

ORIGINAL ARTICLE

Electric currents of 448 kHz upregulate anti-senescence pathways in human dermal fibroblasts

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Funding information

Fundación para la Investigación Biomédica del Hospital Ramón y Cajal, through Project FiBio-HRC, Grant/Award Number: 2015/0050

Abstract

Background: Currently, finding new therapeutic strategies that reduce skin aging is a challenge for dermatologists and aesthetic doctors. In recent years, physical therapies have been included in the options for antiaging treatments; however, the biological bases of such treatments have scarcely been studied. One of these physical therapies is capacitive-resistive electric transfer (CRET) therapy. Previous studies have shown that subthermal treatment with CRET promotes the proliferation and migration of various cell types involved in skin regeneration, such as human ADSC (stem cells), fibroblasts, or keratinocytes.

Objective: This study investigates the effects of in vitro treatment with CRET-Std (standard, non-modulated signal) or CRET-Mod (modulated signal) on cell proliferation and migration, markers of aging, and extracellular matrix production.

Methods: Three types of human dermal fibroblasts were used: neonatal fibroblasts (HFn), replicative senescent fibroblasts (HFs), and adult fibroblasts (HFa). The effects of electric stimulation on cell proliferation and migration were studied through XTT and wound closure assays, respectively. The expression of the aging marker β -galactosidase was assessed using a colorimetric assay, whereas immunoblot, immunofluorescence, and ELISAs were carried out to analyze the expression levels of migration, aging, and extracellular matrix proteins.

Results: The treatment with CRET-Std increased HFn and HFa proliferation, as well as migration in the three types of fibroblasts studied compared to those of the controls. Conversely, CRET-Mod did not modify either of these two processes with respect to the controls. Additionally, CRET-Std also reduced the cellular senescence markers β -gal, vimentin, p53, and p21 in all three types of human skin fibroblasts. In addition, the application of CRET-Std also induced fibronectin production in HFn and was able to stimulate ECM neocollagenesis.

Conclusion: CRET treatment improves a number of functions related to migration and proliferation, and it reduces age-related cellular changes in human dermal fibroblasts. Therefore, the use of this CRET therapy to reduce the signs of dermal aging and to promote tissue regeneration could be of interest.

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KEYWORDS

age, anti-aging products, cell signaling, fibroblasts, radiofrequency

1 | INTRODUCTION

Skin aging is characterized by a progressive loss of its integrity, resulting in an impairment of its thickness, elasticity, and firmness, which generates the appearance of wrinkles. In the maintenance of skin homeostasis, fibroblasts play an essential role since they produce the main components of the extracellular matrix (ECM), such as collagen, elastin, and glycosaminoglycan.¹ During skin aging, dermal fibroblasts decrease their ability to express these ECM components and increase the synthesis of metalloproteinases.

In recent years, the interest in delaying or treating the signs of skin aging has increased. The most common therapies to treat these signs can be grouped in invasive and noninvasive methods. Invasive strategies, despite their effectiveness, are less popular than noninvasive ones because they can cause complications such as the generation of neuropathic pain and/or skin infections in patients. In addition, they require specialist medical personnel for their application, which significantly increases their cost. For this reason, both professionals and patients are looking for new noninvasive antiaging therapies.²

Noninvasive therapies are based on the application of various types of energy, such as laser light, ultrasound, or radiofrequency radiation, to treat the skin. Radiofrequency therapies generate a thermal increase in the target tissues which is induced by the resistance of the tissues to the passage of the electromagnetic signal. These RF therapies have advantages over other common physical therapies, such as those based on laser light, because the electric current is converted into thermal energy at the level of the dermis or subcutaneous tissue, which reduces the complications frequently associated with heating and damage to the epidermis. Additionally, RF-based devices do not lose energy through diffraction, absorption, and scattering as laser light devices do. Furthermore, RF therapies do not interfere with epidermal melanin and therefore can be applied to any type of skin, regardless of its phototype.³ When RF therapies are applied at high power levels (RF ablative therapies), localized thermal lesions are generated which, when healed, promote tissue regeneration. On the other hand, if RF is used at lower power levels it is unable to cause thermal cell necrosis,⁴ and a controlled denaturation of collagen occurs, which activates regenerative processes in the skin. The new generation of RF skin therapies uses even lower power levels, and this has reduced the complications and side effects to a minimum.⁵ An example for such an RF therapy operating at low power levels is the capacitive-resistive electric transfer (CRET) therapy. This therapy induces thermal increases in the target tissues by generating a radiofrequency current of between 0.4 and 0.6 kHz.⁶ In aesthetic medicine, CRET therapy has been used for fatty tissue reduction and body shaping⁷; with regard to skin aging, this CRET stimulation has been shown to be effective in reducing the

appearance of wrinkles.⁸ In addition, previous studies in our laboratory revealed that the effects of CRET therapy may not be limited exclusively to those caused by the hyperthermia generated by the passage of this current. In these studies we could show that in vitro stimulation with a RF current under non-hyperthermic conditions is capable of inducing changes in the proliferation, migration, cytokine production, and differentiation of various types of human cells, both cancerous and noncancerous.^{9–14} However, the mechanisms through which these RF therapies act at the skin level are still poorly understood. For this reason, the objective of this work is to focus on investigating the biological bases that underlie the described clinical effects of CRET therapy on the processes of senescence and tissue regeneration, under subthermal conditions.

2 | MATERIALS AND METHODS

2.1 | Cell culture

As fibroblasts from neonatal individuals express levels of the biomarkers of senescence and tissue regeneration that differ from those of adults,¹⁵ it is relevant to investigate the potential action of CRET therapy on their expression in fibroblasts in different states of senescence.

Therefore, in the present study three types of fibroblasts were used: HFn, human dermal fibroblasts isolated from neonatal (14 days or less) foreskin (C-004-05; Thermo Fisher); HF_s, human senescent fibroblasts obtained by repeated passaging of HFn (replicative senescence); and HF_a, human dermal fibroblasts isolated from adult skin (older than 40 years; C0135C; Thermo Fisher) (intrinsic senescence). To analyze replicative senescence, fibroblasts from neonatal foreskin were subjected to repeated passaging, and divided in young (<50 population doublings), presenescent (>50 doublings), and senescent (>80 doublings) cells. The fibroblasts were fully senescent when their division capacity was almost exhausted, and their morphology changed to flattened and enlarged or very elongated cell shapes. The cultures were maintained in a medium composed of high-glucose DMEM (Biowhittaker, Lonza) supplemented with 10% inactivated fetal bovine serum (Gibco), 1% glutamine, and 1% penicillin–streptomycin (Gibco). The cells were incubated in T75 flasks at 37°C in an atmosphere containing 5% CO₂ and were subcultured once a week.

2.2 | Electric treatment

The procedure for RF exposure has been described in detail elsewhere.¹⁶ Briefly, the cells were plated in 60 mm Petri dishes (Nunc),

except when they were used in immunofluorescence assays; in the latter case, the cells were seeded on glass coverslips placed inside Petri dishes. Three or four days after seeding, depending on the experiment, pairs of sterile stainless steel electrodes designed ad hoc for in vitro stimulation were inserted into all the Petri dishes and serially connected. Only the electrodes corresponding to plates intended for electric stimulation were energized using a signal generator (INDIBA Deep Care ELITE NS model, INDIBA®), while the remaining plates (controls) were sham-treated simultaneously in the same CO₂ incubator. Two different current signals were studied: a standard 448kHz sinusoidal, non-modulated signal (CRET-Std) and a 20kHz, 40% amplitude modulation of the 448kHz signal (CRET-Mod). The intermittent stimulation pattern consisted of 5-min pulses of sine wave current delivered at a subthermal density of 100µA/mm², separated by 4-h interpulse lapses and administered during a total of 6, 12, 24, or 48h.

2.3 | Cell viability/proliferation assay

Cell viability/proliferation was measured using XTT assays (Roche, Switzerland). The HF_n, HF_s, and HF_a cultures were seeded at densities of 5500 cells/cm² and incubated for 3 days. After 48h of CRET-Std, CRET-Mod, or sham treatment, the cells were incubated with the tetrazolium salt XTT (following the manufacturer's instructions) at 37°C in a 6.5% CO₂ atmosphere for 2h. The colored formazan compounds formed were quantified with a microplate reader (TECAN) at a 492nm wavelength.

2.4 | Cell cycle analysis

This procedure has been described in detail elsewhere.^{14,16} Different phases of the cell cycle of cultured HF were analyzed using flow cytometry (FACScan Mod. FACScalibur, Becton Dickinson). At 0, 24, or 48h after the onset of the CRET-Std treatment, the cultures were trypsinized and both the floating and the adherent cells were collected. The samples were centrifuged at 1200rpm for 5min, fixed in 70% ethanol, and stained with a solution containing 3.4mM sodium citrate, 20µg/mL propidium iodide (Boehringer), and 100µg/mL RNase A (Boehringer). CellQuest 3.2 software was used for data acquisition (20000 events per sample) and analysis.

2.5 | Migration assay

Cell migration was measured using a standard wound-closure assay. Briefly, the HF_n, HF_s, and HF_a (31000 cells/cm²) were seeded in Petri dishes and incubated at 37°C. Confluent monolayers of all three cell types were obtained at 4 days post plating. Then, a wound was scratched on the confluent monolayer of each of the plates using a plastic pipette tip and the cells were rinsed with PBS to eliminate debris. Next, the cultures were maintained in completed high-glucose

DMEM and treated with CRET-Std for 6 or 12h, while the remaining dishes were sham-treated for the same intervals. The cells were observed immediately after wounding (0h) and again at 6 and 12h, and phase contrast images at three equidistant points of the scratching area were taken using a digital camera (Nikon DS-Ri2) coupled to an inverted microscope (Nikon Eclipse Ts2R) in the wounds. The wound closure rate was determined by measuring the distance between the edges of the scratches. Each digital image was analyzed using Photoshop software (Adobe Photoshop CS3 Extended version 10.0).

2.6 | β-galactosidase activity analysis

β-galactosidase activity (β-gal) was evaluated using a mammalian β-galactosidase assay kit (Cat no: 75707; Thermo scientific). Cultures of the three cell types were seeded at a density of 31000 cells/cm². After 4 days post-seeding, the cultures were treated with CRET-Std for 48h. At the end of 48h of CRET-Std or sham treatment, the cells were trypsinized and processed for the assay, following the manufacturer's instructions. As the proliferation rate of HF_n is much quicker than that of HF_s, the total enzyme activity values cannot be compared without normalization to the total protein in the sample. For this, an additional analysis was performed to determine the amount of total protein in the samples, using the BCA assay (Pierce™ BCA Protein assay kit; Thermo Scientific). In this study, the β-gal assay values are expressed as values normalized to the amount of protein determined using the BCA assay.

2.7 | Immunofluorescence staining for vinculin, vimentin, p53, and Type I collagen

HF_n, HF_s, and HF_a cultures were seeded at a density of 5500 cells/cm². After 48h of CRET-Std, CRET-Mod, or sham treatment, the samples were fixed with 4% paraformaldehyde. Cells for vinculin, vimentin, and p53 staining were treated with CRET-Std whereas cells for collagen I staining were treated with both signals, CRET-Std or CRET-Mod. The samples were incubated overnight at 4°C with anti-vinculin monoclonal mouse antibody (1:800; Sigma Aldrich), anti-vimentin monoclonal mouse antibody (1:200; Thermo Fisher), anti-type I collagen polyclonal rabbit antibody (1:500; Novocastra), or anti-p53 monoclonal mouse antibody (1:800; Novocastra, Leica). Afterward, the samples were fluorescence-stained with Alexa Fluor® 488 goat anti-rabbit IgG or Alexa Fluor® 568 goat anti-mouse IgG, and simultaneous counterstaining of the cell nuclei and mounting was performed with ProLong-DAPI (Invitrogen). Photomicrographs from monolayers of each of the three cell types were taken, using an inverted fluorescence microscope (Nikon Eclipse Ts2R) coupled to a digital camera (Nikon DS-Ri2) and the images were analyzed using computer-assisted image analysis software (NIS-Elements, NIKON). To assess the collagen staining, RGB fluorescence thresholds (MCH mode) were set before analysis and applied to all the images. The measurement of the fluorescence intensity of the green channel corresponding to the signal provided by the Alexa

green was taken. In the case of p53 analysis, the number of positive cells was also counted, taking into account the previously set fluorescence threshold of the red channel. For both analyses, the values obtained were normalized to the number of cells per field. At least three replicates of each experiment were performed.

2.8 | Immunoblots for p21, p53, vinculin, vimentin, MMP1, and MMP9

HFn, HF_s, and HF_a were plated at a density of 31 000 cells/cm² and incubated at 37°C until the fourth day post-seeding. The cultures received CRET-Std treatment or were sham-treated for 12, 24, or 48 h, and at the end of the electric treatment the cells were lysed for protein extraction. The lysis and immunoblot procedure has been described in detail elsewhere.¹⁷ Briefly, cells were detached with a cell scraper in cold PBS and collected. Cell suspensions were centrifuged at 1200 rpm for 5 min, and the culture media was aspirated carefully. Pellets were resuspended in 10 µL of ice-cold RIPA lysis buffer in microcentrifuge tubes and incubated for 20 min at 4°C. Then, samples were centrifuged at 12000×g for 20 min at 4°C. Supernatants containing the soluble protein were collected in a new tube kept on the ice for further analysis. The protein samples (100 µg protein aliquots) were separated using 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Amersham, Buckinghamshire). The blots were incubated at 4°C overnight in anti-p21 monoclonal rabbit antibody (1:1000, Sigma), anti-p53 monoclonal mouse antibody (1:1000, Novocastra), anti-vinculin monoclonal mouse antibody (1:1000, Sigma Aldrich), anti-vimentin monoclonal mouse antibody (1:1000, Thermo Fisher), anti-MMP1 monoclonal rabbit antibody (1:1000, Abcam plc), and anti-MMP9 monoclonal rabbit antibody (1:1000, Abcam). Anti-GAPDH monoclonal mouse antibody (1:1000, Santa Cruz Biotechnology) was used as a loading control. The membranes were incubated for 1 h at room temperature with Amersham ECL anti-rabbit IgG (1:10000, GE Healthcare) and with Amersham ECL anti-mouse IgG (1:15000, GE Healthcare). The blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Then, the membranes were scanned with a ChemiDoc™ MP Imaging System (ref: 12003154; BioRad). The obtained bands were evaluated using densitometry (PDI Quantity One 4.5.2 software, BioRad). At least five experimental replicates were conducted per protein and cell type. All the values were normalized to the loading control.

2.9 | ELISAs for hyaluronic acid and fibronectin

Hyaluronic acid and fibronectin production were determined using the Quantikine ELISA Hyaluronan immunoassay (Cat no: DHYAL0, R&D systems) and the Quantikine ELISA Human Fibronectin immunoassay (Cat no: DFBN10, R&D systems). Cultures of the three cell types were seeded at a density of 80 000 cells/cm². After 4 days

post-seeding, the cultures were treated with CRET-Std or CRET-Mod or were sham-treated for 48 h. At the end of 48 h of electric treatment, the culture medium was collected and processed for the assay following the manufacturer's instructions. As for the β-galactosidase assay, additional analysis using the BCA assay was performed to determine the amount of total protein in the samples (Pierce™ BCA Protein assay kit; Thermo Scientific), and the values are expressed as normalized to the amount of protein determined using the BCA assay.

2.10 | Statistical analysis

All the procedures and analyses of the treatments were conducted in blind conditions. At least three independent replicates were conducted per experiment, cell type, and treatment; the results are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). Two-tailed unpaired Student's *t*-tests were applied using GraphPad Prism 6.01 software (GraphPad Software). Differences of *p* < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Effects of CRET-Std and CRET-Mod on cell proliferation

3.1.1 | Proliferation assays

Prior to carrying out the proliferation assays on the electrically treated samples, the proliferative capacity under control conditions of the three types of fibroblasts used was compared. In the absence of electric treatment, the HFn showed high proliferation rates, requiring only 67 h to fully double their population. In contrast, the HF_s remained quiescent or had very low rates of proliferation, requiring up to 400 h to double their population. On the other hand, the adult fibroblasts presented a high proliferative capacity, although it was lower than that of the HFn, with a doubling time of 105 h.

CRET treatment with the standard CRET signal (CRET-Std) significantly increased the proliferation of the HFn and HF_a, while it also significantly reduced the proliferation of the HF_s compared to those of the controls. Conversely, the treatment with the modulated signal (CRET-Mod) did not modify the proliferation rate of any of the three types of fibroblasts analyzed (Figure 1). Due to the lack of response to CRET-Mod, the subsequent analyses of the effects on the proliferation, migration, and markers of cellular aging were carried out only with the CRET-Std signal.

3.1.2 | Study of the cell cycle using flow cytometry

After 24 h of CRET-Std treatment, the proportion of cells in the S phase in the HFn increased significantly, while the proportion of

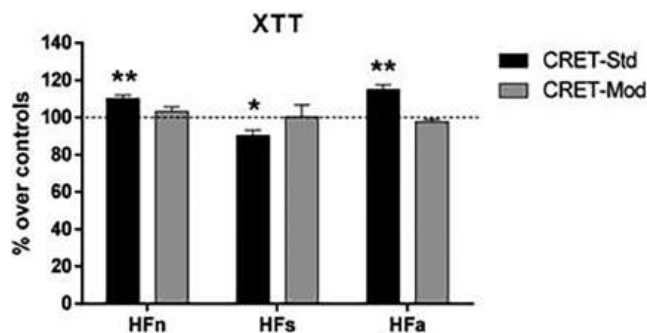


FIGURE 1 XTT assay. Proliferation of HFn, HF, and HFa intermittently treated for 48 h with modulated (CRET-Mod) or standard (CRET-Std). Plot bars show mean $\pm$ SEM normalized to the control. Between three and five experimental replicates per cell type. *: $0.05 > p \geq 0.01$; **: $0.01 > p \geq 0.001$; Student's *t*-test.

cells in G0/G1 decreased. At the end of the treatment ($t=48$ h), a reversal of the effect was observed, with a decrease in the proportion of cells in the proliferative phases S and G2/M and a slight, though not statistically significant, increase in the rate of cells in G0/G1. The increase in apoptosis was statistically significant at both 24 and 48 h of treatment. However, as the absolute percentage of apoptotic cells in the population did not exceed 1%, this effect was not considered relevant to cell viability (Figure 2A,B).

In the HF, the CRET-Std treatment for 24 h significantly increased the proportion of cells in the G0/G1 phases compared to that of the controls and also significantly decreased the rate of cells in the S phase. After 48 h of treatment, no significant differences were observed with respect to the controls. This absence of a response at 48 h could be attributed to the high quiescence of the cells (>90% of the total cells) at the end of the assay. The analysis of the results of the apoptosis in the HF was not conclusive due to the high variability of the data obtained (Figure 2C,D).

Regarding the HFa, after 24 h of CRET-Std treatment, the cell fraction in the G0/G1 phase decreased significantly compared to that of the controls. The cells in the proliferative S and G2/M phases showed a general tendency to increase, although this effect was only statistically significant for those in the S phase. As with the HFn, the treatment induced a very slight proapoptotic effect (<1.5% of the population) and was not relevant in terms of cell viability (Figure 2E,F).

3.2 | Effects of CRET-Std on cell migration

3.2.1 | Wound-healing assay

Migration was studied using a wound-closure assay at 0, 6, and 12 h of CRET-Std treatment. At 6 h, all three cell types revealed a significant acceleration of wound closure compared to the respective controls. At 12 h, as the gap was closed in the HFn and HF cultures, no differences could be observed between the control and the CRET-Std-treated culture. Conversely, in the HFa culture the gap was still

open and the CRET-Std treatment induced an acceleration of its closure compared to that of the control (Figure 3A,B).

3.2.2 | Vinculin expression and distribution

One of the proteins involved in the migratory process is vinculin, which modifies its expression depending on the state of cellular senescence. Under control conditions, the HFn vinculin appeared as numerous, large, elongated plaques, forming focal contacts at the cell periphery. In the HF, the amount of labeling for vinculin was lower, and it was located on the cell membrane in the form of thin plaques, which were shorter than those observed in the HFn, or as small, rounded dots. The HFa cells showed a typical fibroblastic morphology, with highly elongated cytoplasm and multiple extensions. In these cells, vinculin labeling was intense and formed elongated plaques, although in lower quantities than in the HFn; these were more associated with the nucleus and had fewer focal adhesions. The analysis of the immunofluorescence images did not reveal significant changes in cell morphology or in the vinculin labeling pattern in any of the three types of fibroblasts treated for 48 h with CRET-Std when compared to the controls (Figure 3C). On the other hand, the immunoblot analysis of the vinculin expression revealed a statistically significant increase with respect to the control in the treated HFa, but not in the HFn or HF (Figure 3D,E).

3.3 | Effects of CRET-Std on markers of cellular senescence

3.3.1 | β -galactosidase activity

In order to evaluate the potential capacity of CRET-Std to prevent the effects of cellular senescence induced by endogenous factors, the activity of the enzyme β -galactosidase was analyzed. The results revealed that the control cultures with the highest β -gal activity were the HFa, followed by the HF, and then the HFn (Figure 4A). CRET-Std induced a significant decrease in the β -gal activity of the HF and HFa, while in the treated HFn only a very slight and nonsignificant decrease in enzyme activity was observed (Figure 4B).

3.3.2 | Vimentin expression and distribution

In the immunofluorescence images, the labeling for vimentin in the HFn controls appeared as thin fibers creating a network of filaments throughout the cell cytoplasm. The CRET-Std treatment did not change this fibroblastic morphology. In the control HF, the cell labeling for vimentin was considerably higher and showed shorter, thicker, and more disorganized fibers than in the HFn. As with the HFn, CRET-Std did not modify the cell

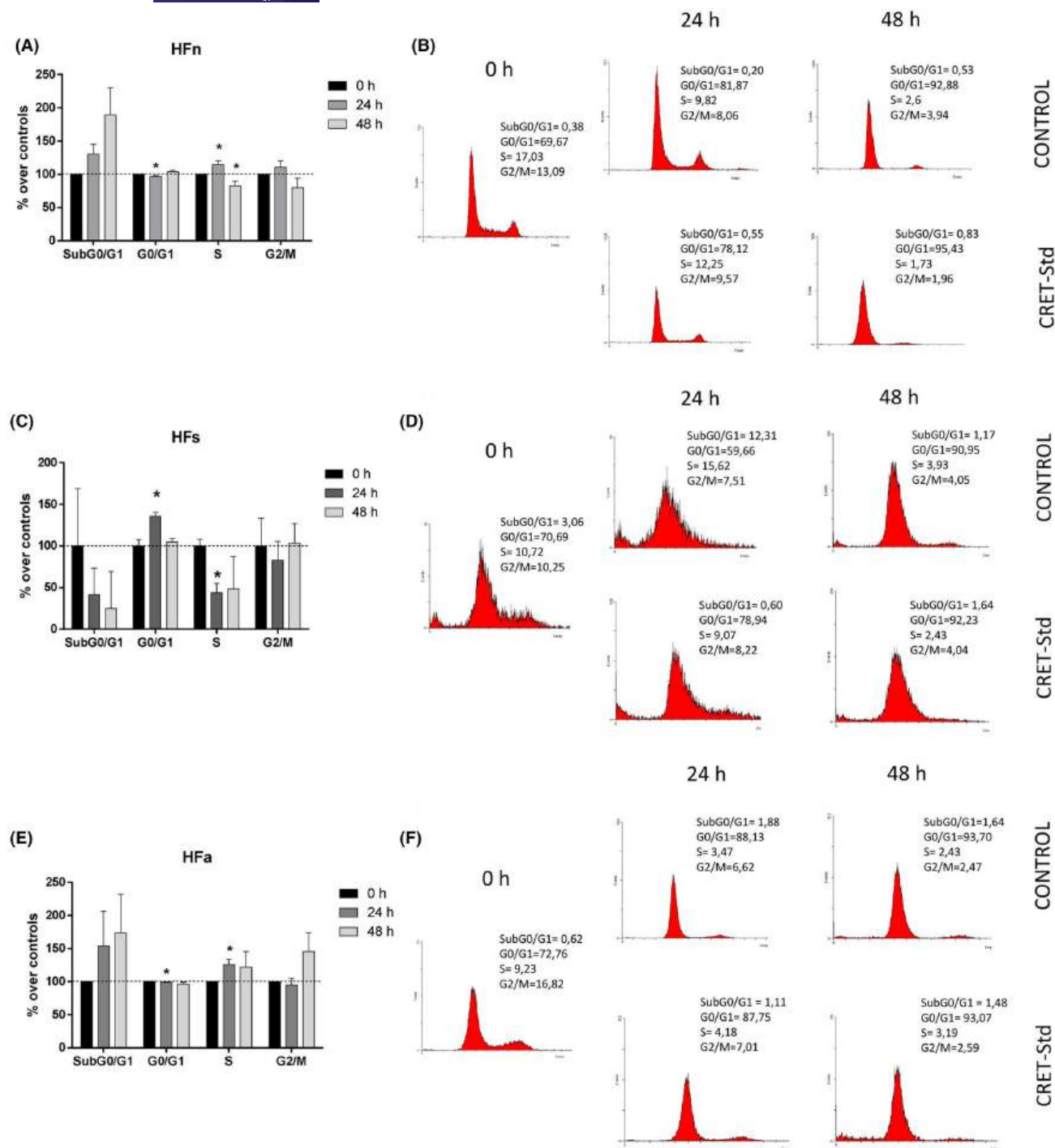


FIGURE 2 Flow cytometry. Effect on apoptosis and the cell cycle of HFn, HF, and HFa at 0, 24, or 48 h of CRET-Std treatment. (A, C, and E) Flow cytometry analysis of HFn, HF, and HFa controls or treated with CRET-Std. Data are presented as percentages of the mean $\pm$ SEM of ≥ 3 experimental replicates and are normalized to the respective controls. *: $0.05 > p \geq 0.01$. (B, D, and F) Representative flow cytometry histograms of HFn, HF, and HFa controls or treated with CRET-Std.

morphology or vimentin labeling pattern in these senescent cultures. In the control HFa samples, the labeling for vimentin was more intense than in the HFn, with a large majority of the cells labeled. While in some of these cells, the cytoplasmic labeling was distributed in very long and thin retiform extensions, in other cells the labeling was located exclusively in the perinuclear region of the cytoplasm. CRET-Std reduced the amount of labeled

cells, preferentially locating the labeling in the perinuclear space or forming long fibers, with weaker labeling than in the controls (Figure 4C).

The immunoblot study of the treated samples did not detect changes with respect to the controls in the expression of vimentin in the HF cells, while it revealed a statistically significant underexpression of the protein in the HFn and HFa (Figure 4D,E).

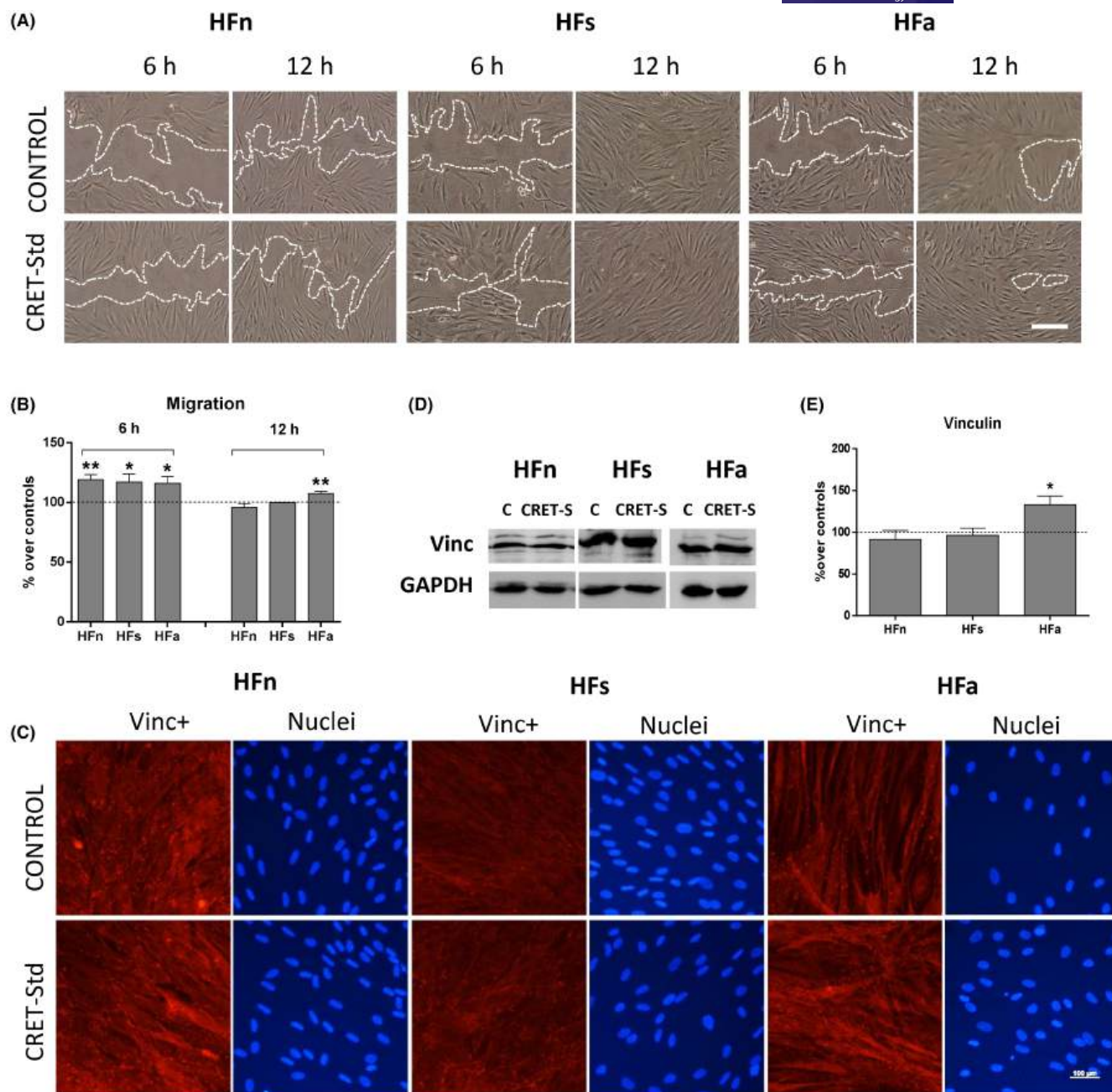


FIGURE 3 Migration analysis. (A) Wound-healing assay; representative micrographs of the wound at $t = 6$ or 12 h of CRET-Std and non-CRET (control) cultures. Scale bar: $50\mu\text{m}$. The dotted black lines indicate the wound's edges. (B) Computational quantification of the gap closure calculated as the mean distance (μm) between the edges at three equidistant points of the gap, using Photoshop software. Data are mean $\pm$ SEM, normalized to the corresponding controls of six experimental replicates per cell type and time interval. *: $0.05 > p \geq 0.01$; **: $0.01 > p \geq 0.001$; Student's t -test. (C) Representative images of immunofluorescence for vinculin in cultures of HFn, HFs, and HFa intermittently treated with CRET-Std for 48 h and respective controls. Scale bar: $100\mu\text{m}$. (D) Representative immunoblot images for vinculin expression of HFn, HFs, and HFa treated intermittently for 48 h with CRET-Std (CRET-S) and respective controls (C). One hundred micrograms protein/lane. Protein/GAPDH ratio. (E) Densitometry of vinculin immunoblot expression. Values are mean $\pm$ SD, normalized with respect to the controls. Between 4 and 6 experimental replicates per cell type; *: $0.01 \leq p < 0.05$. Student's t -test.

3.3.3 | p53 and p21 expression and localization

The analysis of p53 using immunofluorescence revealed a nuclear labeling for p53 in the control samples of all three cell types. The percentage of p53+ cells was low in the HFn (around 5% of the total counted nuclei), slightly higher (7% compared to the total)

in the HFa, and even higher in the HFs (30% compared to the total). In addition, cytoplasmic labeling was found in $11 \pm 3\%$ of the HFs cells, but not in the HFn or HFa. At the end of the CRET-Std treatment, the rate of p53+ nuclei decreased with respect to the controls in all three types of fibroblasts, although in the HFa the difference was not statistically significant. In addition, the

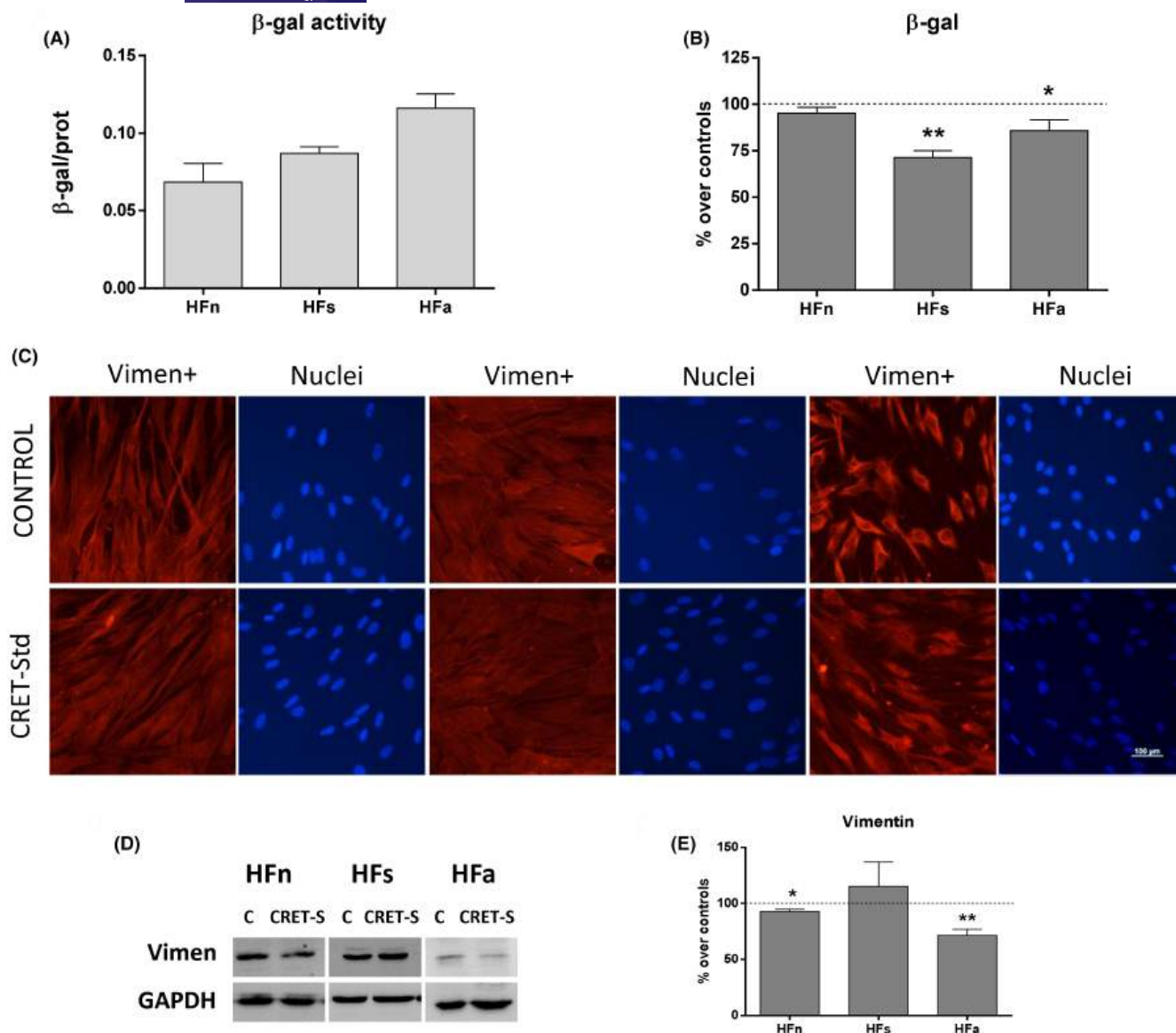


FIGURE 4 Aging markers: β -galactosidase and vimentin. (A) Basal expression of β -galactosidase (β -gal) in HFn, HF_s, and HF_a after 5 days of incubation without electric treatment. Mean $\pm$ SEM. β -gal/total protein ratio. Between four and six experimental replicates per cell type. Raw data. (B) β -gal expression in HFn, HF_s, or HF_a after 48 h of CRET-Std treatment. Mean $\pm$ SEM of samples normalized to the respective controls. Between three and six repeats per cell type. *: $0.01 \leq p < 0.05$. **: $0.01 > p \geq 0.001$; Student's *t*-test. (C) Representative images of immunofluorescence staining for vimentin in cultures of HFn, HF_s, and HF_a intermittently treated with CRET-Std (CRET-S) for 48 h and respective controls (C). Scale bar: 100 μ m. (D) Representative immunoblot images for vimentin expression in HFn, HF_s, and HF_a intermittently treated with CRET-Std for 48 h and respective controls. One hundred micrograms protein/lane. Protein/GAPDH ratio. (E) Densitometry of vimentin immunoblot expression. Mean $\pm$ SD in CRET-Std samples normalized to the respective controls. Between three and four repeats per cell type. *: $0.01 \leq p < 0.05$. **: $0.01 > p \geq 0.001$; Student's *t*-test.

cytoplasmic labeling of the HF_s decreased after treatment, but the difference was also not statistically significant (Figure 5A,B). On the other hand, the p53 immunoblot assays did not show statistically significant differences compared to the control in any of the three cell types (Figure 5B,C).

Regarding p21, its expression in both the HFn and the HF_s decreased significantly compared to that of the control cells after 48 h of CRET-Std treatment, but it did not change in the HF_a (Figure 5C,D).

3.4 | Effects of CRET-Std and CRET-Mod on extracellular matrix: hyaluronic acid, fibronectin, and Type I collagen

As clinical evidence indicates that CRET-Mod could modify the composition of the ECM, the production of hyaluronic acid (HA), fibronectin (FN), and Type I collagen (Col I) was analyzed after treatments with standard or modulated CRET. The ELISA immunoassay showed that in the HFn, CRET-Std increased the FN content while it reduced that of

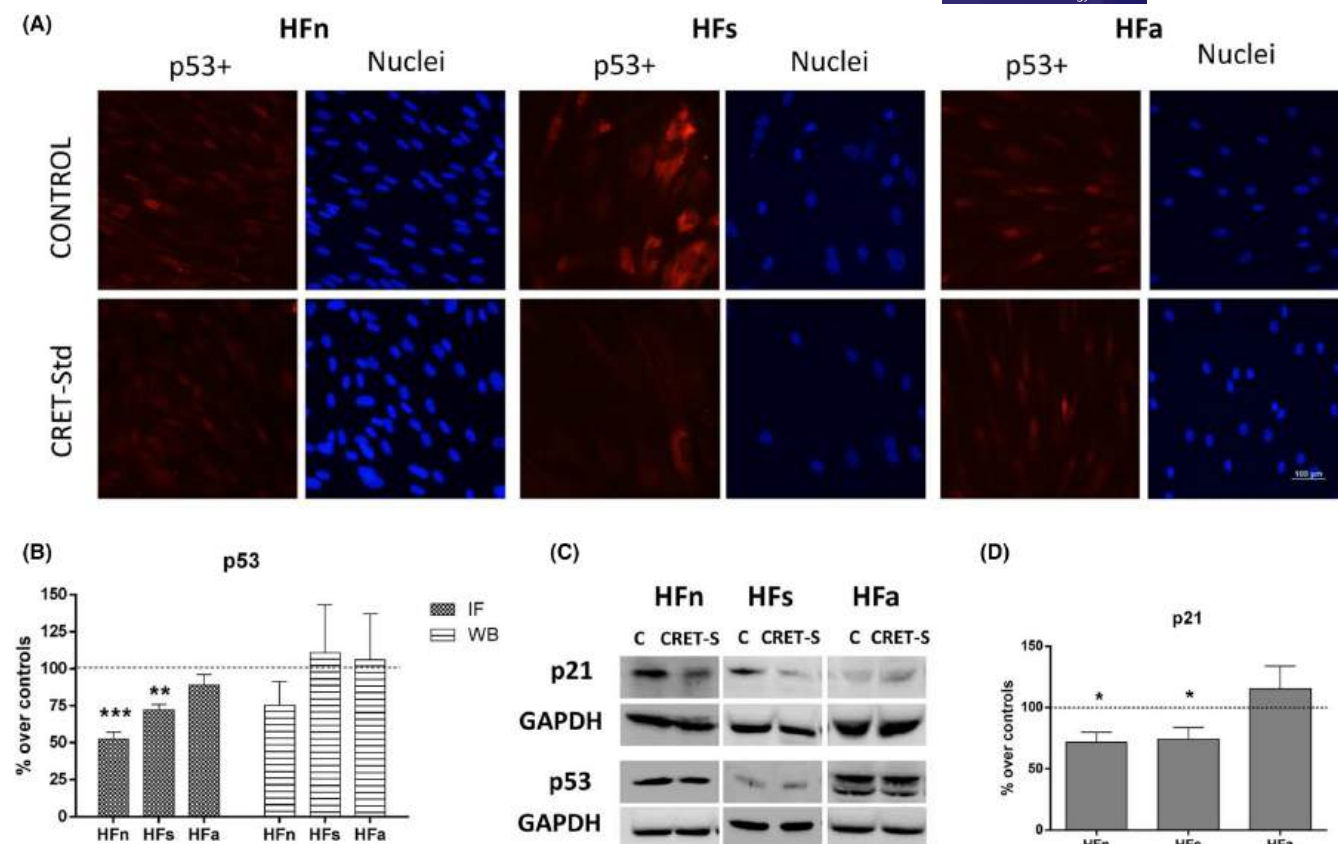


FIGURE 5 Aging markers: p53 and p21. (A) Representative immunofluorescence images for p53 in cultures of HFn, HF, and HFa intermittently treated with CRET-Std for 48h and respective controls. Scale bar: 100 μm. (B) Quantification of p53 expression using image analysis of labeled cells (dotted bars) and immunoblots (striped bars). Values are mean ± SD, normalized with respect to controls. Between 4 and 10 experimental replicates per cell type and assay; **: 0.01 > $p \geq 0.001$; ***: $p < 0.001$; Student's *t*-test. (C) Representative immunoblot images for p21 and p53 expression in HFn, HF, and HFa intermittently treated with CRET-Std (CRET-S) for 48h and respective controls (C). One hundred micrograms protein/lane. Protein/GAPDH ratio. (D) Densitometry of p21 immunoblot expression. Mean ± SD of CRET-Std samples normalized to the respective control. Between 4 and 6 repeats per cell type. *: 0.01 ≤ $p < 0.05$; Student's *t*-test.

HA with respect to the controls. Both results were statistically significant. CRET-Std also significantly reduced the content of Col I in the HFn and HFa samples, as shown by the immunofluorescence results. However, this treatment did not significantly affect the extracellular matrix components in the HF. On the other hand, CRET-Mod significantly reduced FN production in the HF, without significantly affecting the HA or Col I contents of the other fibroblasts studied (Figure 6).

3.5 | Effects of CRET-Std on the metalloproteinases MMP9 and MMP1

The reduction in Type I collagen mentioned above could be due to a possible activation and/or expression of the metalloproteinases induced by CRET. Thus, the expression of MMP1 and MMP9 was analyzed. As only the CRET-Std signal induced significant changes in collagen, the analysis of MMPs was performed only with the unmodulated form of the RF signal. The results revealed a significant MMP9 underexpression in the three types of fibroblasts analyzed, after 12 or 24h of CRET-Std treatment. At the end of electric

treatment (48h), the expression levels were similar to those of their respective controls (Figure 7A,B). On the other hand, the expression of MMP1 increased significantly in the HFa after 12h of electric treatment. In the HFn, the increase in MMP1 occurred after 24h, reversing this effect at the end of the second day of CRET-Std treatment. Regarding the HF, CRET-Std did not affect the expression of MMP1 in any of the intervals tested (Figure 7A,C).

4 | DISCUSSION

Skin aging is a multifactorial process that alters the structure and functionality of the skin at multiple levels, from the molecular to the tissue level. The skin is the largest and most visible organ, and for this reason, human aging manifests itself more obviously. Thus, people who want to look younger than others at the same chronological age request antiaging treatments that are especially focused on the skin. As aging itself cannot be physically prevented, the goal of such treatments is to delay the progress of skin senescence and/or mitigate the severity of age-related changes.¹⁸ Identifying new antiaging

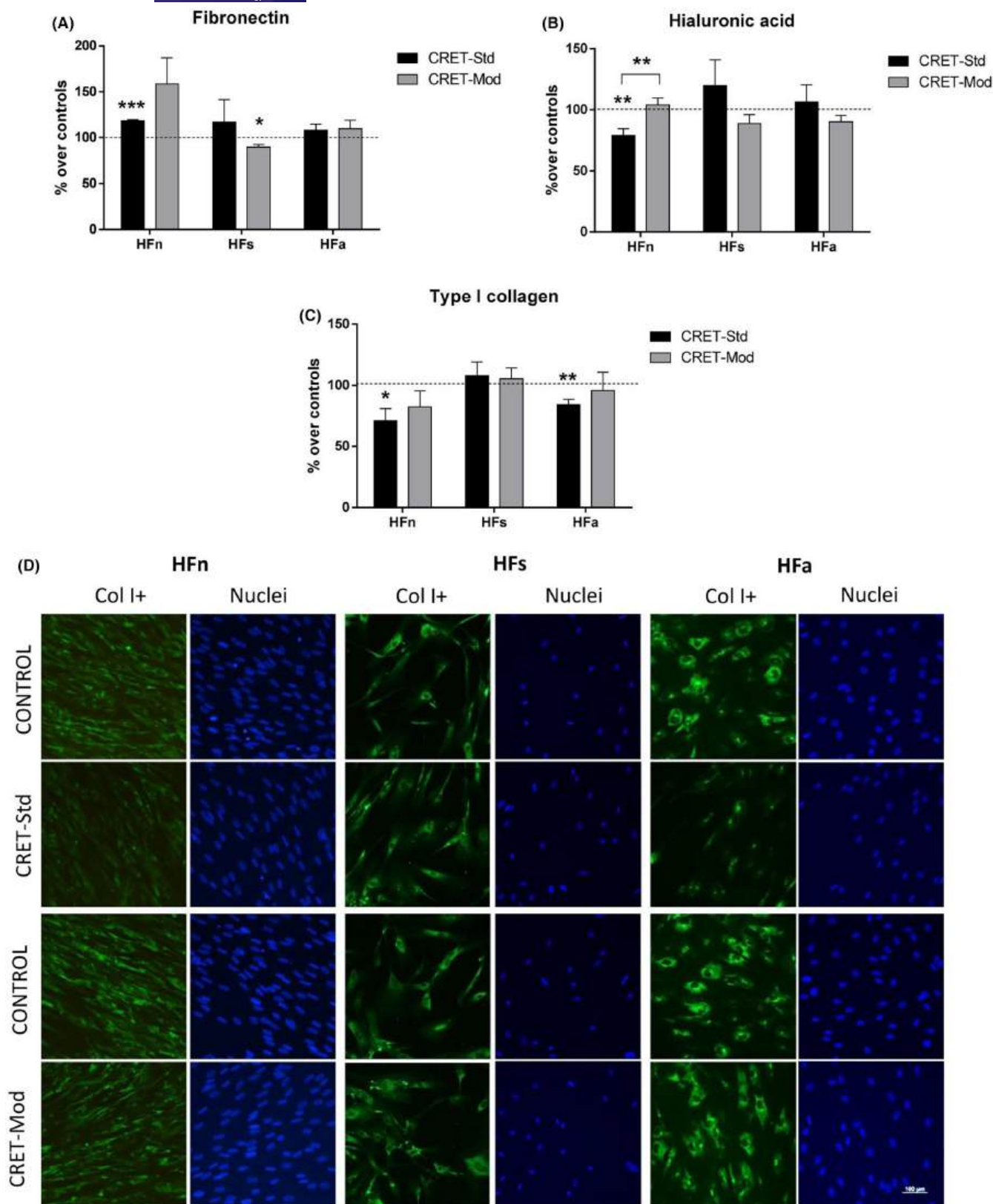


FIGURE 6 Extracellular matrix production. Fibronectin, hyaluronic acid, and Type I collagen expression for HFn, HF, and HFa after 48 h of CRET-Mod or CRET-Std treatment. (A) Fibronectin ELISA. Values are mean $\pm$ SD, normalized with respect to the controls. Three experimental replicates per cell type; *, $0.01 \leq p < 0.05$; ***, $p < 0.001$; Student's *t*-test. (B) Hyaluronic acid ELISA. Values are mean $\pm$ SD, normalized with respect to the controls. Three experimental replicates per cell type; **, $0.01 > p \geq 0.001$; Student's *t*-test. (C) Quantification of expression of Type I collagen using image analysis of labeled cells. Values are mean $\pm$ SD, normalized with respect to the controls. Between three and seven experimental replicates per cell type; *, $0.01 \leq p < 0.05$; **, $0.01 > p \geq 0.001$. (D) Representative images of immunofluorescence staining for Type I collagen (Col I+) in cultures intermittently treated with CRET-Std for 48 h and respective controls. Scale bar: 100 μ m.

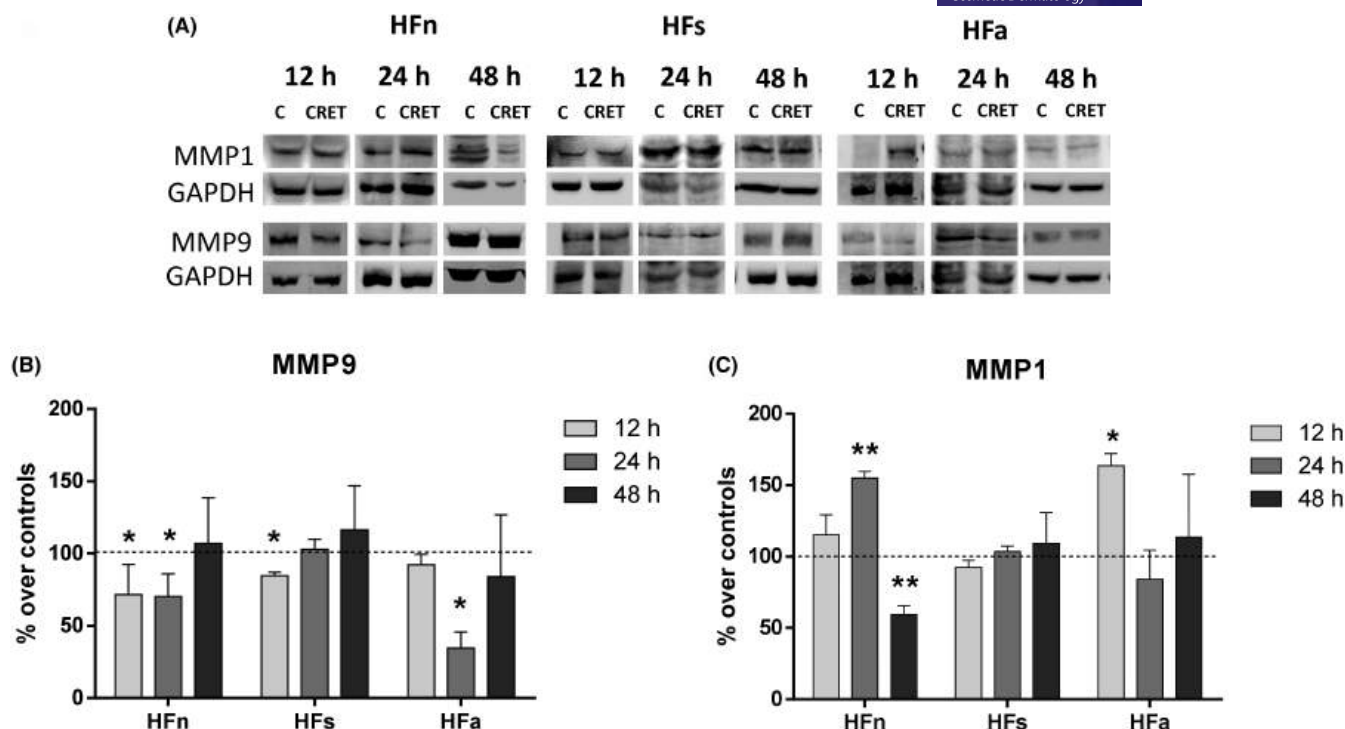


FIGURE 7 MMP1 and MMP9 expression. (A) Representative immunoblot images for MMP1 and MMP9 expression in HFn, HFs, and HFa intermittently treated with CRET-Std (CRET-S) for 12, 24, or 48 h and respective controls (C). One hundred micrograms protein/lane. Protein/GAPDH ratio. (B) Densitometry of MMP9 immunoblot expression. Values are mean $\pm$ SD, normalized with respect to the controls. Between three and five experimental replicates per cell type; *: $0.01 \leq p < 0.05$; Student's *t*-test. (C) Densitometry of MMP1 immunoblot expression. Values are mean $\pm$ SD, normalized with respect to the controls. Between three and four repeats per cell type. *: $0.01 \leq p < 0.05$; **: $0.01 > p \geq 0.001$; Student's *t*-test.

therapies that can slow down the senescence of tissues is of great interest in cosmetic dermatology and aesthetic medicine; however, for their development, it is essential to first reveal the underlying mechanisms of skin aging.¹⁹ Aging has an intrinsic component as it is a genetically programmed and inevitable process, and it has an extrinsic component which is due to external factors such as pollution or solar radiation. Both types of aging share alterations in the functionality of dermal fibroblasts such as telomere shortening and mitochondrial dysfunction, changes in proteostasis and in the secretome, epigenetic alterations, and DNA damage.^{20–22} As a consequence of all these alterations, phenotypic changes appear, such as the appearance of wrinkles or spots, dryness, loss of elasticity, or flaccidity.²³ Among the therapies that can improve the effects of skin aging are physical RF therapies.^{24–27} In this study, the effect of the currents used in noninvasive CRET therapy on the main processes involved in the senescence of human fibroblasts was analyzed.

One of the main characteristics of skin senescence is the decrease in the proliferative capacity of the cells it consists of, mainly keratinocytes and fibroblasts; the decrease in proliferation delays and hinders wound regeneration.²⁸ The loss of keratinocyte proliferative capacity results in less effective skin desquamation, while decreased vascularity and the decreased number of fibroblasts and mast cells reduce the thickness of the dermis.²⁹ In this study, it was observed that subthermal treatment with the currents used in CRET therapy in its unmodulated modality (CRET-Std) increased

the proliferation of HFn and HFa cultures compared to that of the controls. Thus, in the proliferative HFn and HFa cultures, CRET-Std induced increases with respect to the controls in the proliferative phases of the cell cycle, S and G2/M, after 24 h of treatment. These results are similar to those described in previous studies from our laboratory in which CRET-Std induced the proliferation of neonatal fibroblasts, ADSC stem cells, and human keratinocytes.^{10,14} In contrast, the non-proliferating HFs did not change their proliferation rate after treatment with either of the two signal forms (CRET-Std or CRET-Mod). This effect was due to the fact that the HFs, which were aged through replicative senescence, completed their maximum number of replications and were therefore unable to express cell cycle promoters such as cyclin A and cyclin B in response to mitogens,¹ which would otherwise promote their cell cycle.³⁰ Also, the absence of the proliferative effect of CRET-Std on non-proliferating cultures in human PBMC³¹ as well as in stem cells (ADSC) differentiated into chondrocytes¹³ has previously been described by our group. Thus, CRET-Std promotes cell division in proliferating cultures but not in those in which proliferation is inhibited by states of differentiation or senescence. Furthermore, this proliferative effect seems to be dependent on the type of RF signal applied since only the CRET-Std treatment induces such an effect.

In the skin, DNA damage due to aging activates specific response pathways such as p53–p21.^{22,32} p21 and p53 are biomarkers that control the cell cycle, and their activation blocks the progression

from G1 to S in the cell cycle and, therefore, cell proliferation.³³ In proliferating fibroblasts, p53 is very weakly expressed and localized in the nucleus, while in senescent fibroblasts it is overexpressed and partially translocates to the cytoplasm, where it does not induce apoptosis but rather a cell cycle block.³⁰ On the other hand, p21 is a senescent cell biomarker³⁴ that, in addition to blocking the cell cycle, activates immune survival genes by attracting the macrophages that control cells exposed to stress.³⁵ The treatment with CRET-Std decreased the expression of both senescence markers, p53 and p21, in the HFn and HF_s. This underexpression of p53 and p21 in CRET-treated HFn could promote proliferation by unblocking the cell cycle. In HF_s, such decreases would not induce greater proliferation but could be related to a decrease in oxidative stress or the senescent state of fibroblasts.³⁶ Thus, this electrically induced proliferative effect would increase the regenerative capacity of the skin. A similar effect of p53 and p21 underexpression after RF treatments was previously shown in ADSC stem cell cultures aged by replicative senescence.³⁷ Regarding the senescence marker β -gal, this lysosomal enzyme catalyzes the hydrolysis of terminal β -galactose residues from various substrates, including ganglioside GM13 and keratan sulfate,³⁸ two of the main components of the skin that are significantly altered by chronological and extrinsic aging.³⁹ β -gal is the 'gold standard' for the identification of senescent cells both in vivo and in vitro.²⁰ It is expressed preferentially in senescent tissues but not in quiescent, terminally differentiated, or immortalized cells.⁴⁰ RF (2.4 GHz) treatment has been described as being capable of decreasing β -gal activity values in stem cells.³⁷ In our study, CRET-Std treatment significantly reduced β -gal activity in HF_s and HF_a, while the low expression detected in HFn was not modified. Therefore, CRET-Std decreases the activity of three of the main markers of cell senescence.

Another of the characteristics of cell senescence is the decrease in their migration, which directly influences the regenerative capacity of wounds.⁴¹ Migration involves cell anchorage to the extracellular matrix (ECM). One of the proteins involved in the migratory process is vinculin, which participates in the process of the formation and disintegration of adhesive contacts with the ECM. In the skin, the dysfunction of integrin adhesion molecules and adhesion groups causes a loss of communication between the extracellular matrix and the fibroblasts, which contributes to the appearance of signs of aging.⁴² Our results showed that CRET-Std promoted fibroblast migration regardless of the age of the donor and the senescence state of the culture, although the vinculin expression was only increased compared to its controls in the HF_a. This pro-migratory effect of CRET-Std therapy was previously shown in neonatal fibroblasts.¹⁰ In addition to vinculin, vimentin is also involved in cell migration, and it has been shown in fibroblasts from adult donors that a decrease in its expression promotes motility.⁴³ In addition, vimentin is related to the increase in the cell surface of aged fibroblasts through replicative senescence, and, for this reason, its overexpression is commonly used as a marker of cell senescence. In this study, although the morphology of the vimentin filaments did not change in any of the types of fibroblasts studied after electric treatment, its expression decreased in the HFn and HF_a. This electrically induced

decrease in vimentin could also favor the migration of fibroblasts treated with CRET-Std electric current.

In tissue regeneration and the aging processes, the composition and stability of the ECM is essential. Glycosaminoglycans and proteoglycans are an integral part of the skin, and the alteration of their function during intrinsic and extrinsic aging leads to a decrease in skin quality associated with age.³⁹ One mechanism through which senescent cells contribute to tissue dysfunction is the senescence-associated secretory phenotype (SASP) complex, composed of a series of proinflammatory cytokines, chemokines, growth factors, and matrix-remodeling enzymes which are capable of altering their microenvironment.⁴⁴ Due to this mechanism, senescent cells are characterized by a lower content of Type I collagen, hyaluronic acid, and fibronectin, which weakens the structural integrity of the skin, resulting in increased fragility and worse wound regeneration.⁴⁵⁻⁴⁷ Therefore, the capacity of CRET-Std and CRET-Mod to modulate the synthesis of these extracellular matrix components was evaluated. Fibronectin plays a fundamental role in cell adhesion and migration, it is a key protein in tissue regeneration and aging,⁴⁸ and it has been shown that radiofrequency therapies can increase its synthesis.⁴⁹ Indeed, in our assays, the fibronectin content of the HFn increased after they were treated with CRET-Std compared to that of the controls. However, CRET-Mod decreased the fibronectin content in the HF_s compared to that of the controls.

Another of the ECM components analyzed was hyaluronic acid (HA). HA is a non-sulfated glycosaminoglycan; it is a major component of connective tissue and an important structural component of the ECM. The high amounts of water that bind to HA are essential in maintaining adequate hydration within the skin, thereby promoting its correct physiology and maintaining its youthful appearance. In aged skin, HA is reduced in the epidermis and dermis due to the downregulation of CD44, HA synthases (HAS1, HAS2), and the receptor for HA-mediated mobility (RHAMM), contributing to age-induced deterioration.³⁹ It has been shown that its production is sensitive to different types of radiation. Thus, after exposure to ionizing UV radiation, its production is reduced due to the inactivation of HAS1 and HAS2.⁵⁰ Conversely, studies carried out with RF therapies found increases in HA in the dermis after treatments.⁵¹ In our assays, CRET-Std significantly decreased the amount of HA produced by the HFn culture, while CRET-Mod did not alter its content. It is worth noting that HA production in the HF_a and HF_s was not modified by either of the two CRET signals. Thus, CRET treatment could induce a remodeling of the extracellular matrix, which would help to reverse the changes due to aging, but, like the effect on proliferation, it depends on the type of signal applied and the physiological state of the target tissue.

Regarding collagen, it is known that RF therapies promote its production; they are therefore commonly used for antiaging treatments. RF treatments produce collagen denaturation with a thermal increase to 40°C, which induces the initial microinflammation, the production of IL-1 β and TNF- α , and the increased levels of the HSPs and MMPs that degrade the denatured collagen of the ECM. As a consequence of this degradative process, the production of procollagens I and III, tropoelastin, and fibrillin is stimulated in

fibroblasts.^{6,51,52} It has also been shown that CRET treatments under hyperthermic conditions (40–42°C) are capable of inducing neocollagenesis in rats⁵³ and reducing wrinkles in patients⁸ through this same process of thermal increase in tissue. It is interesting to note that, although in our assays the CRET treatment was applied under subthermal conditions, it produced increases in MMP1 and a subsequent decrease in the collagen content of the ECM in a similar manner to the process described under hyperthermic conditions. The mechanism causing this in subthermal conditions is not fully understood; however, it is possible that it is related to the activation of MAPKs. Indeed, it has been shown that MMP1 is activated by MAP-kinase ERK1/2,⁵⁴ and it is known that p-ERK1/2 (the activated form of this protein) is overexpressed due to the CRET effect, at least in HFn cultures.¹⁰ Thus, while CRET probably promotes proliferation through ERK1/2 activation, this same activation induces MMP1 expression, which results in the degradation of collagen in the ECM of HFn and HFa in culture. It would be necessary to carry out new studies with longer CRET treatments to determine whether this collagen degradation induces long-term neocollagenesis in a similar way to that which occurs with hyperthermic CRET treatments.

In summary, it can be concluded that CRET treatment applied under subthermal conditions promotes cell proliferation and migration while reducing the expression of cellular senescence biomarkers such as p21, p53, vimentin, or β -gal in human dermal fibroblasts. In addition, the application of subthermal CRET also induces fibronectin production, while under hyperthermic (and perhaps also sub-thermic) conditions it would stimulate ECM neocollagenesis. Taken together, these results indicate CRET could promote tissue regeneration in the target tissues of the therapy and could be of interest for reducing the signs of dermal aging. However, since this study was carried out in vitro, it would be necessary to delve into the biological bases underlying these responses as well as to investigate the effect of this CRET therapy on regeneration and skin aging processes through clinical trials with patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Not applicable.

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How to cite this article: Toledano-Macías E, Martínez-Pascual MA, Hernández-Bule ML. Electric currents of 448kHz upregulate anti-senescence pathways in human dermal fibroblasts. *J Cosmet Dermatol*. 2024;23:687-700. doi:[10.1111/jocd.16019](https://doi.org/10.1111/jocd.16019)