (stresses/strains) [2]. The tissue formation phase is characterized by re-epithelialization (restoration of the epidermis), the deposition of collagen and ECM proteins (restoration of the dermis), and restoration of the vascular network (angiogenesis). The final stage of healing is remodeling, which is characterized by reorganization of the neomatrix, complete wound closure and improvement of the mechanical properties of the scar [1]. Aging skin suffers analogous processes to healing skin and increasing the rate of skin turnover can improve the appearance and quality of skin [3]. The extreme importance of skin integrity for health and the increase in

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https://doi.org/10.1016/j.biochi.2020.02.008
0300-9084/

pathologies that compromise wound healing have driven a rapid technological advance in this area [4]. The *in vitro* scratch assays [5] is a well-established model used to rapidly screen for molecules that stimulate wound healing or have anti-aging properties. Enzymatic assays or flow cytometry have been used to assess active molecules for proliferative or apoptotic activities, qPCR can identify genes that regulate fibroblast migration/ECM production and electrical cell-substrate impedance sensing (ECIS) assays [6] give a direct measure of cell migration.

CRTAC1 is an ECM protein that was first isolated from human chondrogenic tissue [7] and subsequently described in other vertebrates, non-vertebrate eukaryotes and in some prokaryotes [8,9]. Two splice variants of CRTAC1, with a widespread tissue distribution have been described in humans (a long form hCRTAC1-A and a short form, hCRTAC1-B) [7], which are of interest because of their association with a diversity of diseases of hard and soft tissue [10–12]. The human splice variant, *CRTAC1B*, is specifically expressed in brain tissue and CRTAC1A is produced by human chondrocytes [7,13]. Studies in mice that only possess *CRTAC1B*, revealed that in the brain it is a key factor in lateral olfactory tract (LOT) formation, and it was named LOTUS (for LOT usher substance) [14] *CRTAC1B* is also expressed by mice chondrocytes but gene deletion fails to cause an overt phenotype, although in an osteoarthritic model it significantly inhibited cartilage degradation, osteophyte formation and gait abnormalities in females [15]. Structural analysis and modeling of CRTAC1 suggests it may have several yet unexplored functions, it belongs to a family of β-propeller proteins that share several conserved motifs/domains [8,9,16]. The structure of CRTAC1 has been well conserved during its evolution; it has an N-terminal integrin α chain like-domain, an ASPIC/UnbV domain and a C-terminal calcium-binding epidermal growth factor domain (EGF-Ca). Integrins are cell adhesion receptors that mediate cell-cell and cell-matrix interactions [17] and they interact with ECM proteins via the RGD (Arg-Gly-Asp) motif that is present in ECM proteins such as fibronectin [18], vitronectin [19] and osteopontin [20]. The integrins and their ligands have been implicated in epidermis structure and integrity as well as the regulation of epidermal wound functions and are therapeutic targets to promote wound healing or related pathologies [21,22].

The present study explored the potential function of hCRTAC1-A in human skin using primary normal human dermal fibroblasts (NHDF) and an *in vitro* scratch assay. We investigated the *in vitro* effect of hCRTAC1-A on cell proliferation and migration and extracellular matrix and remodeling processes related to wound healing. A battery of *in vitro* assays (biochemical and PCR-based), immunofluorescence and a biosensor approach were used to characterize the biological activities of hCRTAC1-A.

2. Materials and methods

2.1. Human skin cell culture

Primary normal human dermal fibroblasts (NHDF) isolated from human adult skin were purchased from Lonza Clonetics TM (Lonza, Walkersville, USA) [23]. Cells were cultured and maintained following the manufactures instructions and were grown in a humid 5% CO_2 incubator (Eppendorf, Hamburg, Germany) at 37 °C. Cell growth was performed in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Taufkirchen, Germany) with 4.5 g/l glucose, 110 mg/l sodium pyruvate and L-glutamine supplemented with 0.1% penicillin/streptomycin antibiotic mix (10.000 U:10 mg/ml, Sigma-Aldrich, Taufkirchen, Germany), 250 μg/ml sterile filtered 1:100 amphotericin B solution (Sigma-Aldrich, Taufkirchen, Germany) and 10% FBS (Sigma-Aldrich, Taufkirchen, Germany). For the *in vitro* scratch assays, the cells were maintained in Dulbecco's modified Eagle's medium free of FBS for 2 days before the experiments, in order to obtain arrest of proliferation.

2.2. hCRTAC1-A recombinant protein production

hCRTAC1-A recombinant protein was produced in a prokaryote as described before [8]. In brief, a recombinant clone of *E. coli* BL21 Star(DE3)-hCRTAC1-A was grown in large scale cultures (2 L) of superior LB with ampicillin (100 μg/ml) at 37 °C under constant agitation (250 rpm). Protein expression was induced at 30 °C for 12 h using 0.8 mM IPTG (Sigma-Aldrich, Taufkirchen, Germany) when the *E. coli* BL21 Star(DE3)-hCRTAC1-A cultures had an OD_{600nm} of 0.8. Bacterial protein extracts were prepared, and the insoluble protein fraction was used for protein purification by continuous elution electrophoresis using a Model 491 Prep Cell (Bio-Rad, Hercules, USA). The recombinant protein was refolded by dialysis and its structure was confirmed by circular dichroism and fluorescence. The concentration of the purified hCRTAC1-A used in bioassays was of 0.1 μg/ml.

2.3. Scratch assays

Scratch assays were performed in 6-well tissue culture plates (Sarstedt, Nümbrecht, Germany). Once the cells reached 95%–100% confluence, multiple scratches (four vertical and four horizontal) were created in each well using a 200-μL plastic micropipette tip. The wells were then washed twice with Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} to remove detached cells and refilled with the DMEM growth media free of FBS. Cell migration (scratch closure) was monitored and images were recorded using a Zeiss Axiovert 200 Microscope with a Zeiss AxioCam imaging system (Carl Zeiss MicroImaging, Inc, Thornwood, NY) immediately after the scratch (time 0) and then 24 h and 48 h thereafter.

2.4. Electric cell-substrate impedance sensing (ECIS) scratch assay

The ECIS system was used to electrically record the scratch closure in the presence and absence of hCRTAC1-A (recombinant protein). In brief, when the ECIS measuring electrode (thin gold film that carries a weak AC signal) is cell free then the impedance recorded is low, but as the cells attach and start to cover the electrode their membranes insulate and block the flow of current and this is detected as a rise in impedance. For this reason, impedance increases as the measuring electrode becomes covered by cells. For the scratch assays, an 8W1E device (IBIDI) which is specifically designed for cell migration assays and consists of a single 250 μM circular electrode covered in a thin gold film was used and impedance was recorded using an impedance analyzer (Fluke PM6306, Everett, USA). Briefly, one-hundred thousand NHDF cells maintained in DMEM culture medium were seeded and grown to confluence in the chamber of an 8W1E device. When the NHDF cells reached confluence, a small circular scratch was created by applying an electrical pulse (sigmoidal electrical pulses, 2 V at 40Kzh for 5 sec) using a signal generator (Keysight 33220A Agilent, USA). To remove all detached NHDF cells after application of the electric pulse the chamber of the 8W1E device was washed three times with DMEM free of FBS and then new medium with or without hCRTAC1-A (0.1 μg/ml) was added. Cell migration/circular scratch closure was monitored by measuring the resistance (Ohms (Ω)) at a frequency of 40 kHz for 24 h. Two independent experiments were performed and for each, one measuring electrode was used for the CRTAC1-A treatment and one for the control.

2.5. Immunofluorescent analysis

For immunofluorescence experiments, NHDF cells were grown to confluence on glass coverslips in the base of 24-well tissue culture plates (Sarstedt, Nümbrecht, Germany). The NHDF cell monolayer was scratched with a 250 μM diameter glass fiber and cell migration in the presence of hCRTAC1-A (0.1 μg/ml) or in DMEM medium (control) was monitored by immunofluorescence. Fluorescent compounds for specific cellular structures (Thermofisher, Waltham, USA) and an anti-tubulin antibody were analyzed at several time points: before, immediately after, or 8 and 24 h after inflicting the scratch. Cells were fixed in 4% paraformaldehyde (PFA) for 15 min and then permeabilized with 0.1% Triton™ X-100 for 15 min and blocked with 3% BSA for 1 h at room temperature. The cells were subsequently incubated with a mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich, Taufkirchen, Germany) at 1:300 dilution in 3% BSA for 1 h at room temperature and then labelled with goat anti-mouse IgG (H+L) secondary antibody conjugated to Alexa Fluor 488 (Thermofisher, Waltham, USA) using a 1:1000 dilution in 3% BSA for 1 h at room temperature. Actin filaments were stained for 20 min at room temperature in the dark with Texas Red®-X Phalloidin (Thermofisher, Waltham, USA) 1:40 diluted in PBS and nuclei were stained for 5 min at room temperature in the dark with a solution of 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 300 nM) (Acros organics, Waltham, USA). Preparations were mounted in glycerol mounting media and covered in a glass coverslip. Fluorescence images were obtained with a Leica DM IL microscope coupled to a Visicam HDMI 6 digital camera and photographs were analyzed using ImageJ software for image overlay.

2.6. Quantification of intracellular ATP levels

Intracellular ATP levels were quantified to assess the functional integrity of living cells. ATP determination was performed using a ViaLight HS BioAssay kit (Lonza). NHDF cells were incubated for 24 h with two concentrations (1 μg/ml, 0.1 μg/ml) of hCRTAC1-A and then ATP determined. The results were measured using a GloMax 20/20 single-tube luminometer (Promega). Three independent experiments were performed with eight replicates in each experiment.

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated from NHDF cells using a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany) and DNAse treatment was performed on the column, according to the manufacturer's instructions. The isolated total RNA (1 μl) was run on a 1% (w/v) TAE agarose gel to check integrity and quantified using a Nanodrop (Nanoquant, TECAN, Switzerland). cDNA was synthesized from 34 ng/ml of total RNA using a PrimeScript™ RT kit (Takara Bio Inc, Kusatsu, Shiga, Japan). The reaction mixture was incubated at 37 °C for 15 min and the enzyme was heat inactivated at 85 °C for 5 min. For this study 15 genes were analyzed, however here we present only those that exhibited significant changes.

2.8. RT-qPCR

RT-qPCR was used to characterize the molecular changes associated with hCRTAC1-A bioactivity during wound healing as described before [24]. In brief, target cDNAs were amplified using gene specific primers for selected genes involved in the process. A detailed list of the target genes and the respective gene-specific primer pairs (designed using Primer Express 1.5 software (Applied Biosystems, Darmstadt, Germany) is provided in supplementary Tables 1 and 2 Quantitative RT-PCR reactions were performed using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, Massachusetts, US), gene specific primers at a final concentration of 0.2 μM each and 1 μl of the cDNA as template. Replicate reactions were run for all reactions. The RT-qPCR cycle was performed in a CFX connect™ Real Time System (Bio-Rad Laboratories, Hercules, California, USA) and the cycle used was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer specificity and formation of primer-dimers was monitored by dissociation curve analysis and gel electrophoresis of the reaction products on a 4% (w/v) TBE agarose gel. For the relative quantification of gene expression, a modification of the comparative threshold cycle (Ct) method was used. The ratio of the target gene transcripts (X) in the sample of interest (S), normalized to beta Actin (β-actin) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the target gene transcripts in different treatments (R), was calculated as (1+E)-ΔΔCt, where ΔΔCt was calculated as [(CtX.S-CtACTB.S)-(CtX.R-CtACTB.R)]. All RT-qPCR reactions were performed on three biological samples. The experimental conditions that were analyzed by RT-qPCR and presented here, were: NHDF cells 24 h after scratch (C24h), NHDF cells 24 h after the scratch with hCRTAC1-A (H24h), NHDF cells 48 h after scratch(C48h), NHDF cells 48 h after scratch with hCRTAC1-A (H48h). Gene expression levels were normalized in relation to the intact cells and using the geometric mean of two reference genes (β-actin and GADPH).

2.9. Statistics

For the statistical analysis data is representative of three or more independent experiments. A one-way ANOVA test (one-way analysis of variance) at a 95% level of significance between all groups was performed followed by a two tailed unpaired *t*-test used for comparison between the control and protein supplemented groups. Statistical analysis was performed using GraphPad Prism version 7.0a for MacOSX, GraphPad Software, La Jolla California USA, www.graphpad.comand. $P < 0.01$ (**) and 0.05 (*) were considered significant.

3. Results

3.1. hCRTAC1-A increases fibroblasts viability in vitro

Intracellular ATP levels were used to determine the functional integrity and viability of NHDF cells treated with hCRTAC1-A. NHDF cells were incubated for 24 h in culture medium in the presence of two concentrations of hCRTAC1-A (1 μg/ml, 0.1 μg/ml). NHDF cells treated with 0.1 μg/ml hCRTAC1-A exhibited significantly ($p < 0.05$) increased ATP levels relative to untreated NHDF (control) and NHDF treated with 1 μg/ml (Table 1).

3.2. Electric cell-substrate impedance sensing (ECIS) scratch assay

The Electric cell-substrate impedance sensing (ECIS) system was utilized to study the behavior of hCRTAC1-A treated NHDF cells in real-time, *in situ* by measuring resistance of the cell layer on the sensing electrode. High values of resistance (data not shown) were obtained when the electrode was fully covered with cells, which was the starting point of the impedance measurement. A voltage sine wave was used to detach the cells from the surface of the sensing electrode and resulted in a drastic drop in resistance. Scratch recovery occurred when cells migrated onto the sensing electrode and was assessed by measuring the change in resistance over time. Absolute resistance measurements indicated that scratch recovery occurred approximately 12 h after the scratch

Table 1
Relative ATP levels (LUs) of NHDF cells after 24 h incubation with two concentrations of hCRTAC1-A (1 μg/ml, 0.1 μg/ml). Data are expressed as mean ± SEM for 8 replicates.

Cells treatments	ATP levels (LUs)	Significance compared to control
Untreated NHDF (control)	4,37 E+05 ± 1,32 E+04	NS
NHDF treated with 0.1 μg/ml	6,88 E+05 ± 1,41 E+04	<0.05
NHDF treated with 1 μg/ml	3,91 E+05 ± 1,31 E+04	NS

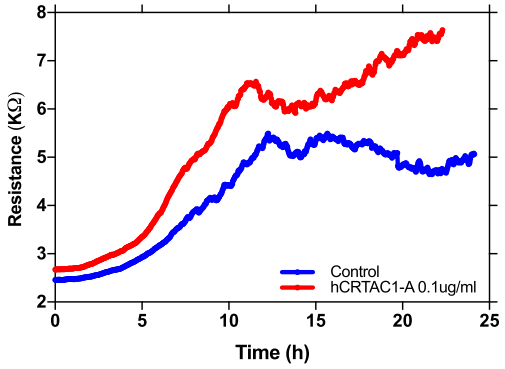

Fig. 1. A representative trace of the full electrical impedance recording as a function of the scratch recovery time of NHDF cells. The change in NHDF cell resistance during cell migration to repair the scratch in the control (medium only) is indicated in blue and in the treatment (medium supplemented with hCRTAC1-A, 0.1 μg/ml) is indicated in red. At least two independent experiments were performed, (results are represented as the mean), and the resistance was measured at a frequency of 40 kHz.

(Fig. 1) and was associated with a gradual increase in resistance across time. Subsequently, the resistance stabilized and reached a plateau with values similar to the resistance measured before scratching confirming that cell confluence was achieved across the sensing electrode (Fig. 1). In the presence of hCRTAC1-A, the increase in resistance was higher and faster than in the control cells (without hCRTAC1-A) and the cell number at confluence was also higher in hCRTAC1-A treated cells indicating a faster scratch repair in the hCRTAC1-A treated cells relative to the control (Fig. 1).

3.3. Immunofluorescent assay

Eight hours after scratch damage, NHDF cells treated with hCRTAC1-A (0.1 μg/ml) started to invade the cell free scratch area while in the control few cells were evident. 24 h after the scratch, NHDF cells treated with hCRTAC1-A and control NHDF cells totally covered the scratch area. However, there was an increase in the intensity of actin fluorescence in hCRTAC1-A treated NHDF cells in comparison with the control NHDF cells. The increase in actin fluorescence intensity was readily appreciated at higher amplifications (Fig. 2 G, H).

3.4. hCRTAC1-A affects the expression of skin-related genes during cell differentiation, cell proliferation, cell migration, extracellular matrix generation and remodeling

To understand, the molecular mechanisms associated with hCRTAC1-A bioactivity, an RT-qPCR platform for an array of key-genes was deployed. Genes involved in primary wound healing related processes in human skin, such as cell proliferation, cell migration, extracellular matrix (ECM) generation and remodeling were assessed. Specific gene transcript levels showed significant changes under the experimental conditions (Fig. 3).

Initially, genes involved in cell proliferation and cell migration related to wound healing were investigated. Analysis of these gene transcripts revealed that tenascin-C (TNC) was down-regulated in hCRTAC1-A treated NHDF cells 24 h after the scratch relative to the control ($p < 0.01$) at the same time point. In contrast, aquaporin 3 (*AQP3*) was significantly up-regulated in hCRTAC1-A treated NHDF cells relative to the control at 24 h after the scratch ($p < 0.05$). Transcripts of chemokine-12 (*CXCL12*) were down-regulated in hCRTAC1-A treated NHDF cells, 24 h after the scratch relative to the control at the same time ($p < 0.05$). Moreover, transcripts of Nitric oxide synthase 2 (*NOS2*) were up-regulated in hCRTAC1-A treated NHDF cells relative to the control 24 h after the scratch ($p < 0.01$). Analysis of extracellular matrix generation and remodeling-related genes revealed that fibronectin 1 (*FN1*) and collagen 3A1 (*COL3A1*) gene transcripts were significantly down-regulated in hCRTAC1-A treated NHDF cells, 24 h after the scratch relative to the control at the same time point ($p < 0.01$). In contrast, gene transcripts of metallopeptidase inhibitor 1 (*TIMP1*) as well as fibromodulin (*FMOD*) were up-regulated in hCRTAC1-A treated NHDF cells relative to the control 24 h after the scratch ($p < 0.05$).

4. Discussion

The aim of the present study was to evaluate the *in vitro* effects of hCRTAC1-A on normal human dermal fibroblasts and an *in vitro* scratch assay.

To start with, our data demonstrated that hCRTAC1-A increased the viability of human dermal fibroblasts *in vitro*. ATP intracellular levels in NHDF cells *in vitro* were increased by hCRTAC1-A indicating it enhanced cell viability, cell proliferation and energy metabolism, even though FBS-free cell culture medium (that normally inhibits cell proliferation) was used. According to previous reports, increased intracellular ATP is associated with higher levels of mitochondrial activity, energy metabolism and cell proliferation [25,26] and is also indicative of a lack of cytotoxicity [25].

ECIS results revealed as previously observed, increased impedance as the scratch closed [27–30] underlining the stimulatory role of hCRTAC1-A on cell migration. Moreover, it allowed the dynamics of scratch repair to be observed in real-time and clearly revealed the stimulatory effects of hCRTAC1-A on cell migration in the FBS-free cell culture medium. The results corroborated previous observations from an *in vitro* fish scale assay that revealed that piscine CRTAC1 accelerated the migration of fibroblasts/epidermal cells onto the cell culture plate [9]. By using immunofluorescence in parallel with the ECIS assays it was possible to demonstrate the cellular changes provoked by the addition of hCRTAC1-A to NHDF cells in the scratch assay (Fig. 2). The increased rate of cell migration in hCRTAC1-A at 24 h was associated with an increase in cytoskeletal actin, which fits well with the notion that F-actin is the main cytoskeletal element controlling cell shape [31–33] and migration [34]. In contrast, tubulin, the staining of which was more evident in control NHDF cells, 24 h post-scratch, has a regulatory role and controls the balance of rho-GTPase activities and therefore, F-actin assembly and actomyosin contractility [35].

In parallel, in order to explore the gene candidates of biological processes related to scratch repair, we performed differential gene transcription analysis. Specifically, we assessed the potential role of hCRTAC1-A in wound healing related pathways such as cell

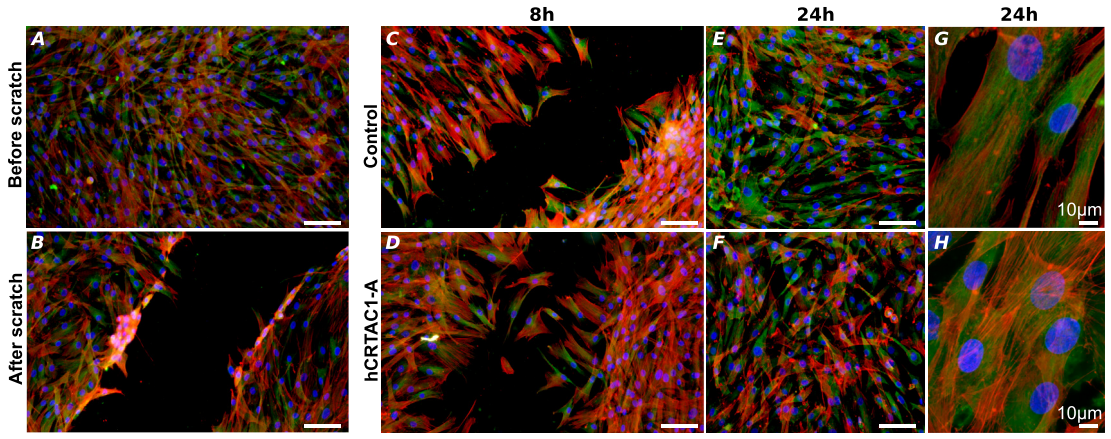

Fig. 2. Immunofluorescent images of NHDF cells during scratch recovery. NHDF cells were labelled with a monoclonal anti-α-tubulin antibody produced in mouse and detected with a goat anti-mouse IgG (H+L) Secondary antibody, Alexa Fluor 488 conjugate (alpha tubulin in green) and stained with Texas Red®-X Phalloidin (actin filaments in red) and DAPI (nuclei in blue). The images are representative of cells before (A) and immediately after scratching (B), 8 h after scratching in normal medium (control) (C) and in medium supplemented with hCRTAC1-A (0.1 μg/ml) (D); and 24 h after scratching in normal medium (control) [E and G (higher amplification)] and in medium supplemented with hCRTAC1-A (0.1 μg/ml) [F and H (higher amplification)]. The scale bars indicate 100 μm unless otherwise indicated. Images were obtained with a Leica DM IL microscope coupled to a Visicam HDMI 6 digital camera and analyzed using image J.

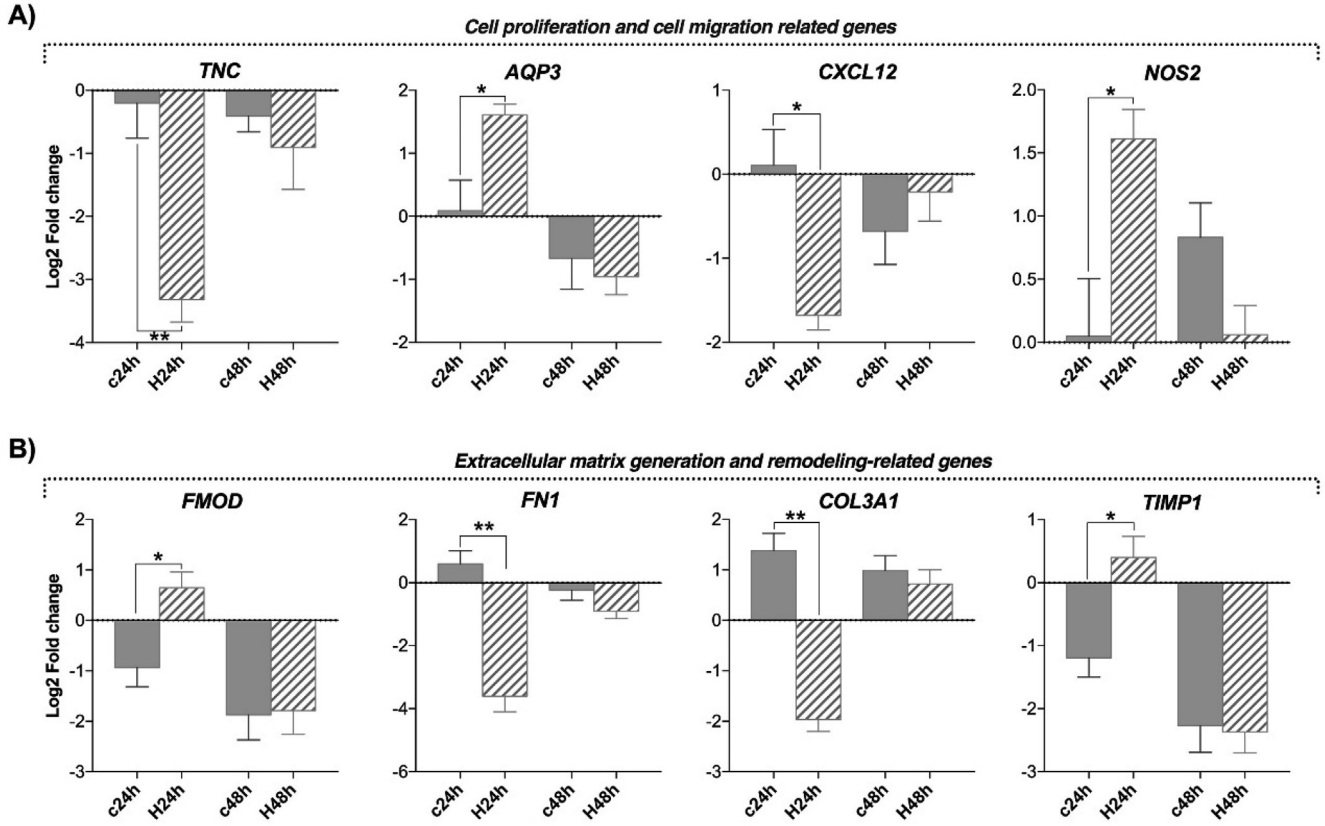

Fig. 3. Expression analysis of candidate genes in the presence and absence of hCRTAC1-A (0.1 μg/ml), in NHDF cells. Transcript levels of genes encoding proteins associated with cell proliferation and cell migration [Tenascin-C (TNC), human aquaporin-3 (AQP3), chemokine-12 (CXCL12) and nitric oxide synthase 2 (NOS2)] are shown in group A. Changes in gene transcripts encoding proteins associated with extracellular matrix regeneration and remodeling [Fibromodulin (FMOD), Fibronectin (FN1), (collagen 3A1 (COL3A1), and tissue inhibitors of metalloproteinases-1 (TIMP1)] are shown in group B. The experimental groups are: NHDF cells control 24 h after the scratch (c24h), hCRTAC1-A treated NHDF cells 24 h after the scratch (H24h), NHDF cells control 48 h after the scratch (c48h), hCRTAC1-A treated NHDF 48 h after the scratch (H48h). Data corresponds to the mean ± SEM of at least three independent experiments with six replicates each time. Gene expression levels were normalized in relation to intact cells and using the geometric mean of two reference genes (β-actin and GADPH). * and ** denote significantly different from C24h of $p < 0.05$ and $p < 0.01$ (two tailed unpaired student t-test), respectively.

differentiation, cell proliferation, cell migration, extracellular matrix generation and remodeling. Transcript analysis revealed that the presence of hCRTAC1-A modified the expression of a battery of genes that are associated with these pathways. In our study, we observed that *CXCL12* transcripts were down-regulated in hCRTAC1-A treated NHDF compared to the control 24 h after the scratch while transcripts of *NOS2* were up-regulated at the same time point suggesting that hCRTAC1-A potentially induces cell

proliferation in a *NOS2* related manner. According to previous studies, *NOS2* induces reepithelialization which is a cell proliferation-related to wound healing process [36–38]. On the other hand, *CLXL12*, is a cytokine that promotes fibroblast proliferation related to wound healing [39,40].

In terms of cell migration, our results revealed that *AQP3* transcripts were up-regulated 24 h after the scratch while transcripts of *TNC* were significantly down-regulated in the same cells at the same time point. These results potentially underline the stimulatory role of hCRTAC1-A in cell migration in an *AQP3*-dependent manner. Transcripts of *AQP3* and *TNC* have previously been reported to promote cell migration [41,42]. Specifically, Cao et al. [42] demonstrated that the up-regulation of *AQP3* plays an important role in wound healing through facilitating fibroblast migration by EGF/EGFR signaling. On the other hand, it has been reported that the up-regulation of *TNC* inhibits fibroblast migration [43,44].

In terms of the extracellular matrix and remodeling, *FMOD* has been reported to play important roles in modulating ECM organization [45] and collagen fibrillogenesis [46,47]. FN1 induces cell adhesion as well as extracellular matrix (ECM) formation during wound healing [48–50]. COL3A1 is a major component of ECM, during the wound healing process and is subsequently replaced by type I collagen which has a higher tensile strength [51], while TIMP1 is responsible for the inhibition of degradation of matrix-associated material not involved in the remodeling process [52]. In our study, transcripts of *COL3A1* were down-regulated in hCRTAC1-A treated NHDF 24 h after the scratch, suggesting that the protein may be involved in the degradation of collagen type III as part of the final stage of the repair process. *FN1* gene transcripts followed a similar trend to *COL3A1*, corroborating the previously observed interdependence of FN1 and collagens [53,54]. The up-regulation of *FMOD* and *TIMP1* 24 h post-scratch appears to indicate a positive role for hCRTAC1-A in ECM generation and may explain the more rapid closure of the scratch by the NHDF cells.

5. Conclusions

In conclusion, our data support, for the first time, the significant *in vitro* effects of hCRTAC1-A on NHDF cells. The actions of hCRTAC1-A on a diversity of processes linked to wound healing lead us to speculate that this matrix protein is a regulatory molecule involved in maintaining tissue homeostasis. The *in vitro* findings of this study pave the way for further studies into the effects of hCRTAC1-A on diverse aspects of skin function. Future analysis directed at global changes in gene transcription using RNA-Seq analysis should provide greater insight into CRTAC1-A actions and will be a fruitful area of research in the future.

Authors contributions

DMP and SL conceived the work, planned and oversaw the experiments. SL, JCRC carried out the collection of the data of the experiments with the scratch assay and the RT-qPCR analysis. LA produced the native recombinant CRTAC1-A. SL, RCF, AM and HG carried out the collection of data of the experiments on ECIS. RCF carried out collection of data of the immunofluorescence-based experiments. SL, RCF, JCRC and DMP analyzed and integrated the datasets and drafted the manuscript. All the other authors critically read and contributed to improve the MS.

Funding

This work was supported by the H2020-MSCA-RISE-2015, ALGAE4A-B, grant agreement number 691102 and EMBRIC (European Union's Horizon 2020 research and innovation programme under grant agreement No 654008). The Portuguese Foundation for Science and Technology (FCT) provided CCMARs core funding (UIDB/04326/2020) and additional funds came from the operational programmes CRESC Algarve 2020 and COMPETE 2020 through the project EMBRC.PT ALG-01-0145-FEDER-022121. JCRC was supported by FCT (UIDB/04326/2020). RCF and LA were funded by FCT, under the "Norma Transitória" -DL57/2016/CP1361/ project CT0020 and CT0011, respectively.

Declaration of competing interest

We have no conflict of interest to declare.

Acknowledgements

Special thanks to Ana Patrícia Gago Mateus, PhD and Rita Costa, PhD for their help on the execution of qPCR analysis.

Appendix A. Supplementary data

Supplementary data of this article can be found online at https://doi.org/10.1016/j.biochi.2020.02.008.

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