Antiadipogenic effects of subthermal electric stimulation at 448 kHz on differentiating human mesenchymal stem cells

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Received November 11, 2015; Accepted February 15, 2016

DOI: 10.3892/mmr.2016.5032

Abstract. The 448 kHz capacitive-resistive electric transfer (CRET) is an electrothermal therapy currently applied in anticellulite and antiobesity treatments. The aim of the present study was to determine whether exposure to the CRET electric signal at subthermal doses affected early adipogenic processes in adipose-derived stem cells (ADSC) from human donors. ADSC were incubated for 2 or 9 days in the presence of adipogenic medium, and exposed or sham-exposed to 5 min pulses of 448 kHz electric signal at 50 μA/mm^2 during the last 48 h of the incubation. Colorimetric, immunofluorescence, western blotting and reverse transcription-quantitative polymerase chain reaction assays were performed to assess adipogenic differentiation of the ADSC. Electric stimulation significantly decreased cytoplasmic lipid content, after both 2 and 9 days of differentiation. The antiadipogenic response in the 9 day samples was accompanied by activation of mitogen-activated protein kinase kinase 1/2, decreased expression and partial inactivation of peroxisome proliferator-activated receptor (PPAR) γ, which was translocated from the nucleus to the cytoplasm, together with a significant decrease in the expression levels of the PPARG1 gene, perilipin, angiopoietin-like protein 4 and fatty acid synthase. These results demonstrated that subthermal stimulation with CRET interferes with the early adipogenic differentiation in ADSC, indicating that the electric stimulus itself can modulate processes controlling the synthesis and mobilization of fat, even in the absence of the concomitant thermal and mechanical components of the thermoelectric therapy CRET.

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Key words: adipose-derived stem cell, adipogenesis, radiofrequency, electric stimulation, PPARγ, p-MEK

Introduction

Obesity, whose incidence is rapidly growing in developed societies, is a risk factor involved in a number of severe diseases, including diabetes, heart conditions and different cancer types (1,2). A variety of physical techniques, including ultrasound, laser and electrical and/or magnetic stimuli, ranging from direct current (DC) and low or ultralow frequencies to high frequencies or radiofrequencies (RF), have been assayed in antiobesity treatments (3-5).

Concerning RF, treatments with electromagnetic fields or electric currents within the 100 kHz-3 GHz frequency spectrum, which induces tissue heating through molecular friction, have been reported effective in therapies for adipose tissue reduction (6-9). The antiadipogenic or lipolytic effects of such electrothermal therapies, which are dependent on the signal frequency and power, have received support from *in vivo* and *in vitro* experimental studies (10-12). Specifically, in capacitive-resistive electric transfer (CRET) therapy, the target tissues are heated through exposure to 448 kHz sine wave currents. The non-invasive therapy is applied manually, exerting pressure with capacitive or resistive electrodes over the skin, so that the underlying target tissues receive two simultaneous stimuli: One thermal, electrically-induced, and one mechanical (8). Previous experimental studies applying 448 kHz CRET currents at densities of 400-800 μA/mm^2 that increased the temperature of the culture medium up to 42˚C, induced a significant reduction in intracellular lipid depot in OP9 mouse preadipocytes differentiated into mature adipocytes (13).

These above results supported the hypothesis that the antiadipogenic or lipolytic effects of CRET treatment are due to the cellular or tissular response to the electrically-induced hyperthermia. However, previous studies by our group have shown that *in vitro* exposure to CRET currents in the 448-570 kHz range significantly affects essential cellular functions, including the control of proliferation in different human cell types, even when the currents are administered at subthermal doses (14-19). Specifically, when applied at the frequency of 448 kHz, the subthermal stimulus significantly affected the cell cycle and proliferation of adipose-derived human stem cells (ADSC) through upregulation of proliferating cell nuclear antigen and extracellular signal-regulated kinases 1 and 2 (ERK1/2). These results revealed that, at least

at the cellular level, CRET currents can exert an electrostimulatory action that is independent of the electrically-induced hyperthermia. Therefore, the aim of the present study was to determine if, besides the antiadipogenic effects induced by thermal treatment with 448 kHz CRET (13), electric stimulation with the same signal can also interfere, by itself and under conditions of normothermia, with the adipocytic differentiation. The present study investigated the effect of *in vitro* exposure to short pulses of CRET current at 448 kHz, administered at a subthermal density of 50 μA/mm^2, on the early adipogenic differentiation of human ADSC. The present study was focused primarily on the comparative analysis of fatty acid content in ADSC exposed or sham-exposed to CRET at early stages of chemically-induced adipogenic differentiation. The results revealed a significant decrease in fatty acids in the CRET exposed samples. The present study subsequently delved into the molecular basis of the observed response. Therefore, the action of CRET on the enzyme, mitogen-activated protein kinase kinases 1 and 2 (MEK1/2), and on the transcription factor, nuclear peroxisome proliferator-activated receptor (PPAR)γ, both of which serve important roles in the regulation of adipogenesis (20-22). Additionally, since PPARγ directly activates a number of genes involved in lipid synthesis and/or storage in adipocytes, the action of CRET on the expression levels of selected genes was assessed in ADSC at the early stages of adipogenic differentiation. Throughout, PPARγ refers to the expression of PPAR protein, whereas PPARG refers to PPAR gene expression.

Materials and methods

Cell culture. The stromal-vascular fraction of adipose tissue is the source of both new adipocytes *in vivo* (23) and mesenchymal cells *in vitro*, termed ADSC (24,25). Human ADSC were our biological model of choice for two fundamental reasons: i) ADSC are responsive to subthermal treatment with CRET (15); ii) ADSC are an optimal model for investigating phenomena involved in adipogenic processes. The use, as an alternative model, of immortalized preadipocytes from human origin was unsuitable for the present study, due to the genetic alterations typical of such established cell lines. ADSC were isolated from subcutaneous adipose tissue surgically obtained from healthy donors (2 men and 2 women; 29-69-years-old). All participants provided written informed consent to participate in the present study. Protocols for informed consent, and for collection and processing of the samples met the ethical standards applicable in the European Union, and were evaluated and approved by the Ethics Committee for Clinical Trials of the Ramón y Cajal University Hospital (Madrid, Spain). The isolation protocol was performed, as described previously (15). Briefly, ADSC were isolated from 0.5-1 cm^3 pieces of fat, free of blood vessel debris and fibrotic tissue, sliced into 1-2 mm^3 fragments. These fragments were digested with 1 mg/ml collagenase A (Roche Applied Science, Basel, Switzerland) for 40 min at 37˚C. The digested tissue was dissociated using a P1000 pipette with filtered tips. The resulting cell dispersion was subsequently centrifuged at 300 x g for 5 min at room temperature to isolate the vascular-stromal fraction. The resulting pellet was resuspended in culture medium (MesenPro-RS medium; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 1% glutamine (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and seeded into a 75 cm^2 T-flask. After 24 h incubation at 37˚C in an atmosphere containing 5% CO_2 and 100% humidity, the flask was rinsed twice with Hank's balanced salt solution (HBSS; Gibco; Thermo Fisher Scientific, Inc.) and fresh MesenPro was added. After 4 days, the culture medium was renewed, and following another 3 days, when confluent, the cells were subcultured. For subculturing, the cells were detached using 0.05% trypsin plus 0.02% ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO, USA) in HBSS and seeded into a new flask at a density of 670 cells/cm^2. All experiments were performed with cells between passages 3 and 7, which were seeded into 60 mm Petri dishes at a density of 2,270 cells/cm^2. Plating was performed directly on the dish surface, with the exception of immunofluorescence studies, in which the cells were seeded onto glass coverslips placed inside the dish.

Adipogenic differentiation of ADSC. After 4 days of growing in Petri dishes, the cultures were incubated in adipogenic differentiation medium composed of high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc.), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc.), 1% glutamine and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific Inc.), 250 μM 3-isobutyl-1-metylxanthine (Gibco; Thermo Fisher Scientific Inc.), 200 μM indomethacin (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich) and 1 μM dexamethasone (Sigma-Aldrich). The cultures were maintained in this medium for periods of 2-9 days, with the medium being renewed every 3-4 days. The samples were CRET- or sham-exposed during the last 48 h of the adipogenic treatment. Therefore, the electric stimulation was applied to cells at early or intermediate stages of the adipogenic differentiation. This procedure was adopted on the basis of pilot data indicating that early adipogenic phases can be particularly sensitive to subthermal treatment with CRET, whereas mature adipocytes are susceptible to thermal doses of CRET (13).

CRET exposure. The exposure system was described previously (14,18). Briefly, the exposure to electric current was performed by means of pairs of sterile stainless steel electrodes designed ad hoc for *in vitro* stimulation, which were fitted inside all Petri dishes, CRET-exposed and sham-exposed. Only cells grown on the rectangular area located within the electrode gap were used in the present study, with the cells on the remaining surface being discarded. For CRET exposure, the electrode pairs were connected in series to a signal generator (INDIBA Activ HCR 902; INDIBA®, Barcelona, Spain). For sham-exposure, the electrode pairs inserted in control dishes were also connected to the generator, however, were not energized. The stimulation pattern consisted of 5 min pulses of 448 kHz, sine wave current at a subthermal density of 50 μA/mm^2, separated by 4 h interpulse lapses, for a total period of 48 h. Such exposure parameters have been previously shown by our group to affect human cell proliferation (14-19). During the 48 h treatment interval, the CRET- and

sham-exposed cultures were grown in two separate, identical CO_2 incubators (Thermo Fisher Scientific, Inc.). Stimulation parameters, as well as atmospheric conditions inside the incubators (37˚C, 90% humidity and 5% CO_2) were constantly monitored. The electromagnetic environment inside the incubators was monitored using specific magnetometers for three frequency ranges of interest: Static, power frequency and RF. The recorded values coincided with those reported in previous studies (15) and corresponded to field levels typically found in laboratory environments.

Oil red O staining and quantification of lipid content. To assess the adipogenic differentiation of ADSC, the quantity of fatty acids synthesized by cultures grown for 2 or 9 days in differentiating medium were quantified and compared with those in samples maintained in basal medium for the same intervals (n=4 dishes/condition). After 48 h of CRET- or sham-exposure during the last two days of incubation in the presence of the corresponding media, the samples were washed with phosphate-buffered saline (PBS) and were fixed in 4% paraformaldehyde at 4˚C for 20 min. The cells were subsequently permeabilized with 60% isopropanol for 3 min and stained with Oil Red O (Sigma-Aldrich) for 30 min. The stained fatty acids were extracted by stirring of the samples in 99% isopropanol for 5 min, and the fatty acid content was assessed by spectrophotometry at 510 nm using a CE 2021 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK).

Immunoblotting. ADSC cultured in adipogenic medium for 2 or 9 days were CRET- or sham-exposed for the last 48 h of incubation, following the above described protocol. The cells growing on the dish surface between the electrode pairs were harvested in PBS and centrifuged at 200 x g for 5 min. The pellet was lysed for 45 min at 4˚C in a buffer containing 10 mM Tris-HCl, 10 μM KCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF, 10 mg/ml leupeptin, 5 μg/ml pepstatin, 100 mM NaF, 20 mM β-glycerophosphate, 20 mM sodium molybdate, 0.5% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS). The lysates were centrifuged at 12,000 x g for 15 min at 4˚C and the protein concentration in the supernatant was determined through Bradford's method. Sample protein concentrations were estimated from a standard curve using serial dilutions of bovine serum albumin ranging from 1-20 μg/ml and Coomassie Brilliant Blue G-250 working solution. Final concentrations in the Bradford reagent were 0.4% Coomassie Brilliant Blue G-250 (w/v), 0.2% ethanol (v/v) and 0.4% phosphoric acid (v/v; all Sigma-Aldrich). Protein concentrations were measured at 595 nm using a CE 2021 spectrophotometer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Odyssey® nitrocellulose membranes (LI-COR Biosciences, Nebraska, USA) using a semi-dry transfer method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked in PBS containing 5% non-fat dry milk for 1 h at room temperature. The membranes were subsequently incubated overnight at 4˚C in antibodies against rabbit monoclonal anti-human anti-PPARγ (81B8) (1:1,000; 2443), rabbit monoclonal anti-human anti-phosphorylated (p-)MEK1/2 (1:1,000; 9154; both Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse monoclonal anti-human anti-β-actin (1:5,000; A5441; Sigma-Aldrich) as a loading control, in the blocking buffer. PPARγ antibody enables detection of the two PPARγ isoforms of interest for the present study: PPARγ1 and PPARγ2. Following primary antibody incubation, the membranes were washed four times with PBS-Tween. Membranes were subsequently incubated for 1 h at room temperature with IRDye 800CW-conjugated goat polyclonal anti-rabbit immunoglobulin (Ig)G (1:10,000; 926.32211) and with IRdye 680LT-conjugated goat polyclonal anti-mouse IgG (1:15,000; 926.68020; both LI-COR Biosciences). The fluorescent intensity of the blots was measured with a LI-COR Odyssey scanner (LI-COR Biosciences) and evaluated using Quantity One software, version 4.6.7 (Bio-Rad Laboratories, Inc.).

Immunofluorescence. Cultures between passages 3 and 5 were seeded onto coverslips and incubated in differentiation medium for 2-9 days. The cells were CRET- or sham-stimulated during the last 48 h of differentiation. The cells were fixed with 4% paraformaldehyde for 20 min at 4˚C, and permeabilized with ethanol/acetic acid at -20˚C for 20 min. Afterwards, the cells were incubated overnight at 4˚C with mouse monoclonal anti-human anti-PPARγ antibody (1:50; sc-81152 Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) and fluorescently stained with anti-mouse IgG conjugated to Alexa Fluor 488 (1:500; A27012; Molecular Probes; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The cell nuclei were counterstained with bisBenzimide H 33258 (Sigma-Aldrich) at a concentration of $1x10^{-5}$ M.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA from ADSC was extracted with TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. The cells were homogenized in 1 ml TRI Reagent containing 1 μl glycogen (20 mg/ml; Sigma-Aldrich) as a carrier for nucleic acid precipitation. A total of 500 ng total RNA was used to generate cDNA by RT using a Primer Script RT™ reagent kit (Takara Bio Inc., Shiga, Japan). RT-qPCR amplification was performed using the SYBR Green I Master kit and LightCycler 480 II (Roche Applied Science). The initial denaturation step was 95˚C for 5 min, followed by 45 cycles of amplification at 95˚C for 10 sec, 60˚C for 15 sec, and 72˚C for 15 sec. The melting curves were evaluated and the PCR reaction products were separated on a 2% agarose gels and stained with ethidium bromide to confirm the presence of a single product. The efficiency of the reaction was evaluated by amplifying serial dilutions of cDNA (1:10, 1:100, 1:1,000, and 1:10,000). The association between the threshold cycle (Ct) and the log [RNA] was linear (-3.6< slope <-3.2). The relative quantities of target genes were normalized against the expression of the housekeeping gene, ribosomal protein large P0, according to the ΔCt method (26). The primers used in RT-qPCR are shown Table I.

Statistical analysis. All data are expressed as the mean ± standard error of the mean. Unless stated otherwise, differences between treated and control samples were analyzed by two-tailed unpaired Student's t-test, using Graph-Pad Prism 6.01 software (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Sequence of primers used in reverse transcription-quantitative polymerase chain reaction.

Primer	GeneBank ID	Sequence (5'→3')
RPLP0	NM_001002	Forward: CCTCATATCCGGGGGAATGTG Reverse: GCAGCAGCTGGCACCTTATTG
PPARG1	NM_138712	Forward: AAGGCCATTTTCTCAAACGA Reverse: AGGAGTGGGAGTGGTCTTCC
PPARG2	NM_138712	Forward: CCATGCTGTTATGGGTGAAA Reverse: TCAAAGGAGTGGGAGTGGTC
FABP4	NM_001442	Forward: AGCACCATAACCTTAGATGGGG Reverse: CGTGGAAGTGACGCCTTTCA
SCD	NM_005063	Forward: TCTAGCTCCTATACCACCACCA Reverse: TGTCGTCTTCCAAGTAGAGGG
PLIN	NM_002666	Forward: GTGGAGTACCTCCTCCCTG Reverse: GGTGTATCGAGAGAGGGTGTT
ANGPTL4	NM_139314	Forward: GGCTCAGTGGACTTCAACCG Reverse: CCGTGATGCTATGCACCTTCT
SREBP1c	NM_001005291	Forward: ACCGACATCGAAGGTGAAGT Reverse: AGCATGTCTTCGAAAGTGCA
FASN	NM_004104	Forward: TACGTACTGGCCTACACCCAGA Reverse: TGAACTGCTGCACGAAGAAGCATAT

RPLP0, ribosomal protein large P0; PLIN, perilipin; ANGPTL, angiopoietin-like; FASN, fatty acid synthase; PPAR, peroxisome proliferator-activated receptor; FABP, fatty acid binding protein; SCD, stearoyl-CoA desaturase; SERBP, sterol regulatory element-binding protein.

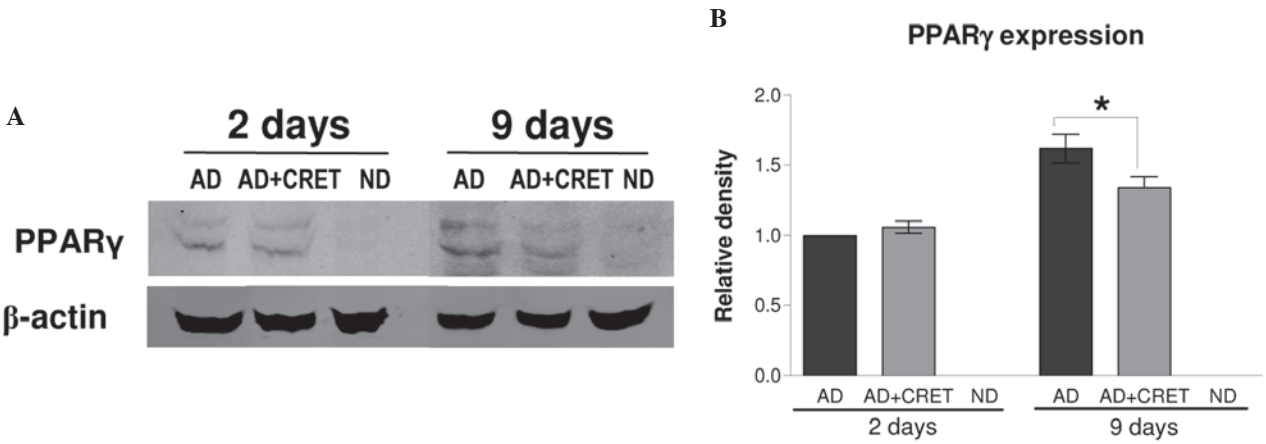

Figure 1. Immunoblotting to assess the expression of PPARγ. (A) Representative western blot analysis of PPARγ using β-actin as a loading control. A total of 30 μg protein was added per lane. (B) Densitometric analysis of the immunoblot analysis for PPARγ (PPARγ/β-actin ratio) corresponding to the bands for PPARγ1 and PPARγ2. The data are presented as the mean ± standard deviation of seven repeats per treatment interval (12 samples per repeat), normalized over controls grown in differentiating medium for 2 days (*P<0.05 by paired t-test). The PPARγ expression levels in the ND controls were vestigial and non-quantifiable, therefore, were assigned a value of 0. PPAR, peroxisome proliferator-activated receptor; CRET, capacitive-resistive electric transfer; AD, control samples incubated in adipogenic medium for 2 or 9 days and sham-exposed to CRET; AD + CRET, samples grown in adipogenic medium and exposed to CRET during the final 48 h; ND: samples grown in basal, non-differentiating medium.

Results

CRET effect on lipid content. Quantitative analysis of Oil Red O staining revealed that intermittent exposure to CRET during the last 48 h of incubation significantly reduced cell lipid content with respect to sham-exposed controls, in samples grown in differentiating medium for 2 days (61.32±15.05% below controls; P<0.05) or 9 days (8.48±2.37% below controls; P<0.05) (data not shown).

CRET effects on the expression of PPARγ. From the above results, and since the ligand-activated transcription factor PPARγ is crucial in lipid metabolism and in adipocyte differentiation, the potential effects of CRET on the expres-

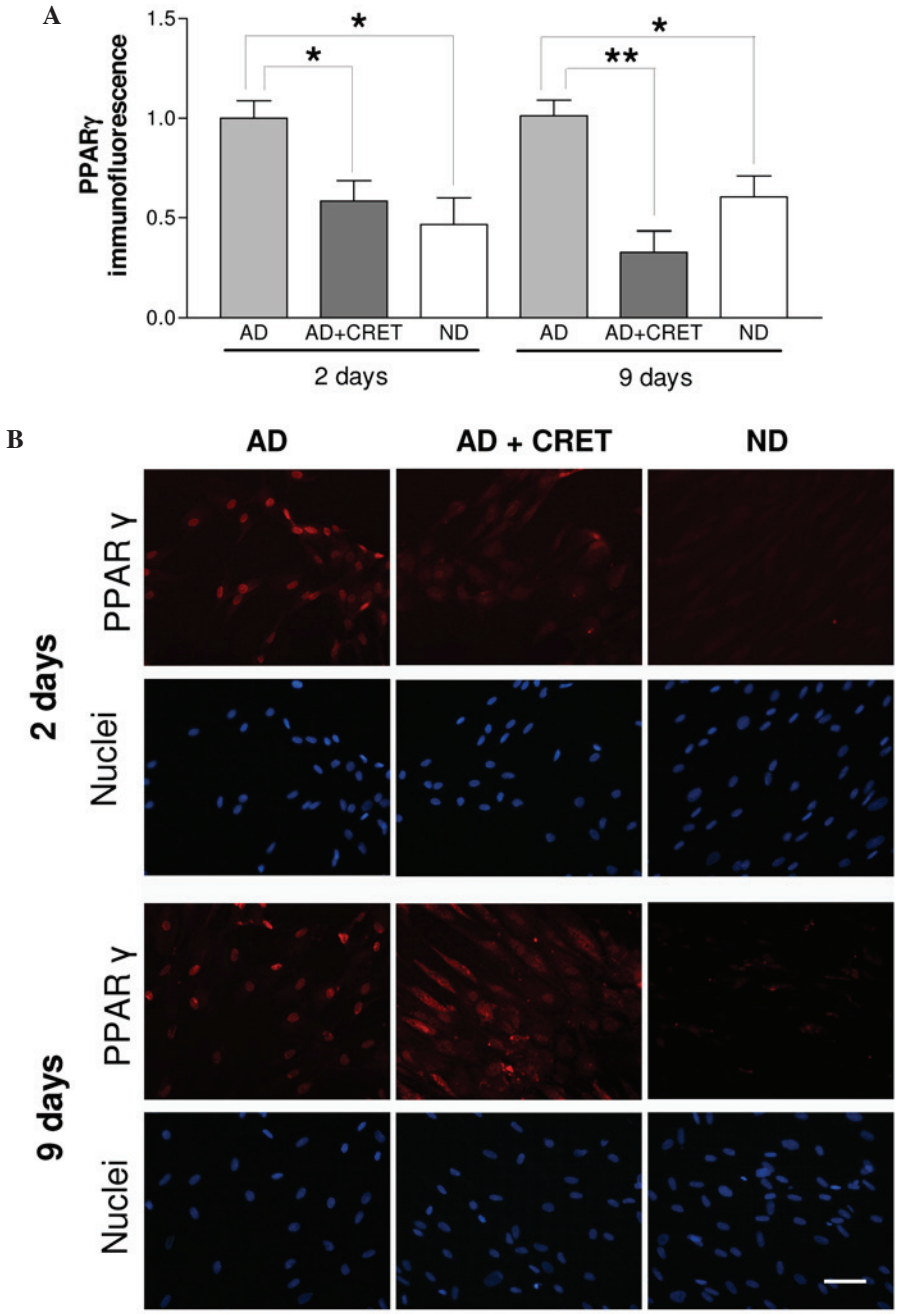

Figure 2. PPARγ immunofluorescence. (A) Immunofluorescence analysis of the expression of PPARγ. Only cells showing nuclear PPARγ were scored as positive. The data presented as the mean ± standard error of the mean of three repeats per experimental time, each with four coverslips per experimental group, normalized over controls differentiated for 2 or 9 days and sham-exposed to CRET (*P<0.05; **P<0.01 by Student's t-test). (B) Representative micrographs of immunofluorescence for PPARγ antigen (Scale bar, 100 μm). The DNA in the nuclei of the entire cellular population was blue stained with bisBenzimide and Alexa Red staining identifies those cells expressing PPARγ. PPAR, peroxisome proliferator-activated receptor; CRET, capacitive-resistive electric transfer; AD, control samples incubated in adipogenic medium for 2 or 9 days and sham-exposed to CRET; AD + CRET, samples grown in adipogenic medium and exposed to CRET during the final 48 h; ND: samples grown in basal, non-differentiating medium.

sion of PPARγ was assessed by immunoblotting analysis. As expected, samples incubated in basal medium expressed no PPARγ at either at 2 or 9 days of incubation (Fig. 1). The increased expression of PPARγ induced by the adipogenic medium was unaffected by CRET when the electric stimulus was applied during the first two days of differentiation. By contrast, when applied from incubation day 7 to 9, CRET significantly reduced the expression of PPARγ, 17% below the sham-exposed, differentiated controls. These results indicated that CRET affected the expression of PPARγ at intermediate adipogenic phases, however, not at the earliest differentiation stages.

Effects of CRET on the nuclear expression and intracellular location of PPARγ. The number of cells exhibiting nuclear expression of PPARγ in CRET-exposed samples was significantly reduced with respect to sham exposed, differentiated controls, both in samples with 2 or 9 days of incubation in differentiating medium, (42 and 67% reduction, respectively; Fig. 2A). In addition, in samples with 9 days of differentiation

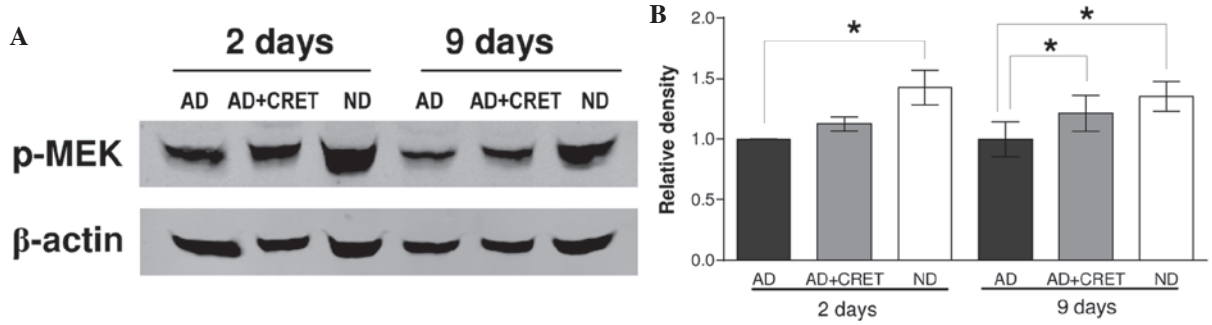

Figure 3. p-MEK1/2 immunoblot. (A) Representative western blot analysis of p-MEK using β-actin as loading control. A total of 30 μg protein was added to each lane. (B) The results of the densitometric analysis of the immunoblots for p-MEK1/2 (p-MEK/β-actin ratio) are shown. The data are presented as the mean ± standard deviation of six experimental repeats per treatment interval, with 12 samples per repeat, normalized over controls grown in differentiating medium for 2 days (*P<0.05 by Paired t-test). p-MEK, phosphorylated mitogen-activated protein kinase kinase; CRET, capacitive-resistive electric transfer; AD, control samples incubated in adipogenic medium for 2 or 9 days and sham-exposed to CRET; AD + CRET, samples grown in adipogenic medium and exposed to CRET during the final 48 h; ND: samples grown in basal, non-differentiating medium.

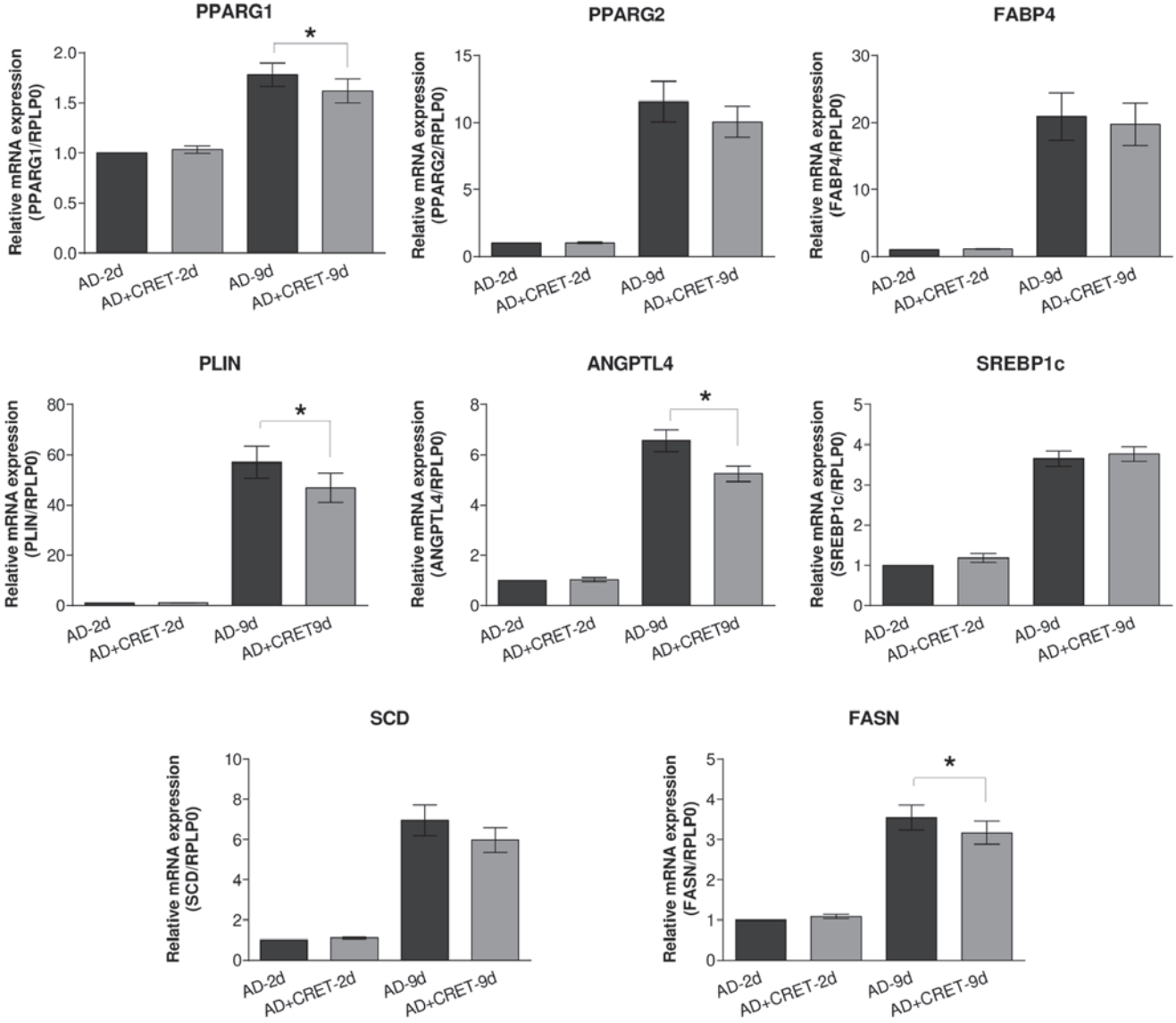

Figure 4. Analysis of mRNA expression levels of multiple proteins. Reverse transcription-quantitative polymerase chain reaction analysis of the effects of CRET stimulation on the expression levels of PPARG1, PPARG2, FABP4, PLIN, ANGPTL4, SREBP1c, SCD and FASN. The data are presented as the mean ± standard deviation of six replicates per experimental time, with 12 samples per replicate, normalized over samples differentiated for 2 days and sham-exposed to CRET (*P<0.05 by Paired t-test). PPARG, peroxisome proliferator-activated receptor gamma; FABP, fatty acid binding protein; PLIN, perilipin; ANGPTL, angiopoietin-like; SREBP, sterol regulatory element-binding protein; SCD, stearoyl-CoA desaturase; FASN, fatty acid synthase; CRET, capacitive-resistive electric transfer; AD, control samples incubated in adipogenic medium for 2 or 9 days and sham-exposed to CRET; AD + CRET, samples grown in adipogenic medium and exposed to CRET during the final 48 h; ND: samples grown in basal, non-differentiating medium.

and treated with CRET, the immunostaining revealed a predominantly cytoplasmic location of PPARγ, which is in contrast with the preferential nuclear location of PPARγ in sham-exposed differentiated controls and in undifferentiated cells (Fig. 2B). The electric stimulation induced no such response in samples with 2 days of adipogenic differentiation.

Effect of CRET on the expression of p-MEK. It has been previously reported that mitogenic stimuli can induce increased nuclear expression of MEK1, whose interaction with PPARγ results in the inactivation and translocation of PPARγ to the cytoplasm (20-22). The possibility that CRET exposure can induce changes in the expression of p-MEK that are associated with the observed cytoplasmic expression of PPARγ was investigated by immunoblotting of the expression of p-MEK. The results revealed that in the absence of electrical stimulation, incubation in adipogenic medium for 2 or 9 days induced a statistically significant decrease in the expression of p-MEK (42 and 35%, respectively, below non-differentiated controls; Fig. 3). In differentiated cultures, CRET stimulation induced overexpression of p-MEK with respect to the sham-exposed controls (12 and 21% at 2 and 9 days, respectively), the effect being statistically significant in samples differentiated for 9 days.

Effects of CRET on the regulation of genes intervening in adipocyte differentiation. As expected, incubation for 2 or 9 days in the presence of adipogenic medium significantly increased the mRNA expression of PPARG and of the other selected genes intervening in lipid storage and PPARγ activation (data not shown). The results in Fig. 4 demonstrated that when applied during the two initial days of differentiation, CRET exposure failed to induce significant changes in the mRNA expression levels of the studied genes. By contrast, when the electric stimulus was administered from day 7-9 of adipogenesis, the mRNA expression of PPARG1 was significantly reduced by 9%, with respect to sham-exposed controls. A similar trend, though not significant statistically, was also observed for the expression of PPARG2. In concomitance with the reduced mRNA expression of PPARG1, CRET also induced significant decreases in the mRNA expression of genes located downstream of PPARG, including perilipin (18%), angiopoietin-like (ANGPTL)4 (20%) and fatty acid synthase (FASN; 11%).

Discussion

ADSC can differentiate to form mature adipocytes, maintaining this ability throughout life. Since restrictive dieting is known to induce a reduction in size, but not in number, of adipocytes in adipose tissue, it has been postulated that cytoplasmic accumulation of fat during adipogenic differentiation is crucial in human obesity (27). Therefore, the experimental study of the effects of antiobesity therapies on the regulation of the adipogenic differentiation can provide relevant information for optimizing the effectiveness of therapies, including those applying electrothermal currents. For instance, when applied at thermal doses, stimulation with 448 kHz CRET has been reported to cause a significant decrease in the quantity of fatty deposits in OP9 mouse stromal cells in advanced stages of differentiation (13). In the present study, exposure to short, subthermal pulses of 448 kHz CRET currents resulted in a significant and consistent reduction of lipid content in human ADSC at the early stages of the adipogenic differentiation. Taken together, these results suggested that, when administered simultaneously, the thermal stimulus, together with mechanical pressure applied in the CRET therapy, the electric stimulus is able to significantly reduce the large quantities of lipids present in advanced stages of adipogenesis. Additionally, subthermal electric stimulation can induce, by itself and in the absence of the thermal factor, an antiadipogenic action in the earlier stages of adipogenesis.

The present study hypothesized that the early antiadipogenic action of the subthermal stimulus is exerted through a chain of responses at the molecular level, rather than through a more direct effect of emptying of the lipid vesicles. In order to investigate the processes potentially involved in the subthermal effects, the protein and mRNA expression levels of PPARγ, the key nuclear transcription factor in adipogenesis control, were determined. Immunoblotting assay failed to detect significant changes with respect to the controls in the expression of PPARγ in the samples at the earlier stages, grown in differentiation medium for 2 days only and exposed simultaneously to electric stimulation. By contrast, in samples differentiated for 9 days, the subthermal treatment induced a significant decrease in PPARγ with respect to sham exposed controls. On the other hand, an immunofluorescence assay revealed that CRET treatment significantly decreased the number of cells displaying nuclear PPARγ, both in samples differentiated for 2 or 9 days, the latter showing a predominantly cytoplasmic location of PPARγ (Fig. 2B).

Although little is known about its intracellular distribution, experimental evidence locates PPARγ in the nucleus of the quiescent cells, where it exerts its predominant function as a transcription factor for adipogenesis. On this basis, the apparent discordance between the responses obtained at 2 days in the immunoblot vs. immunofluorescence assays, may be attributable to the fact that immunoblotting quantifies the total PPARγ protein in the sample, regardless of its cellular location. By contrast, immunofluorescence exclusively quantifies PPARγ that, being present in the cell nucleus, acts as an adipogenic transcription factor. Therefore, the mentioned discordance would not occur if only the nuclear, active PPARγ protein was susceptible to the electric stimulus. As a whole, these results were coherent with the observed reduced lipid content in CRET-treated ADSC and indicate that such an effect can be mediated by electrically-induced changes in the expression of PPARγ.

It has been reported that when the cell receives a mitogenic stimulus, p-MEK, the kinase enzyme of the MAPK-ERK1/2 pathway, is overexpressed and binds physically to PPARγ, causing its translocation from the nucleus to the cytoplasm (20-22) and preventing PPARγ from exerting its function as an activator of adipogenic effector genes. To elucidate whether this is a potential mechanism involved in the observed translocation of PPARγ to the cytoplasm of the samples differentiated for 9 days and treated with CRET, the expression of p-MEK was analyzed. While in undifferentiated samples immunoblotting revealed a high expression of p-MEK, typical of proliferating cultures, the differentiation

medium induced significant low expression of p-MEK (Fig. 3). Compared with differentiated, sham-exposed controls, CRET treatment induced the overexpression of p-MEK, although this effect only reached statistical significance in cultures with 9 days of differentiation. These results suggested that this overexpression of p-MEK may result in the downregulation of the genomic activity of PPARG. Therefore, the 448 kHz electric signal may be interpreted by the cell as a mitogenic stimulus, which induces the overexpression of p-MEK. With regards to this, previous studies by our group have shown that 448 kHz CRET can stimulate mitosis in undifferentiated mesenchymal ADSC by activating the expression of p-ERK, another member of the same MAPK proliferative pathway (15). In cultures undergoing adipogenic differentiation, early activation of this pathway may not result in changes in cell proliferation, however, only in the translocation of PPARγ towards the cytoplasm, and subsequent hindering of the adipogenic differentiation. In other words, CRET stimulation may result in at least one non-genomic interaction of PPARγ with different protein partners (e.g. those associated with the cytoskeleton, lipid droplets and kinases), leading to alternative cytoplasmic signaling, which would result in partial inhibition of early adipogenic differentiation.

Regarding the influence of CRET on the expression of genes involved in the adipogenic metabolism and differentiation, no significant changes were detected in the gene expression levels in ADSC differentiated for 2 days and treated with CRET. By contrast, in samples differentiated for 9 days, CRET caused decreased expression of PPARG1, ANGPTL4, perilipin and FASN, without significantly affecting the other genes analyzed (Fig. 4). These results revealed that CRET stimulation not only induces decreased protein expression of PPARγ, but also results in the downregulation of PPARG, which leads to the reduced expression of a number of genes whose transcription directly requires PPARγ protein, including ANGPTL4 and perilipin, or indirectly, including FASN (28). ANGPTL4 is known to irreversibly inactivate lipoprotein lipase (LPL) (29). Therefore, it was hypothesized that the decreased expression of this gene may result in increased lipolytic activity of LPL. Perilipin is a protein covering lipid vesicles, protecting them against the action of cytosolic lipases (30). Therefore, decreased gene expression of perilipin in response to CRET may promote mobilization of intracellular reservoirs of triacylglycerol. Finally, FASN is a multi-enzyme protein that, in the presence of NADPH, catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA to long chain saturated fatty acids (31). Therefore, the CRET-induced decrease in FASN expression may also contribute to reduced quantities of intracellular lipids. Taken together, these results are consistent with those from the other tests performed in the present study, and indicated that the hindering effects of CRET on early adipogenic stages would be mediated, at least in part, by electrically induced low expression of genes involved in the control of the synthesis and mobilization of fatty acids during adipogenesis.

In conclusion, the 448 kHz CRET therapy simultaneously applies three stimuli: Electrical, thermal and mechanical, which may act in synergy in antiobesity treatments. The present study identified and described potential molecular mechanisms through which the assayed 448 kHz electric signal can, by itself and in the absence of the accompanying thermal and mechanical components of the CRET treatments, interfere with processes controlling the synthesis and mobilization of fat at early adipogenic stages. The affected processes included the activation of MEK1/2, reduced expression of PPARγ protein and downregulation of the gene expression levels of PPARG1, perilipin, ANGPTL4 and fatty acid synthase. The possibility cannot be disregarded that these effects also contributed to the antiadipogenic action exerted on mature adipocytes by thermal treatment with CRET (13), even if the extent of such potential contribution remains to be determined.

Acknowledgements

The authors would like to thank Dr María Antonia Martínez-Pascual and Mrs. Lorena Crespo-Toro (both IRYCIS, University Hospital Ramón y Cajal) for their valuable technical contribution. This work was financially supported by the Fundación para la Investigación Biomédica del Hospital Ramón y Cajal (no. 2012/0032).

References

1. Hurt RT, Kulisek C, Buchanan LA and McClave SA: The obesity epidemic: Challenges, health initiatives, and implications for gastroenterologists. Gastroenterol Hepatol (NY) 6: 780-792, 2010.
2. Lavie CJ, Milani RV and Ventura HO: Obesity and cardiovascular disease: Risk factor, paradox, and impact of weight loss. J Am Coll Cardiol 53: 1925-1932, 2009.
3. Abraham MT and Mashkevich G: Monopolar radiofrequency skin tightening. Facial Plast Surg Clin North Am 15: 169-177, 2007.
4. Belenky I, Margulis A, Elman M, Bar-Yosef U and Paun SD: Exploring channeling optimized radiofrequency energy: A review of radiofrequency history and applications in esthetic fields. Adv Ther 29: 249-266, 2012.
5. Mulholland RS, Paul MD and Chalfoun C: Noninvasive body contouring with radiofrequency, ultrasound, cryolipolysis, and low-level laser therapy. Clin Plast Surg 38: 503-520, 2011.
6. Alexiades-Armenakas M, Dover JS and Arndt KA: Unipolar radiofrequency treatment to improve the appearance of cellulite. J Cosmet Laser Ther 10: 148-153, 2008.
7. Brightman L, Weiss E, Chapas AM, Karen J, Hale E, Bernstein L and Geronemus RG: Improvement in arm and post-partum abdominal and flank subcutaneous fat deposits and skin laxity using a bipolar radiofrequency, infrared, vacuum and mechanical massage device. Lasers Surg Med 41: 791-798, 2009.
8. Valentim da Silva RM, Barichello PA, Medeiros ML, de Mendonça WC, Dantas JS, Ronzio OA, Froes PM and Galadari H: Effect of capacitive radiofrequency on the fibrosis of patients with cellulite. Dermatol Res Pract 2013: 715829, 2013.
9. van der Lugt C, Romero C, Ancona D, Al-Zarouni M, Perera J and Trelles MA: A multicenter study of cellulite treatment with a variable emission radio frequency system. Dermatol Ther 22: 74-84, 2009.
10. Boisnic S, Divaris M, Nelson AA, Gharavi NM and Lask GP: A clinical and biological evaluation of a novel, noninvasive radiofrequency device for the long-term reduction of adipose tissue. Lasers Surg Med 46: 94-103, 2014.
11. Hamida ZH, Comtois AS, Portmann M, Boucher JP and Savard R: Effect of electrical stimulation on lipolysis of human white adipocytes. Appl Physiol Nutr Metab 36: 271-275, 2011.
12. Trelles MA, van der Lugt C, Mordon S, Ribé A and Al-Zarouni M: Histological findings in adipocytes when cellulite is treated with a variable-emission radiofrequency system. Lasers Med Sci 25: 191-195, 2010.
13. Kato S, Saitoh Y and Miwa N: Repressive effects of a capacitive-resistive electric transfer (CRet) hyperthermic apparatus combined with provitamin C on intracellular lipid-droplets formation in adipocytes. Int J Hyperthermia 29: 30-37, 2013.

14. Hernandez-Bule ML, Cid MA, Trillo MA, Leal J and Ubeda A: Cytostatic response of HepG2 to 0.57 MHz electric currents mediated by changes in cell cycle control proteins. Int J Oncol 37: 1399-1405, 2010.
15. Hernández-Bule ML, Paíno CL, Trillo MÁ and Úbeda A: Electric stimulation at 448 kHz promotes proliferation of human mesenchymal stem cells. Cell Physiol Biochem 34: 1741-1755, 2014.
16. Hernández-Bule ML, Roldán E, Matilla J, Trillo MA and Ubeda A: Radiofrequency currents exert cytotoxic effects in NB69 human neuroblastoma cells but not in peripheral blood mononuclear cells. Int J Oncol 41: 1251-1259, 2012.
17. Hernández-Bule ML, Trillo MA, Bazán E, Martinez-Pascual MA, Leal J and Ubeda A: Nonthermal levels of electric currents applied in capacitive electric transfer therapy provokes partial cytotoxic effects in human neuroblastoma cultures. Neurocirugia (Astur) 15: 366-371, 2004.
18. Hernández-Bule ML, Trillo MA, Cid MA, Leal J and Ubeda A: In vitro exposure to 0.57-MHz electric currents exerts cytostatic effects in HepG2 human hepatocarcinoma cells. Int J Oncol 30: 583-592, 2007.
19. Hernández-Bule ML, Trillo MÁ and Úbeda A: Molecular mechanisms underlying antiproliferative and differentiating responses of hepatocarcinoma cells to subthermal electric stimulation. PLoS One 9: e84636, 2014.
20. Burgermeister E, Chuderland D, Hanoch T, Meyer M, Liscovitch M and Seger R: Interaction with MEK causes nuclear export and downregulation of peroxisome proliferator-activated receptor gamma. Mol Cell Biol 27: 803-817, 2007.
21. Burgermeister E and Seger R: MAPK kinases as nucleo-cytoplasmic shuttles for PPARgamma. Cell Cycle 6: 1539-1548, 2007.
22. Burgermeister E and Seger R: PPARgamma and MEK interactions in cancer. PPAR Res 2008: 309469, 2008.
23. Hollenberg CH and Vost A: Regulation of DNA synthesis in fat cells and stromal elements from rat adipose tissue. J Clin Invest 47: 2485-2498, 1969.
24. Van RL, Bayliss CE and Roncari DA: Cytological and enzymological characterization of adult human adipocyte precursors in culture. J Clin Invest 58: 699-704, 1976.
25. Zannettino AC, Paton S, Arthur A, Khor F, Itescu S, Gimble JM and Gronthos S: Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. J Cell Physiol 214: 413-421, 2008.
26. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45, 2001.
27. Cristancho AG and Lazar MA: Forming functional fat: A growing understanding of adipocyte differentiation. Nat Rev Mol Cell Biol 12: 722-734, 2011.
28. Lowe CE, O'Rahilly S and Rochford JJ: Adipogenesis at a glance. J Cell Sci 124: 2681-2686, 2011.
29. Mattijssen F and Kersten S: Regulation of triglyceride metabolism by Angiopoietin-like proteins. Biochim Biophys Acta 1821: 782-789, 2012.
30. Brasaemle DL, Subramanian V, Garcia A, Marcinkiewicz A and Rothenberg A: Perilipin A and the control of triacylglycerol metabolism. Mol Cell Biochem 326: 15-21, 2009.
31. Jayakumar A, Tai MH, Huang WY, al-Feel W, Hsu M, Abu-Elheiga L, Chirala SS and Wakil SJ: Human fatty acid synthase: Properties and molecular cloning. Proc Natl Acad Sci USA 92: 8695-8699, 1995.