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Molecular Mediators Involved in *Ferulago campestris* Essential Oil Effects on Osteoblast Metabolism

Maria Giovanna Sabbieti,[1] Dimitrios Agas,[1] Filippo Maggi,[2] Sauro Vittori,[2] and Luigi Marchetti[1*]

[1] *School of Biosciences and Biotechnology, University of Camerino, I-62032 Camerino (MC), Italy*

[2] *School of Pharmacy, University of Camerino, I-62032 Camerino (MC), Italy*

ABSTRACT

This study was performed to investigate the effects of the essential oil obtained from fruits of *Ferulago campestris* (FC) on primary calvarial mouse osteoblasts (COBs). The composition of the oil was dominated by monoterpene hydrocarbons (78.8–80.3%), with myrcene (33.4–39.7%), α-pinene (22.7–23.0%), and γ-terpinene (8.1–10.9%) as the major components. Owing to their lipophilic properties, these compounds easily cross cell membranes and affect bone cell function by stimulating or inhibiting specific molecular pathways. We demonstrated, for the first time, that FC oil increased osteoblast proliferation by MAP kinase activation; in addition, oils enhanced the protein kinase AKT, which is known to be critical for control of cell survival, also in presence of the MEK-1 inhibitor PD98059, and this effect was accompanied with a down-regulation of pro-apotoic molecules such as Bax and caspases. Interestingly, FC oil significantly increased Runx2 (Runx2/Pebp2αA/AML3) and phospho-Smad1/5/8 protein level, the master regulators of osteoblast differentiation, and their nuclear localization. PD98059 pre-treatment further improved Runx2/phospho-Smads up-regulation. Thus, FC oils influence osteoblast metabolism probably using alternative signaling pathways depending also on the maturation stage of the cells. Taken together our data delineate a positive function of FC oil on osteoblast metabolism, suggesting its possible use as a dietetic integrator in the prevention or in the therapy of pathologies due to impaired bone remodeling. J. Cell. Biochem. 112: 3742–3754, 2011.

KEY WORDS: ESSENTIAL OIL; *FERULAGO CAMPESTRIS*; OSTEOBLASTS; OSTEOPOROSIS

Recently, natural products have gained more increased interest as alternative treatments for osteoporosis and for the maintenance of bone health in spite of their negligible adverse side effects. A wide variety of natural compounds can influence the process of bone remodeling, particularly by enhancing osteogenic differentiation, and inhibiting bone resorption, thus having beneficial effects on the skeleton [Putnam et al., 2007]. There is increasing interest in the use of plant-derived estrogens, also known as phytoestrogens such as isoflavones (e.g., genistein and daidzein) and lignans, derived from a large number of plant sources. They are very promising in their role as supplements in preventing and treating post menopausal osteoporosis, because of their similarity in structure to estradiol and therefore as potential replacements for estrogen deficiency [Appleton and Lockwood, 2006].

On the other hand, recent studies demonstrated that also essential oils and their monoterpene components affect bone metabolism when added to the food of rats [Mühlbauer et al., 2003]. Essential oils are volatile, natural mixtures of complex compounds, mainly monoterpenoids and sesquiterpenoids, characterized by a strong odor and playing an important role in the protection of the plants.

Being widely distributed in the plant kingdom and active on bone, essential oils could constitute an ideal candidate for a dietary approach to osteoporosis. Considering the disadvantages of conventional treatment, the use of natural additives to the diet could be an important means of reducing the incidence of osteoporosis, or at least reducing the extent of mineral loss from bones. In addition, many of these constituents already form part of a normal diet, are known to be safe. Monoterpenes, the major components of essential oils, belong to the group of isoprenoids containing 10 C-atoms. They are present in some herbs commonly used in human nutrition. Owing to their lipophilic properties, essential oils and monoterpenes easily cross cell membranes and affect cell function. For instance, various monoterpene components (thujone, eucalyptol, camphor, and borneol) occurring in sage oil (*Salvia officinalis* L.) were found to strongly inhibit bone resorption in vivo and in vitro [Mühlbauer et al., 2003]. Pine oil (*Pinus spp.*),

Maria Giovanna Sabbieti and Dimitrios Agas equally contributed to this study.

*Correspondence to: Luigi Marchetti, School of Biosciences and Biotechnology, University of Camerino, Via Gentile III da Varano, I-62032 Camerino (MC), Italy. E-mail: luigi.marchetti@unicam.it

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rich in α-pinene, β-pinene, and bornylacetate, prevented bone loss in an osteoporosis model, by reducing the rate of trabecular bone mineral density (BMD) in aged ovariectomized (OVX) rat. *Cis*-verbenol, a metabolite of α-pinene occurring in human urine, inhibited osteoclastic resorption activity [Eriksson and Levin, 1996]. 3-Carene, a bicyclic monoterpene occurring in pine trees has been found to stimulate the mineralization in osteoblastic cells and its activity could result from its potential to promote the activation of mitogen-activated protein (MAP) kinases signaling pathways and the transcript levels of osteoblast mineralization-related genes such as osteopontin and type I collagen [Jeong et al., 2008]. It has been suggested that monoterpenes act directly on bone cells, not via the stimulation of calciotropic hormones, and the mode of action is thought to be via inhibition of the mevalonate pathway and the prenylation of small G proteins such as Ras, Rho, and Rac [Clegg et al., 1982; Crowell et al., 1991; Ren et al., 1997]. This effect is normally exerted by modulation of transcription factors, particularly NF-κB, and enzymes, cytokines, cell cycle proteins, receptors and cell-surface adhesion molecules [Aggarwal and Shishodia, 2004].

The formation of bone involves a complex series of events, such as the proliferation and differentiation of osteoprogenitor cells that eventually result in the formation of a mineralized extracellular matrix [Stein et al., 1990; Bresford et al., 1993; Inoue et al., 1996]. The sequential expression of type I collagen, alkaline phosphatase (ALPase) and osteocalcin, and the deposition of calcium are known to be markers for osteoblastic differentiation. It is now well known that many regulations various systemic and local factors modulate bone turn-over among which, insulin-like growth factor-I (IGF-I), bone morphogenetic factors (BMPs), and Wnt proteins are potent bone anabolic factors [Reddi, 1998; Chen et al., 2004; Niu and Rosen, 2005; Krishnan et al., 2006]. A serine-threonine kinase Akt, also called protein kinase B (PKB), is known as a potent signal transducer of these bone anabolic factors [Ghosh-Choudhury et al., 2002; Hanada et al., 2004; Kawamura et al., 2007].

Studies have shown that Runt-related transcription factor 2 (Runx2) also known as cbfa1/pebp2aA/AML3 plays an important role in osteoblast differentiation. Mice with disruption of Runx2 gene fail to develop mineralized bone [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997], while haploinsufficient mice develop cleidocranial dysplasia, which is characterized by delayed ossification [Otto et al., 1997]. Several studies showed that Runx2 regulated the expression of genes associated with osteoblast differentiation [Banerjee et al., 1997; Ducy et al., 1997; Ji et al., 1998]. Runx proteins have been shown to interact through their C-terminal segment with Smads, a family of signaling proteins that regulate a diverse array of developmental and biological processes in response to transforming growth factor (TGF)/bone morphogenetic protein (BMP) family of growth factors [Massague, 1998; Derynck et al., 1998; Alliston et al., 2001]. On the other hand, it is well known that inhibitory Smads, Smad6, and Smad7, (I-Smads) inhibit phosphorylation of R-Smads by competing with R-Smads for binding to activated type I receptors.

Importantly, in vivo osteogenesis requires the C terminus of Runx2 containing the overlapping subnuclear targeting signal and the Smad interacting domain [Zaidi et al., 2002]. The Runx and Smad proteins are jointly involved in the regulation of phenotypic gene expression and lineage commitment. Gene ablation studies have revealed that both Runx proteins and Smads are developmentally involved in haematopoiesis and osteogenesis [Derynck et al., 1998; Goumans and Mummery, 2000].

We previously demonstrated that PGF2α and PTH regulate osteoblast metabolism by involving fibroblasts growth factor-2 (FGF-2) and its receptors (FGFRs) [Hurley et al., 1999; Sabbieti et al., 1999; Agas et al., 2008; Sabbieti et al., 2009; Sabbieti et al., 2010].

In order to find natural additives to the diet, potentially useful as means of reducing the incidence of osteoporosis, or at least reducing the extent of mineral loss from bones, in this work we investigated the effects of monoterpenes-containing essential oil of *Ferulago campestris* L. (FC) (Apiaceae) on osteoblast growth and differentiation. To the best of our knowledge, there have been no reports regarding the effects of FC oil on osteoblasts in vitro.

MATERIAL AND METHODS

PLANT MATERIAL

Fruits of FC were collected in August 2008 in the following localities: site 1, Madonna di Val Povera (Camerino, central Italy), 900 m above sea level, N 43°06′33″ E 13°00′06″; site 2, Madonna del Sasso (Pergola, central Italy), 300 m above sea level, N 43°30′56″ E 12°49′13″. The plants were botanically confirmed by Dr. Maggi using available literature [Pignatti, 1982; Cannon, 1993]. The voucher specimens were deposited in the Herbarium Camerinensis (included in the online edition of Index Herbariorum: http://sweetgum.nybg.org/ih/) [Holmgren and Holmgren, 1998] of School of Environmental Sciences (University of Camerino, Italy) under the accession codes CAME 13399 (site 1) and CAME 13442 (site 2); they are also available at the following website: http://erbariitaliani.unipg.it.

EXTRACTION OF ESSENTIAL OILS

Fresh fruits of FC were hydrodistilled in a Clevenger-type apparatus for 3 h. The essential oils were dried over anhydrous sodium sulfate, and then stored in sealed vials protected from the light at −20°C before gas chromatographic and biological study. Three oils were obtained from each sample by hydrodistillation and subsequently analyzed by GC-FID and GC-MS. The oil yield ($n = 3$), expressed in percentage, was estimated on a dry weight basis, by calculating the water content prior to the distillation by leaving plant material in a stove at 110°C for 24 h.

GC–FID AND GC–MS ANALYSIS

For gas chromatographic separations, an Agilent 4890D instrument coupled to an ionization flame detector (FID) was used. Volatile components were separated on a HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm id; 0.17 μm film thickness) (J & W Scientific, Folsom, CA, USA), with the following temperature program: 5 min at 60°C, subsequently 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min, for a total run of about 65 min. Injector and transfer line temperatures were 280°C. Helium was used as the carrier gas, at a flow rate of 1.4 ml/min. Split ratio: 1:34. A mixture of aliphatic hydrocarbons (C_8–C_{30}) (Sigma,

Milano, Italy) in hexane was directly injected into the GC injector under the above temperature program, in order to calculate the retention index (as Kovats index) of each compound. All samples were diluted 1:200 in hexane for GC analysis and the volume injected was 1 μl. Data were collected by using HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01).

GC-MS analysis was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer using a HP-5MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm id, 0.1 μm film thickness) (J & W Scientific, Folsom). The temperature program was the same with that reported above. Injector and transfer line temperatures were 280°C. Helium was used as the carrier gas, at a flow rate of 1 ml/min. Split ratio: 1:50; acquisition mass range: 29–400 *m*/*z*. All mass spectra were acquired in electron-impact (EI) mode with a ionization voltage of 70 eV. All samples were diluted 1:100 in hexane and the volume injected was 2 μl. Data were analyzed by using MSD ChemStation software (Agilent, Version G1701DA D.01.00).

IDENTIFICATION AND QUANTIFICATION OF VOLATILES

Ten out of twenty-seven volatile components detected in fruits of *F. campestris* were identified by comparing the retention time, retention index, and mass spectrum of the chromatographic peaks with that of standards analyzed under the same conditions. The peak assignments of the other volatile components was based on computer matching of the mass spectra obtained with the WILEY275, NIST 08 (National Institute of Standards and Technology), and ADAMS libraries, taking into account the coherence of the retention indices of the analyzed compounds with those reported by Adams [2007] and NIST 08 [2008] libraries. Quantification of volatile components was obtained by means of FID peak-area internal normalization. Response factors were calculated on the basis of a previous procedure adopted for the quantification of volatiles in *M. melissophyllum* L. [Maggi et al., 2010].

EXPERIMENTAL ANIMALS

Harlan Sprague-Dawley ICR (CD-1) male mice (Harlan, Italy) were used. Mice were sacrificed by CO_2 narcosis and cervical dislocation in accordance with the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators.

PRIMARY CALVARIAL OSTEOBLASTS (COBs)

COBs were obtained from newborn mice by sequential digestion with 0.1% collagenase (Roche Diagnostic, Milano, Italy), as previously described [Montero et al., 2000]. Cells were pooled and cultured to confluence in 100-mm dishes.

ASSESSMENT OF VIABLE COBs (MTS)

Cells viability was determined by both MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and BrdU assay. Briefly, COBs were plated at the density of 5000 cells/well in 96 culture plates (Costar Corp., Celbio, Milano, Italy) and grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Milano, Italy) supplemented with 10% heat inactivated foetal calf serum (FCS; Invitrogen, Milano, Italy) penicillin, and streptomycin to approximately 80% confluence. Cells were serum deprived for 24 h and treated with FC oil samples (from 10^{-3} to 10^{-6} M) or vehicle for additional 24 h. MTS assay was performed, by incubating the cells with 20 μl/well of CellTiter 96 Aqueous One Solution Reagent (Promega Italia srl, Milano, Italy) and incubated for 2 h in a humidified, 5% CO_2 atmosphere. The quantity of formazan product was directly proportional to the number of living cells in culture. The colored formazan was measured by reading the absorbance at 490 nm using a 96-well plate reader. For BrUd incorporation, BrdU assay was performed using "Cell Proliferation ELISA, BrdU (colorimetric)" (Roche Diagnostics). Cells were cultured 24 h in presence and absence of FC oil samples (from 10^{-3} to 10^{-6} M), then were labeled with BrdU following the manufacturer's instructions. Photometric detection was done with an ELISA reader at 370 nm wavelength. The experiment was run in triplicate.

CELL CULTURES FOR WESTERN BLOTTING

COBs were plated at 15 000 cells/cm^2 in 6-well culture plates (Costar Corp.) in DMEM (Sigma–Aldrich, Milano, Italy), supplemented with 10% heat inactivated FCS (Invitrogen, Milano Italy), penicillin and streptomycin. Cells were grown to confluence, then cultures were serum deprived for 24 h and treated with FC oil samples (10^{-4} M), or vehicle for selected time periods. Other cultures were pre-treated with the MEK1 inhibitor, PD98059 (4×10^{-5} M) (Cell Signaling Celbio, Milano, Italy), for 1 h before incubation with FC oils. Proteins were extracted with cell lysis buffer (Cell Signaling Celbio, Milano, Italy) and concentration was determined by the BCA protein assay reagent (Pierce, Rockford, Celbio, Milano, Italy). After SDS–polyacrylamide gel electrophoresis (PAGE) on 12% gels, proteins were transferred to PVDF membranes (Amersham Biosciences, Europe, GMBH). The next steps were performed by ECL Advance Western Blotting Detection Kit (GE Healthcare, Milano, Italy). Briefly, membranes were blocked with Advance Western Blotting Agent (GE Healthcare, Milano, Italy) in PBS-T (PBS containing 0.1% Tween-20) for 1 h at room temperature. Then, membranes were incubated with the following antibodies: mouse anti-phospho-p44/42, rabbit anti-cyclin D1, mouse anti-cyclin E, rabbit anti phospho-Smad 1/5/8, rabbit anti-cleaved caspase 9, rabbit anti-cleaved caspase 3, rabbit anti-caspase 9, rabbit anti caspase 3, rabbit anti-phospho-AKT^{ser308}, rabbit anti-AKT (Cell Signaling, Celbio, Milano, Italy) all diluted 1:800; mouse anti-c-myc, rabbit anti-Pebp2aA (Runx-2), rabbit anti-Bcl-2, rabbit anti-Bax, rabbit anti-phospho-p21^{thr145}, rabbit anti Smad6/7 antibodies (Santa Cruz Biotechnology, TebuBio, Milano, Italy) all diluted 1:200. All membranes were incubated with the above primary antibodies for 2 h at room temperature. After washing with PBS-T, the blots were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (GE Healthcare, Milano, Italy) or with HRP-conjugated rabbit anti-mouse IgG antibody (GE Healthcare, Milano, Italy) diluted 1:100 000 in blocking solution for 1 h at room temperature.

After further washing with PBS-T, immunoreactive bands were visualized using luminol reagents and Hyperfilm-ECL film (GE Healthcare, Milano, Italy) in accordance with the manufacturer's instructions. To normalize the bands, filters were stripped and re-probed with mouse anti-α-tubulin antibody (Sigma–Aldrich, Milano, Italy), with rabbit anti-p44/42 (Cell Signaling, Celbio,

Milano, Italy) or with rabbit anti-p21 (Santa Cruz Biotechnology, TebuBio, Milano, Italy). Band densities were quantified densitometrically.

CELL CULTURES FOR IMMUNOFLUORESCENCE

COBs were plated at 5000 cells/cm^2 in 6-well culture plates (Costar Corp., Celbio, Italy) containing coverslips, previously cleaned and sterilized, in DMEM with 10% heat inactivated FCS, penicillin, and streptomycin. For Runx2 and Smad 1/5/8 labeling, cells were grown to approximately 80% confluence. For osteocalcin (OC) labeling, cells were cultures for 14 days in differentiation medium (DMEM, 10% FCS, 8 mM β-glycerophosphate, 50 μg/ml ascorbic acid). Then, cultures were treated for 24 h with FC oil (10^{-4} M) and labeled for Runx2 and Smad 1/5/8 other cultures were treated for 3 days with FC oil (10^{-4} M) before OC labeling. Control cultures were pulsed with only vehicle. Then, cells were briefly rinsed with PBS 0.1 M, pH 7.4, and fixed in 4% paraformaldeyde (PFA) diluted in PBS for 25 min at room temperature. After washing three times in PBS, cells were permeabilized with 0.3% Triton X-100 for 30 min and incubated with 0.5% BSA diluted in PBS for 20 min at room temperature. Then, cells were incubated for 2 h at room temperature with goat anti-Pebp2aA (Runx-2) or rabbit anti-osteocalcin antibodies (Santa Cruz Biotechnology, TebuBio) both diluted 1:50 in PBS; other cells were incubated with rabbit anti-phopho-Smad1/5/8 antibody (Cell Signaling, Celbio) diluted 1:80 in PBS. After rinsing, cells were incubated with rabbit anti-goat IgG Alexa Fluor 594 conjugated (Molecular Probes, Invitrogen, Milano Italy) or chicken anti-rabbit IgG Alexa Fluor 488 conjugated (Molecular Probes) both diluted 1:100 in PBS for 1 h at room temperature. After washing, coverslips were mounted on slides with PBS/glycerol (1:1). Control experiments were performed by omitting the appropriate primary antibodies. Slides were imaged using a Leica DM 2500 fluorescent microscopy.

Fluorescence analysis was performed by a fluorimeter Tecan Infinite with excitor filter 590 nm and emission 635 nm for Alexa Fluor 594, or 485 and of 535 nm for Alexa Fluor 488. The amount of Alexa Fluor 594-labeled anti-Runx-2 and Alexa Fluor 488-labeled anti-phospho-Smad 1/5/8 or anti-osteocalcin was quantified by a Tecan Infinite fluorescence reader.

ALKALINE PHOSPHATASE (ALP) ASSAY AND ALIZARIN RED S HISTOCHEMICAL STAINING

COBs were plated at 5×10^4 cells/well in 6-well culture plates and cultured for 6 days in differentiation media in the presence or absence of FC oil (10^{-4} M). ALP staining was performed with a commercial kit (Sigma–Aldrich) according to the manufacturer's instructions.

Using Alizarin red S histochemistry, the cultured cells were stained on days 14 and 21 for assessing the mineralized matrix. The medium was removed, and the cell layers were rinsed with PBS and fixed in 4% PFA diluted in PBS for 25 min at room temperature. Then, cultures were washed with PBS and stained with 2% Alizarin red S pH 7.2 (Sigma–Aldrich) for 20 min at 37°C. Stain was desorbed and the collected solutions were distributed as 100 μl/well on 96-well plates for absorbance reading at 590 nm by spectrophotometry (Tecan Infinite reader). Cultures were observed at light microscope level.

STATISTICAL ANALYSIS

Where applicable, the significance of difference between two groups was evaluated with an unpaired two-tailed Students *t*-test by using Microsoft Excel.

RESULTS

COMPOSITION OF FC OIL

Twenty-seven volatile components were identified in the essential oil obtained by hydrodistillation from fruits of FC, corresponding to 95.7–97.5% of the total oils (Table I). Their compositions were based on monoterpene hydrocarbons (78.8–80.3%) such as myrcene (33.4–39.7%), α-pinene (22.7–23.0%), and γ-terpinene (8.1–10.9%), which accounted for 64.2–73.6% of their total composition (Fig. 1). Aromatics constituted the second fraction of the oil (9.4–9.8%), with (2,3,6)-trimethyl benzaldehyde (8.6–9%) being the most abundant. Oxygenated monoterpenes and sesquiterpene hydrocarbons gave a little contribute with 4.3–4.8 and 2.4–2.5%, respectively. No significant quali-quantitative differences were detected on the basis of the different origin of the samples.

OSTEOBLAST PROLIFERATION

Cell viability was incremented by treatment with FC oil samples for 24 h, in a dose-dependent manner reaching a maximum at 10^{-4} M of concentration (about 28% respect to vehicle treated cells), while oil concentration of 10^{-3} M, $10^{-3.5}$, $10^{-5.5}$, and 10^{-6} M did not exert significant effects (Fig. 2A). Similar results were obtained by using BrdU assay (Fig. 2 B).

REGULATION OF PROTEINS INVOLVED IN CELL CYCLE

Western blotting data showed that treatment with FC oil samples at 10^{-4} M increased phospho-p44/42 levels (Fig. 3A) and, of relevance, c-Myc, cyclins D1, and E levels (Fig. 3B). A MEK1 inhibitor, PD98059, blocked enhanced c-Myc and cyclins by FC oil sample 1 (Fig. 3C) and sample 2 (data not shown). Pooled data from three independent experiments clearly showed a statistically significant increment of pospho-p44/42 after 15 and 30 min of FC oil samples treatment as well as of c-Myc and cyclins after 6 and 24 h of treatment, only in absence of PD98059 (Fig. 3D–F). In addition, an increase level of phospho-p21 was found after treatment with FC oil samples, while p21 protein levels were not significantly modify (Fig. 3G and H).

MODULATION OF COBs SURVIVAL/APOPTOSIS

AKT is serine/threonine protein kinase activated by phosphatidylinositol 3 kinase (PI3K) phosphorylation. To investigate the activation of AKT by FC oils, COBs were treated with the effectors for 2, 6, and 24 h. Western blotting analysis revealed that FC oil sample 1 increased level of phospho-AKT starting after 2 h and maintained until 24 h. Interestingly, pretreatment with PD98059 up-regulated phospho-AKT levels both in absence and in presence of FC oil (Fig. 4A and B). Overlapping results were also obtained using FC oil sample 2 as effector (data not shown).

TABLE I. Volatile Constituents (Quantitative Values, Expressed in Percentages, are Average of Three Determinations ± Standard Deviation) of Fruits of *F. campestris* (Besser) Grecescu

Component[a]	RI HP-5[b]	RI LIT[c]		Fruits		ID[e]
		ADAMS	NIST 08	Site 1[d] (%)·[f]	Site 2[d] (%)·[f]	
Aromatics				9.8	9.4	
(2,3,4)-Trimethyl benzaldehyde	1316	1314		0.8 ± 0.05	0.8 ± 0.13	MS,RI
(2,3,6)-Trimethyl benzaldehyde	1354	1355		9.0 ± 2.21	8.6 ± 0.74	MS,RI
Monoterpene hydrocarbons				80.3	78.8	
α-thujene	926	930	925	0.1 ± 0.01	0.2 ± 0.08	MS,RI
α-pinene	931	939	934	22.7 ± 1.82	23.0 ± 1.32	Std
Camphene	947	954	949	0.2 ± 0.01	0.2 ± 0.02	Std
Sabinene	973	975	975	0.5 ± 0.02	0.6 ± 0.05	MS,RI
β-pinene	975	979	975	1.1 ± 0.06	1.2 ± 0.06	Std
Myrcene	992	990	992	39.7 ± 1.17	33.4 ± 2.07	Std
α-phellandrene	1004	1002	1002	1.0 ± 0.06	1.1 ± 0.43	Std
α-terpinene	1019	1017	1018	0.1 ± 0.01	0.2 ± 0.09	MS,RI
p-cymene	1028	1024	1028	2.1 ± 0.71	3.2 ± 1.18	Std
Sylvestrene	1031	1030	1032	2.2 ± 0.07	2.2 ± 0.02	MS,RI
(Z)-β-ocimene	1044	1037	1041	1.8 ± 0.15	1.2 ± 0.09	MS,RI
(E)-β-ocimene	1054	1050	1052	0.6 ± 0.02	0.4 ± 0.04	MS,RI
γ-terpinene	1062	1059	1063	8.1 ± 0.52	10.9 ± 1.53	Std
α-terpinolene	1089	1088	1088	0.1 ± 0.03	0.4 ± 0.03	Std
δ-3-carene	1011	1011	1011	0.5 ± 0.10	0.6 ± 0.07	MS,RI
Oxygenated monoterpene				4.3	4.8	
Umbellulone	1165	1171	1165	0.4 ± 0.00	0.5 ± 0.07	MS,RI
(Cis)-chrysantenyl acetate	1264	1265		3.9 ± 0.24	4.3 ± 0.26	MS,RI
Sesquiterpene hydrocarbons				2.4	2.5	
δ-elemene	1334	1338	1334	0.2 ± 0.05	0.3 ± 0.09	MS,RI
β-elemene	1390	1390	1390	0.2 ± 0.05	0.1 ± 0.02	MS,RI
(E)-caryophyllene	1414	1419	1412	0.8 ± 0.09	1.1 ± 0.14	Std
γ-elemene	1433	1436		0.1 ± 0.02	0.1 ± 0.01	MS,RI
α-humulene	1450	1454	1449	0.1 ± 0.01	0.1 ± 0.03	Std
Germacrene D	1478	1485	1477	0.6 ± 0.08	0.7 ± 0.14	MS,RI
Selina-3,7(11)-diene	1539	1546	1538	0.2 ± 0.02	0.1 ± 0.01	MS,RI
Germacrene B	1554	1561	1555	0.2 ± 0.09	0.1 ± 0.03	MS,RI
Oil yield (%)				7.1	5.9	
Total identified (%)				97.5	95.7	

[a]Compounds belonging to each class are listed in order of their elution from a HP-5 column; percentage values are means of three determinations ± standard deviation.
[b]Retention index on HP-5 column, experimentally determined using homologous series of C8-C30 alkanes.
[c]Relative retention index taken from Adams [2007] and/or NIST 08 [2008] for DB-5 and HP-5 capillary column, respectively.
[d]Sites: 1 Madonna di Val Povera; 2, Madonna del Sasso.
[e]Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams [2007] and NIST 08 [2008]; RI, by comparison of RI with those reported in literature; [Demirci et al., 2000] std, by comparison of the retention time and mass spectrum of available authentic standard.
[f]Relative percentage obtained from peak area internal normalization.

It has been reported that AKT activation leads to over expression of the anti-apoptotic proteins Bcl-2 which compete with the pro-apoptotic proteins Bax to regulate apoptosis. Our Western blotting data revealed that FC oils enhanced Bcl-2 protein levels at 6 and 24 h, decreased Bax protein particularly at 24 h (Fig. 4C) and did not significantly affect both proteins after 2 h of incubation (data not shown). Pooled data from three independent experiments confirmed the above results (Fig. 4D). Therefore, the Bcl-2/Bax ratio was clearly increased, as shown in Fig. 4E, above all after 24 h of treatment. Furthermore, treatment with FC oil samples for 6 and 24 h, decreased both cleaved caspase 9 and 3 levels with a maximal reduction at 24 h (Fig. 4F and G).

EFFECTS ON COBs MATURATION

To determine whether FC oils interfere with osteoblast differentiation, we evaluated their effects on Runx-2 and phospho-Smads 1/5/8 levels,

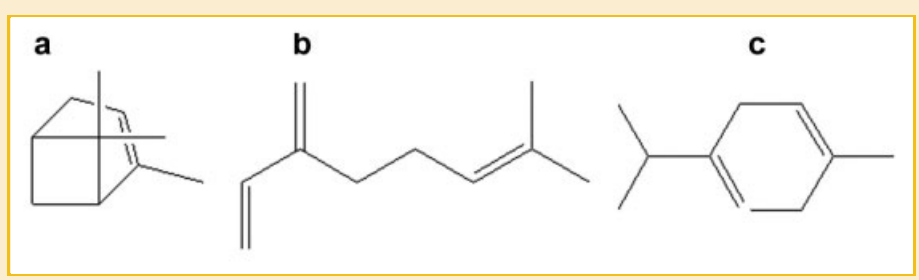

Fig. 1. Structures of major compounds identified in the essential oil of *Ferulago campestris* fruits: (a) α-pinene; (b) myrcene; (c) γ-terpinene.

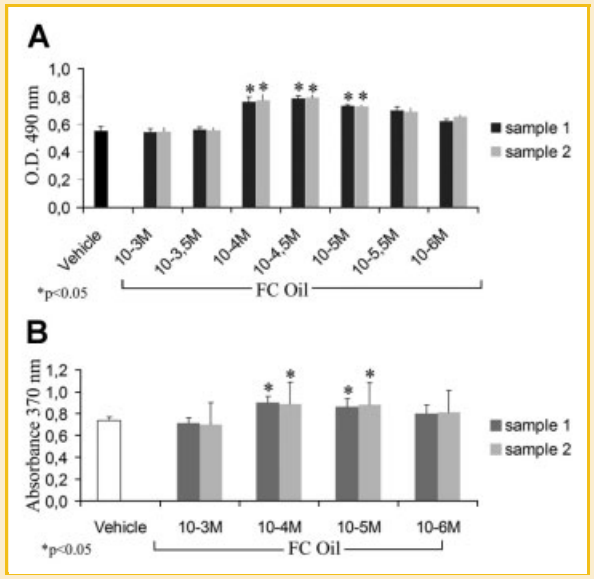

Fig. 2. MTS assay (A); BrdU assay (B) – Cells were 24 h serum deprived and treated with FC oil from 10^{-3} to 10^{-6} M or vehicle for 24 h. Values from three different experiments were referred as means ± standard deviation (SD). Note the significant increase of cell viability particularly at the concentration of 10^{-4} M FC oil; $^{*}P < 0.05$ versus correspondent unstimulated control.

which are critically involved in this process. Interestingly, western blotting results evidenced an increase of Runx2 in COBs, treated for 24 h with FC oil, and an up-regulation of phospho-Smads 1/5/8 in COBs treated for 6 and 24 h; in contrast, Smads 6/7 levels were not significantly modified (Fig. 5A and B). Moreover, data from immunolabeling experiments, in addition to support western blotting results, indicated that FC oil sample 1 at 10^{-4} M (Fig. 5C) and sample 2 at 10^{-4} (data not shown) induced accumulation of Runx2 and phospho-Smads 1/5/8 proteins in the nucleus. Pooled data from four different fluorescence analysis indicated that FC oil statistically increased the mean fluorescence intensity of phospho-Smads 1/5/8 and Runx2 when compared to vehicle treated COBs (Fig. 5D). Interestingly, a statistically significant increase of OC labeling was found in COBs treated with FC oil sample 1 at 10^{-4} M (Fig. 5C) and sample 2 at 10^{-4} M (data not shown). Pooled data from four different fluorescence analysis indicated that FC oil statistically increased the mean fluorescence intensity of OC when compared to vehicle treated COBs (Fig. 5F).

In line with the above results, after 6 days of culture, an increase of ALP positive colonies was found in COBs oil treated compared with vehicle treated osteoblasts, as better shown in the graphic derived from a pool of three independent experiments (Fig. 6A).

In addition, Alizarin red staining on days 14 and 21 revealed a slight, but significant, increase in calcium nodules after treatment with FC oil samples (Fig. 6B).

INVOLVEMENT OF ERK ON RUNX2-SMADS MODULATION

The involvement of ERK on Runx2-Smads regulation by FC oil using the MEK1 inhibitor PD98059 was also examined. In particular, pre-treatment with the MEK-1 inhibitor increases the positive effects, on both differentiating proteins for oil sample 1 (Fig. 7A and B) and oil sample 2 (data not shown).

DISCUSSION

Hydrodistillation of fruits of FC gave high oil yields (5.9–7.1%), among the highest obtained for other *Ferulago* species [Khalighi-Sigaroodi et al., 2005], and especially in comparison with that obtained for the same taxon from Turkey (1.3%) [Demirci et al., 2000]. For better understanding, most of the commercialized essential oils extracted from Apiaceae and employed in pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industries, namely *Pimpinella anisum*, *Foeniculum vulgare*, *Anethum graveolens*, *Coriandrum sativum*, *Carum carvi*, *Cuminum cyminum*, are obtained with the following yields: 1–4, 2–6, 2–5, 0.4–1.0, 4–7, and 2–5%, respectively [Leung and Foster, 1996]. Therefore, the high yields obtained make FC oil employable by the above industries, also on the basis of its composition. With respect to the previous investigation, which considered the volatile compounds extracted by microdistillation from fruits [Başer, 2002], our analysis showed a different chemical profile. The major volatiles in our study resulted myrcene, α-pinene, and γ-terpinene that were completely lacking in Turkish samples. On the other hand, trans-chrysanthenyl acetate, the main volatile (17.2%) microdistilled from Turkish fruits, was not detected in essential oils from Italian plants. A-Pinene (31.8%), sabinene (15.8%), α-phellandrene (6.3%), and limonene (6.6%) were the major components of fruit oils obtained by hydrodistillation of Turkish samples [Demirci et al., 2000], while in our study we detected only the former in similar amounts (22.7–23.0%), sabinene and α-phellandrene in little amounts (≤1%) and the latter completely missing. Among major compounds (>10%) detected in the essential oils of *Ferulago* species [Khalighi-Sigaroodi et al., 2005], only β-phellandrene, carvacrol, ferulagone, (2,4,5)-trimethyl benzaldehyde, and spathulenol were missing in *F. campestris*, whereas γ-terpinene detected in our sample has never been revealed as major component of fruits oil in other species of the genus.

To perform our study we used primary calvarial osteoblasts from newborn mice, which at no step of preparation or culture were exposed to phenol red; cells were serum deprived for 24 h before treatment, thus excluding the presence of any contaminating mitogen. Moreover, FC oil samples were tested at concentration ranging from 10^{-3} to 10^{-6} M, and the most active concentration was applied. We, initially, demonstrated that FC oils increased COBs viability at 10^{-4} and 10^{-5} M, in dose-dependent manner with a maximal response at 10^{-4} M. Higher concentrations of FC oil (10^{-3} and $10^{-3.5}$ M) had no effects probably because of their slight toxicity. In particular, for the first time we demonstrated, by BrdU assay, that FC oils exert a positive influence on osteoblast proliferation.

In attempt to determine the molecular basis underlining the increased cell viability and proliferation, first we evaluated the expression of proteins which play an important role in the progression of the G1-cell cycle [Jansen-Durr et al., 1993; Liu et al., 1995; Berns et al., 1997; Ussar and Voss, 2004]. Interestingly, our

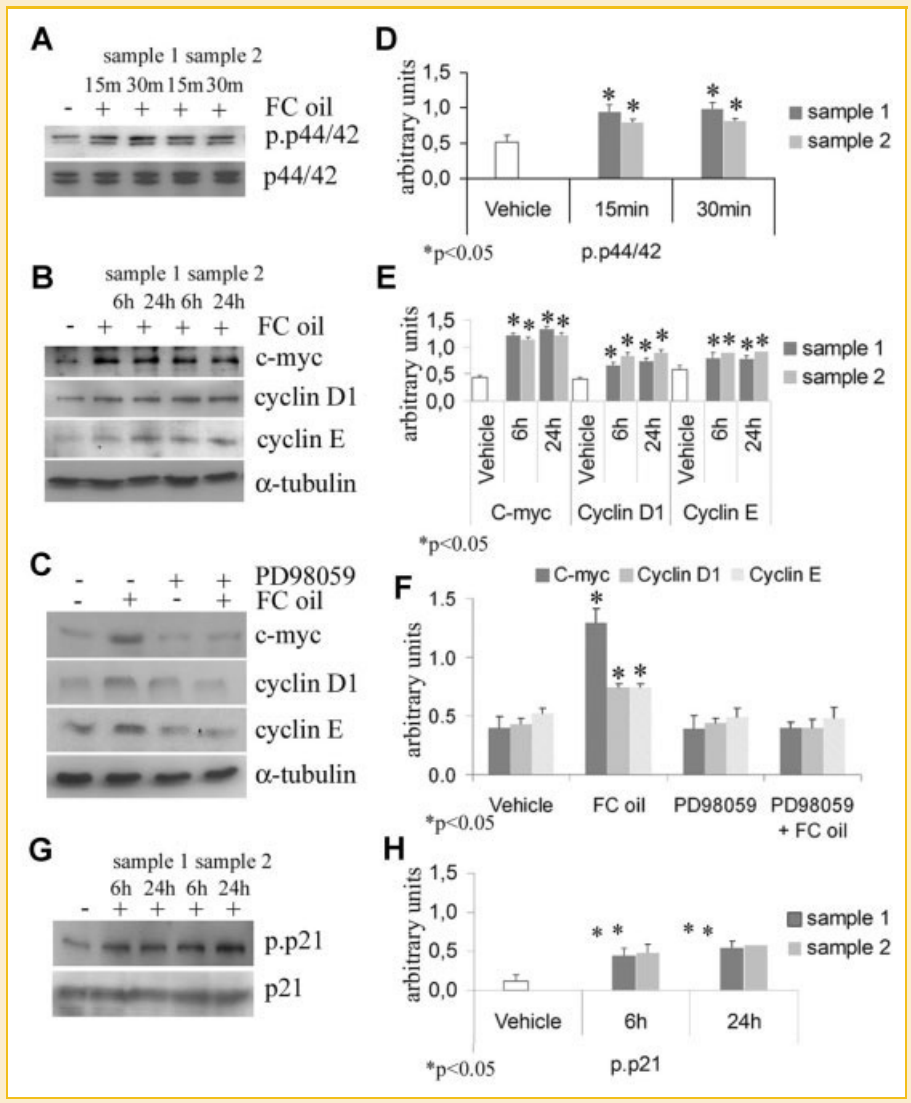

Fig. 3. Phospho-p44/42 levels. Cells were serum deprived for 24 h and treated with FC oil (10^{-4} M) for 15 and 30 min. Proteins were extracted, subjected to SDS–PAGE, transferred to PVDF membrane and probed with mouse anti-phospho-p44/42 antibody; then, filters were stripped and re-probed with rabbit anti-p44/42 antibody to show equal amount of loading (A). c-Myc and cyclins syntheses in absence (B) or in presence (C) *of PD-98059.* Cells were serum deprived for 24 h and treated with vehicle or FC oil (10^{-4} M) for 6 and 24 h. Other cultures were pretreated with PD98059 (4×10^{-5} M) before treatment with FC oil (sample 1) for 24 h. Filters were probed with mouse anti-c-Myc, rabbit anti-cyclin D1, mouse anti-cyclin E antibodies; then, filters were stripped and re-probed with mouse anti-α-tubulin antibody to show equal amount of loading (B, C). Graphics represent results of three independent experiments. Values were referred as means ± standard deviation (SD); $^{*}P < 0.05$ versus correspondent unstimulated control (D, E, F). Phospho-p21 and p21 levels. Cells were serum deprived for 24 h and treated with vehicle or FC oil (10^{-4} M) for 6 and 24 h. Filters were probed with rabbit anti-phospho-p21 or rabbit anti-p21 antibodies (G). Graphic represents results of three independent experiments. Values were referred as means ± standard deviation (SD); $^{*}P < 0.05$ versus correspondent unstimulated control (H).

data, demonstrated an up-regulation of phospho-p44/42 and c-Myc protein levels by FC oil, in addition to an increased synthesis of cyclin D1 and cyclin E.

The cell cycle progression is negatively regulated by two families of cyclin-dependent kinase inhibitors (CKIs) [Sherr and Roberts, 1999], which include p21Cip/WAF1 protein [Chen et al., 1995; Fotedar et al., 1996]. However, when phosphorylated, p21 is retained in the cytoplasm with consequent loss of cell cycle inhibition and protection from apoptosis [Asada et al., 1999; Huang et al., 2003]. AKT/PKB, a known survival protein, has been shown to phosphorylate a consensus threonine 145 residue of p21 inhibiting its ability to bind PCNA [Rossig et al., 2001; Zhou et al., 2001; Li et al., 2002]. Moreover, AKT confers survival signals, at least in part, by direct or indirect up regulation of Bcl-2 [Pugazhenthi et al., 2000; Bratton et al., 2010].

Since we have shown, in this study, an increased level of both phospho-p21 and phospho-AKT as a result of treatment with FC oils, it may be possible that the effectors promote osteoblast survival by AKT activation. Indeed, Bcl-2/Bax ratio was increased by the treatment, while active caspases 9 and 3 were decreased, consistently with the change in Bcl-2 and Bax which are known to balance apoptotic signals through caspase activation.

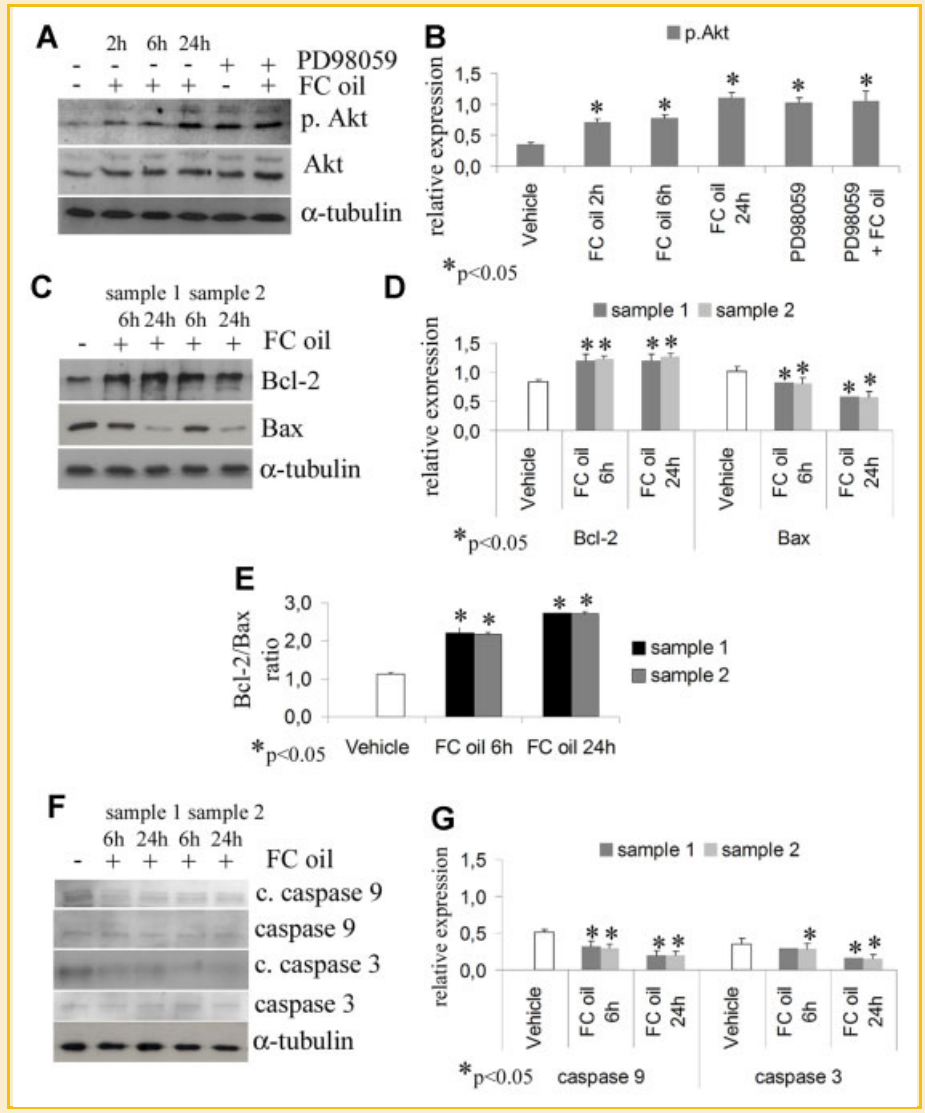

Fig. 4. Phospho-AKT and total-AKT levels in absence or in presence of PD98059. Cells were serum deprived for 24 h and treated with FC oil sample 1 (10^{-4} M) for an additional 2, 6, and 24 h. Other cultures were pretreated with PD98059 (4×10^{-5} M) before treatment with FC oil sample 1 for 24 h. Filters were probed with rabbit anti-phospho-AKT^{ser308} or rabbit anti-AKT antibodies; then, filters were stripped and re-probed mouse anti-α-tubulin antibody to show equal amount of loading (A). Graphic represents results of three independent experiments. Values were referred as means ± standard deviation (SD); $^{*}P < 0.05$ versus correspondent unstimulated control (B). Bcl-2/Bax ratio. Cells were serum deprived for 24 h and treated with FC oils for 6 and 24 h. Filters were incubated with rabbit anti-Bcl-2 or rabbit anti-Bax antibodies; then filters were stripped and re-probed with mouse anti-α-tubulin antibody to show equal amount of loading (C). Graphics represent results of three independent experiments. Values are referred as means ± standard deviation (SD); $^{*}P < 0.05$ versus correspondent unstimulated control (D). Statistical analysis from a pool of three different experiments showed that FC oil increased Bcl-2/Bax ratio at 6 h and, particularly, at 24 h; (mean ± SD, $^{*}P < 0.05$) (E). Cleaved caspase 9 and 3 levels. Cells were serum deprived for 24 h and treated with FC oils (10^{-4} M) for 6 and 24 h. Proteins, transferred to PVDF membrane, were probed with rabbit anti-cleaved caspase 9, rabbit anti-cleaved caspase 3, rabbit anti-caspase 9, or rabbit anti-caspase 3 antibodies; then filters were stripped and re-probed with mouse anti-α-tubulin antibody to show equal amount of loading (F). Statistical analysis from a pool of three different experiments showed that FC oil decreased cleaved caspase 9 and 3 with a maximal response at 24 h (Mean ± SD, $^{*}P < 0.05$) (G).

Moreover, it is reasonable to hypothesize that active AKT is an upstream regulator of apoptosis-associated proteins in FC oils signal, since its increase is already evident after 2 h of treatment, while apoptosis-associated proteins are modulated after 6 and 24 h. In addition, phospho-AKT was up-regulated also in presence of MEK1 inhibitor PD98059, thus excluding an ERK role in AKT activation. Also, AKT activation has been correlated with an increased synthesis of Runx2 [Raucci et al., 2008]. Hence, AKT activation plays two major roles required for osteoblastogenesis: protection from apoptosis and increased differentiation.

As previously stated, Runx2 and Smads play a crucial role for the differentiation of osteoblast from mesenchymal precursors and bone formation. Hence, the next question was whether Runx2 and phospho-Smads 1/5/8 were modulated by FC oils. Interestingly, western blotting results demonstrated an up-regulation of both proteins induced by the effectors, and immunocytochemical studies

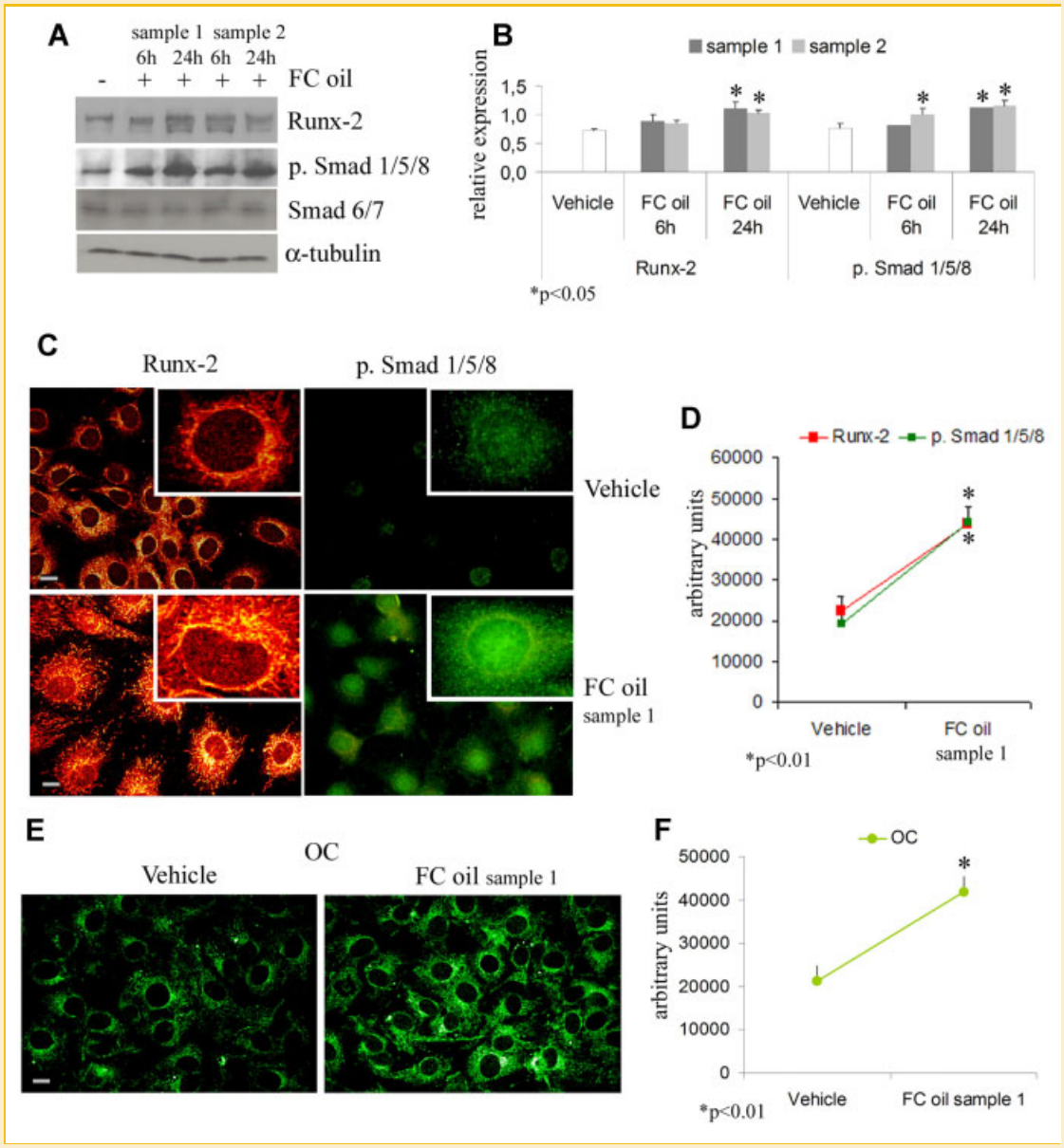

Fig. 5. *Runx2* and *Smads* levels. Cells were serum deprived for 24 h and treated with FC oils (10^{-4} M) for 6 and 24 h. Filters were probed with the rabbit anti-Pebp2aA (Runx2), with the rabbit anti-phospho-Smad 1/5/8 or with rabbit anti-Smad 6/7; then, membranes were stripped and re-probed with mouse anti-α-tubulin to show equal amount of loading (A). Graphics represent results of three independent experiments. Values are referred as means ± standard deviation (SD); $^{*}P < 0.05$ versus correspondent unstimulated control (B). *Runx2* and *phospho-Smad* 1/5/8 subcellular localization. Localization of *Runx2* and *phospho-Smad* 1/5/8 was analyzed by fluorescent microscope, using goat-anti-Runx2 antibody (red: Alexa Fluor 594 staining) and rabbit anti-phospho-Smad 1/5/8 antibody (green: Alexa Fluor 488 staining). Bar, 50 μm (C). Fluorescence analysis from a pool of four different experiments was quantified by a Tecan Infinite fluorescence reader and values were analyzed by Magellan v4.0 software. Values are reported as means ± standard deviation (SD) and statistically analyzed with *t*-test; $^{*}P < 0.01$ (D). Labeling for OC was analyzed by fluorescent microscope, using the rabbit-anti-OC antibody (green: Alexa Fluor 488 staining). Bar, 50 μm (E). Fluorescence analysis was quantified as described in Figure 5 D (F). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

showed an increment of Runx2 and phospho-Smads 1/5/8 particularly at nuclear level, which correlate with their role as transcription factors. In particular, Runx2 binds and increases the activity of the osteocalcin promoter [Ducy et al., 1997; Karsenty and Wagner, 2002; Salingcarnboriboon et al., 2010]. In line, we found an increase of osteocalcin labeling in osteoblasts treated with FC oils. These data, in parallel with the increased ALP and Alizarin red staining which indicate mineralization processes, suggest a positive role of FC oil on osteoblast maturation. Moreover, by using the MEK-1 inhibitor, we found that MAP kinase signaling interferes with FC oil- Smads/Runx2 up-regulation, and Smads/Runx2 increase was improved with inhibition of ERK activity. There are several studies showing that ERK is essential for the early stages of osteoblast differentiation and is involved in the stimulation of osteoblast related gene expression by extracellular matrix–integrin receptor interaction, BMP-2 and growth factors [Lai et al., 2001;

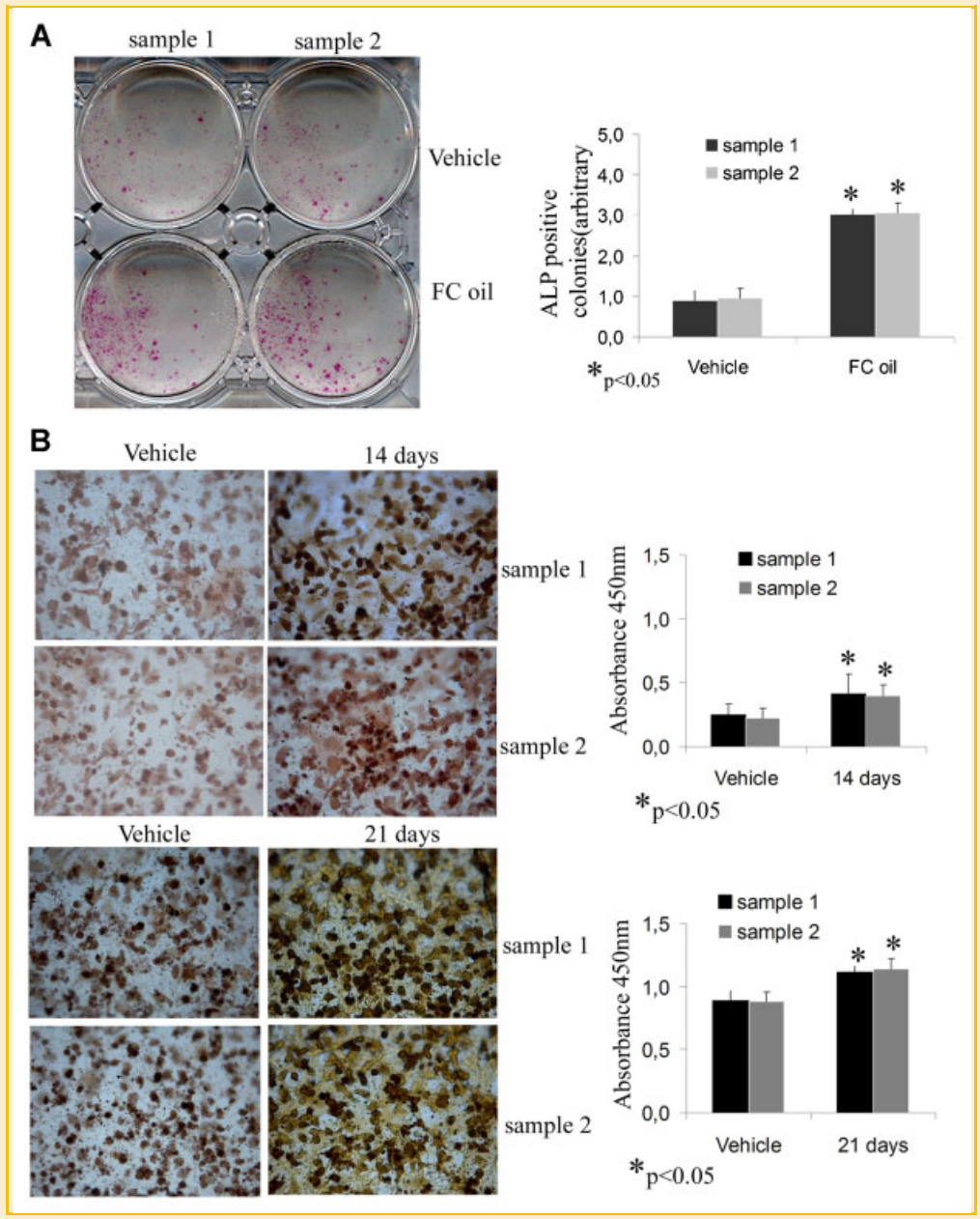

Fig. 6. ALP staining. COBs were cultured for 6 days in differentiation media in the presence or absence of effectors. Colony area was measured by NIH Image; $^{*}P < 0.05$ versus correspondent unstimulated control (A). COBs were cultured for 14 and 21 days in differentiation media in the presence or absence of effectors. Results of quantitative analysis of Alizarin red staining ($^{*}P < 0.05$ versus correspondent unstimulated control) were in accordance with Alizarin red-S staining observed by light microscope (B). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Ziros et al., 2002; Naganawa et al., 2008]. Conversely some other reports suggest that ERK plays a negative role in BMP-2 and growth factor-induced osteoblast differentiation [Hu et al., 2003; Nakayama et al., 2003; Chow et al., 2007]. In our study, may be also possible that other signaling molecules, modulated by MAP kinase, negatively affect the up-regulation of Smads/Runx2 induced by FC oils. Indeed, it was demonstrated that signals that promotes osteoblasts differentiation activate AKT, while signals that promote osteoblasts proliferation and inhibit their differentiation activate ERK, indicating a cross-talk between the AKT and ERK pathway [Raucci et al., 2008]. In this regard, it is also to be noted that the effect of ERK inhibition on the levels of Runx2 and phospho-Smads was not as striking as the effect of ERK inhibition on the levels of c-Myc and cyclins, suggesting that the primary role of ERK activation might be to increase osteoblasts proliferation.

Assumptions about the mechanism of action of FC oil on COBs could be made on the basis of its monoterpene hydrocarbons richness and expected lipophilicity. As reported in literature, monoterpene hydrocarbons easily cross cell membranes and affect bone cell metabolism, by inhibiting resorption in vivo and in vitro

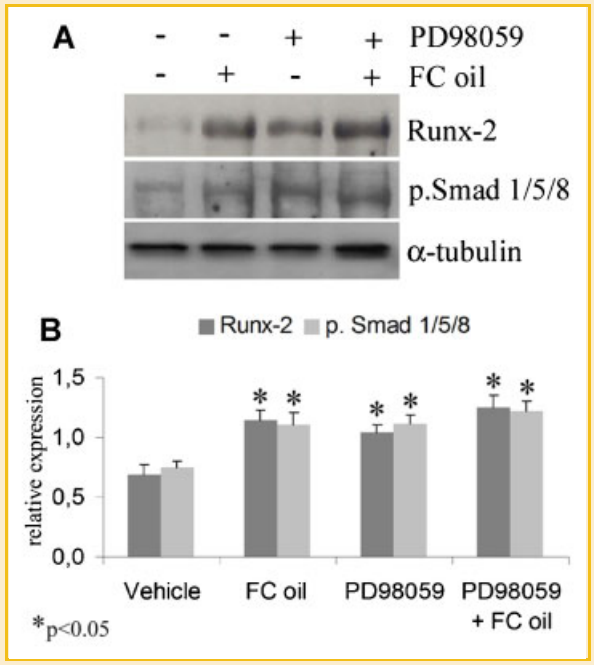

Fig. 7. *Runx2* and *phospho-Smad* 1/5/8 levels in presence of PD98059. Cells were serum deprived for 24 h and treated with FC oil (10^{-4} M) for 24 h. Other cultures were pretreated with PD98059 (4×10^{-5} M) before treatment with FC oil sample 1 for 24 h. Filters were probed with rabbit-anti-Runx2 or rabbit anti-phospho-Smad 1/5/8 antibodies; then, membranes were stripped and re-probed with mouse anti-α-tubulin antibody to show equal amount of loading (A). Graphics represent results of three independent experiments. Values are referred as means ± standard deviation (SD); $^{*}P < 0.05$ versus correspondent unstimulated control (B).

and preventing bone loss in osteoporosis models [Mühlbauer et al., 2003]. Hence, this study suggest the existence of alternative intracellular pathways by which FC oils regulate osteoblast metabolism, probably dependent on the maturation stages of osteoblasts; although future studies are necessary to explore the cross-talk between the various signaling pathways, FC oil may represent an interesting candidate for a dietary approach to osteoporosis.

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