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Fibroblast contributes for osteoblastic phenotype in a MAPK-ERK and sonic hedgehog signaling-independent manner

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Abstract We hypothesized that a crosstalk between osteoblast and fibroblast (FB) exists, which contributes to bone as a dynamic tissue. Cell-free supernatants were harvested from fibroblast cultures and later subject pre-osteoblasts to investigate there capacity to modulate cell viability and differentiation mechanisms, reporting the possible involvement of Shh signaling as a paracrine mechanism. By exploring immunoblotting technology, we have shown that FB-released factors interfere with osteoblast metabolism by up-regulating the phosphorylation of FAK and Rac-1 proteins at the early stage and later contribute to osteoblast differentiation by up-modulating alkaline phosphatase (ALP) and in vitro mineralization. We also found that Shh signaling was not required during osteoblastic differentiation promoted by the FB-released factors as well as MAPK-ERK phosphorylation, while pre-osteoblast cultures subjected to osteogenic medium (O.M.) require downstream transducers of Shh, such as Patched and Gli-1, and MAPK-ERK. Altogether, our results indicate for the first time a possible mechanism involved in the crosstalk between fibroblasts and osteoblasts, as it was possible to observe trophic factors released by fibroblasts interfering decisively in osteoblast metabolism in a Shh-independent manner. This study collaborates the body of work that indicates paracrine signaling molecules participate in the crosstalk among bone-resident cells and explains, at least partially, the biological mechanisms responsible for bone tissue dynamism, opening new avenues to understand etiologies of bone diseases.

Keywords Bone · Fibroblast · Osteoblast · Crosstalk · Cell signaling

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Introduction

The perfect crosstalk among cells has been proved to be extremely decisive during bone development [1, 2] and responsive to physiologic requirements [3, 4]. In bone tissue, the crosstalk between the two main bone cells (osteoblasts and osteoclasts) has been well characterized [5]. It is known that bone marrow presents a wide variety of host interacting with each other, but very little information has been reported about the crosstalk between fibroblast (FB) and osteoblast [6]. As observed elsewhere, osteoblasts are cells originated from mesenchymal tissue and are nearly indistinguishable from fibroblasts [7–9].

In addition, morphogenetic proteins play important roles during tissue development, remodeling, and repair processes. Among others, bone morphogenetic protein (BMP) and Hedgehog (Hh) members are extremely decisive for bone homeostasis [10–13], because they modulate and compromise cell differentiation at a bone marrow niche. Indian Hedgehog (Ihh) was found to be an important factor during mineralized tissue development. It has been proposed, using chick and mouse models, to be largely expressed in pre-hypertrophic chondrocytes [14].

In this present study, we examined if fibroblast is able to modulate osteoblast metabolism. Our main hypothesis tested was that fibroblast-released molecules modulate osteoblasts performance by requiring Sonic Hedgehog (Shh) signaling transducers, because hedgehog members

are physiological factors that support tissue development and integrity. In order to address this issue, fibroblast cultures were conducted, and the fibroblast-conditioned medium was used to subject pre-osteoblast. First, our results showed that fibroblast-released molecules interfere in early pre-osteoblast metabolism and viability by up-regulating both FAK and Rac-1 phosphorylations (up to 24 h) and later contribute to osteoblast differentiation by up-modulating alkaline phosphatase (ALP) and in vitro mineralization. In addition, all stages assayed in this work showed to have a Shh-independent manner. In addition, we believe these results support better understanding of the relevance of the paracrine signaling between bone-resident cells, which open new avenues to understand bone disease etiologies, such as osteoporosis.

Materials and methods

Antibodies

The following antibodies were purchased from Cell Signaling (Danvers, MA, USA): Shh Antibody (#2287, 19, 42, 45 kd), GLI Antibody (#2553, 160 kd), Ptch 1 (#2468, 180-210 kd), Integrin β1 (#34971, 115-135 kd), Rac1/cdc42 Antibody (#4651, 21 kd), Phospho-Rac1/cdc42 (Ser71) Antibody (#2461, 28 kd), and GAPDH Rabbit (#5174, 37 kd). From Abcam (Cambridge, MA, USA): Anti-Fak antibody (ab61113), anti-Fak (phpspho Y576 + Y577) antibody [EP1832Y] (ab76244), anti-Cofilin antibody (ab42824), anti-Cofilin (phospho S3) antibody (ab12866), Anti-ERK1 + ERK2 Antibody [ERK-7D8] (ab54230), and Anti-ERK1/2 (phospho-Thr202/Tyr204) antibody (ab214362).

Cell cultures

We used NIH-3T3-E1 (FB, fibroblasts) and (MC3T3-E1, subclone 4) pre-osteoblasts. During all experiments, the cells were maintained and cultured in Alpha-MEM medium containing antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin), Ribonucleosides, and Deoxyribonucleosides, supplemented with 10% Fetal Bovine Serum (Nutricell, Campinas, SP, Brazil). Cells were maintained at 37 °C and 95% humidity, in 5% CO_2.

Cell viability

For the cell viability assay, the medium was conditioned for 3 days by the fibroblast cells and then used to subject the pre-osteoblasts, which were previously plated (96-well plates) at the density of 5×10^4 cells/ml. After 24 h exposed to the FB-conditioned medium, cell viability was assessed by the MTT assay, when MTT solution (1 mg/ml) was added and maintained in an incubator for an additional 3 h. The MTT solution was removed, and 100 µl of DMSO was added for solubilizing the dye formed by viable cells. Finally, the absorbance was measured at 570 nm using a microplate reader (SYNERGY-HTX multi-mode reader, Biotek, USA).

Immunoblot

After 3, 6, and 24 h of seeding (for adhesion test) or 10 days (for differentiation test), the cells were lysed [50 mM Tris–HCl, pH 7.4, 1% Tween 20, 0.25% Sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM O-Vanadate, 1 mM NaF, and protease inhibitors (1 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM aminoethyl fluorosilicon 4-fluoride hydrochloride)] and the samples sonicated (1 pulse s^{-1}—SONICS Vibra-Cell). The protein extracts were pooled by centrifugation, and the protein concentration determined by the Lowry method. To the extracts was added sample buffer [1:1 ratio, sample buffer: 2X sodium dodecyl sulfate (SDS), 100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue, and 20% glycerol].

Alizarin red and alkaline phosphatase staining

Pre-osteoblasts were plated (5×10^4 cells/ml) in 24-well plates and were treated at semi-confluence with the FB-conditioned medium up to 10 days. The medium was changed every 3 days. ALP staining was used exactly as recommend by the manufacturer (SIGMAFAST BCIP/NBT tablet). Previously, cells were fixed with 10% formalin for 1 min, and then washed with Wash Buffer (0.05% Tween in 20 ml in PBS free of Ca^{2+} and Mg^{2+}). Afterwards, they were kept for 10 min in the dark in ALP solution, and the cells were washed with PBS and photographed in an inverted microscope. For Alizarin red S staining, the cells were fixed with 10% formalin for 30 min at room temperature, then 2% Alizarin-Red S dye was added to the cultures, and the plate was maintained into a dark chamber for 45 min. Finally, the wells were washed with PBS, and the plate was photographed using an inverted microscope (Zeiss, Germany).

Statistical analysis

Results were represented as mean ± standard deviation (SD). They were verified using student's t test (2-tailed) with $p < 0.05$ considered statistically significant and $p < 0.001$ considered highly significant. In an experiment in which there were >2 groups, we used one-way ANOVA (non-parametric) with post-test of Bonferroni, in order to

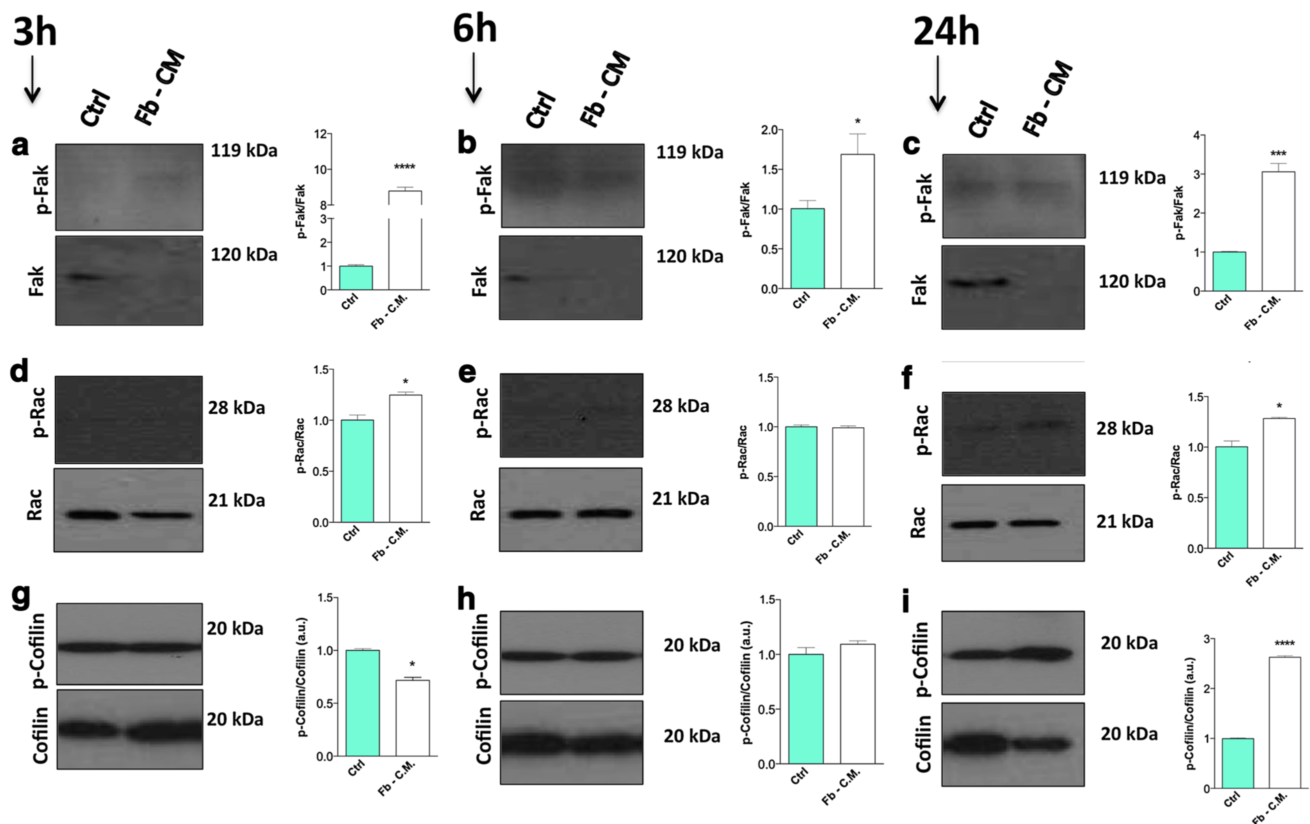

Fig. 1 Fibroblast interferes with osteoblast viability by modulating crucial signaling proteins. The cells were cultured under routine classic conditions. In the semi-confluence, the cells were treated with fibroblast-conditioned medium, and after 24 h, the cells were trypsinized, counted, and re-seeded. After 3 (**a**, **d**, **g**), 6 (**b**, **e**, **h**), and 24 h (**c**, **f**, **i**) of seeding, the cells were lysed using standard lysing buffer (described in "Materials and methods"). The pooled protein was resolved on SDS-PAGE gel, and after PVDF membrane transfer. These were identified specifically by using specific primary antibody in Western blotting protocol. Representative blotting is shown, and the graphs represent arbitrary values obtained by densitometric analysis of bands normalized by the average values of the respective GADPH bands (housekeeping control). The differences were considered significant when $*p < 0.01$; $***p < 0.0002$; and $****p < 0.0001$

compare all pairs of groups. In this case, the significance level was considered when alpha = 0.05 (95% confidence interval). The software used was GraphPad Prism 6.

Results

Fibroblasts affect osteoblasts viability by modulating crucial signaling proteins

As detailed in the "Materials and methods," fibroblasts were maintained up to 72 h, and then the conditioned medium was collected to be used later. In the meantime, cultures of pre-osteoblasts (MC3T3-E1) were grown and, at the semi-confluent stage, were treated with the FB-conditioned medium, as previously mentioned. After 24 h, the pre-osteoblasts were trypsinized, counted, and re-plated. After 3, 6, and 24 h of seeding, the pre-osteoblasts were scraped in lysis buffer. These samples were resolved into SDS-PAGE approach for evaluating specific intracellular proteins using a specific antibody. We investigated crucial proteins involved in cell viability such as Rac-1, FAK, and Cofilin. Our results showed that FAK was progressively phosphorylated from 3 to 24 h (Fig. 1a–c), which suggests an effect of FB on pre-osteoblast adhesion mechanism and viability. In addition, Rac-1 phosphorylation was also reported, as presenting a significant profile at 24 h (Fig. 1d–f). Interesting, we also found that cofilin presented changeable phosphorylation profile: decreasing at 3 h (Fig. 1g) and significantly increasing at 24 h (Fig. 1i). Osteoblast metabolism was modified by FB-released factors requiring a transient cofilin phosphorylation at up to 24 h of treatment.

In addition, we observed few variations on the integrin-ß1 expression profile (Fig. 2), while the Patched-1 (Ptch) was significantly down-regulated in response to FB-released factors (Fig. 3).

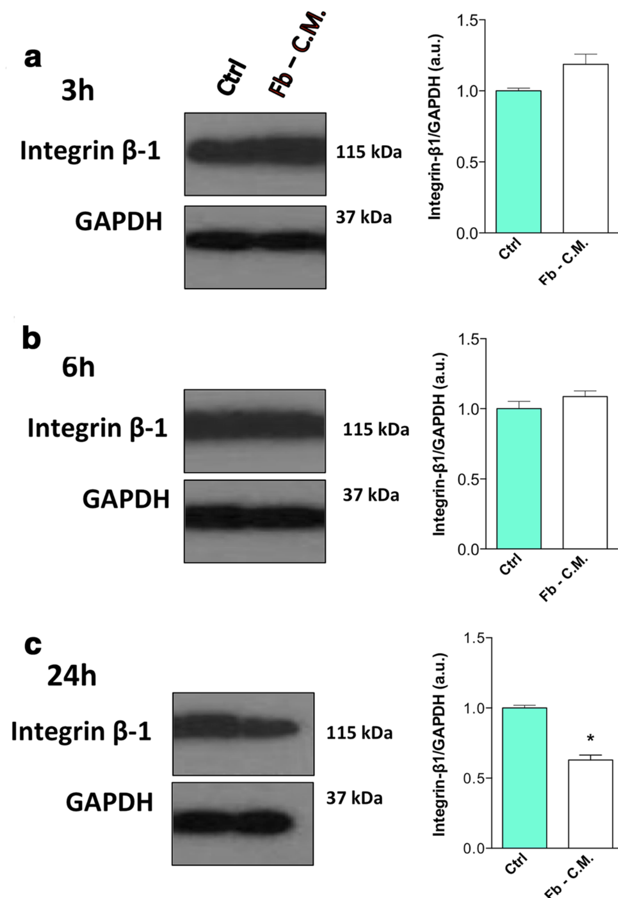

Fig. 2 Integrin-ß1 profile in response to fibroblast-conditioned medium. Briefly, the cells were cultured respecting the details listed previously. Representative blotting is shown and the graphs represent arbitrary values obtained by densitometric analysis of bands normalized by the average values of the respective GADPH bands (housekeeping control). The differences were considered significant when $*p < 0.01$

Fibroblasts contribute to osteogenic phenotype in a MAPK-ERK and Shh-independent manner

Later, we evaluated the effect of the FB-conditioned medium on osteoblast differentiation up to 10 days by assaying 2 classical methodologies: Alkaline phosphatase (Fig. 4a–c); Alizarin red (Fig. 4d–f) staining. Our results showed that FB-conditioned medium was able to stimulate osteogenic differentiation because those two parameters were up-regulated (Fig. 4g, h) in response to FB-conditioned factors and when compared to 2 other control (Ctrl) groups. Two control groups were included in this experiment as follows: (1) Crtl: pre-osteoblasts were maintained under a classical condition; (2) Positive Ctrl: pre-osteoblast were subjected to osteogenic medium (O.M.), as described in "Materials and methods".

In addition, we investigated the involvement of sonic hedgehog signaling by evaluating Shh, Patched (Ptch), and Gli-1 expressions by immunoblotting. Curiously, our results revealed that the O.M. promoted osteogenic phenotype acquisition by governing Shh signaling because Shh, Gli-1, and Ptch (Fig. 4i–n) were up-expressed. In contrast, FB-released factors also promoted osteogenic phenotype, but it was not dependent on Shh signaling, because Shh, Ptch, and Gli-1 were down-expressed (Fig. 4i–n). Moreover, MAPK-ERK involvement was also investigated, because MAPK-ERK has been reported to guide osteoblast differentiation. In this regard, our results showed that MAPK-ERK presented a different phosphorylation profile in response to O.M. and FB-released conditioned medium: while O.M. promoted an increase on MAPK-ERK phosphorylation, FB-released factors promoted its significant decrease (Fig. 4o, p).

Discussion

We are interested to understand how the trophic factors released from the host cells in the bone niche could be involved during osteoblastic phenotype. Here, we explored the possibility of fibroblast to modulate pre-osteoblast metabolism by investigating in vitro approaches. In order to explore this issue, first we collected conditioned medium from fibroblasts cultured up to 3 days and used to subject pre-osteoblast cells; thereafter, the subjected pre-osteoblasts were investigated regarding two important stages: adhesion (3, 6, and 24 h after seeding) and differentiation (up to 10 days in culture).

First, we found that FB modulated osteoblast viability by up-modulating crucial signaling proteins such as FAK and Rac-1 activations, during early stages of pre-osteoblast adhesion. These results are very interesting if we consider that both signaling proteins are involved with cytoskeleton rearrangement during pre-osteoblast adhesion [15, 16]. The mechanism involving the synchrony of fibroblast/osteoblast crosstalk could enhance bone repair and might be involved during infectious-induced bone remodeling. In this regard, we reported elsewhere the attenuation of both FAK and Src phosphorylations in osteoblast during the response to TNF-α [17]. On the other hand, we can suppose that fibroblast-released factors could contribute to the osteoblast survival signaling, as it requires activation of FAK and Rac-1, while TNF-α has been suggested to attenuate both FAK and Src phosphorylations, culminating in impair of osteoblast performance. In addition, it is reasonable to suggest that the balance of the crosstalk between osteoblast and fibroblast, and considering the intensity of the TNF-α signaling, could guide the maintenance of the periodontal ligament healthiness and alveolar bone tissue repair, in bone commitment during periodontal diseases. FAK and Src activations are important parameters to

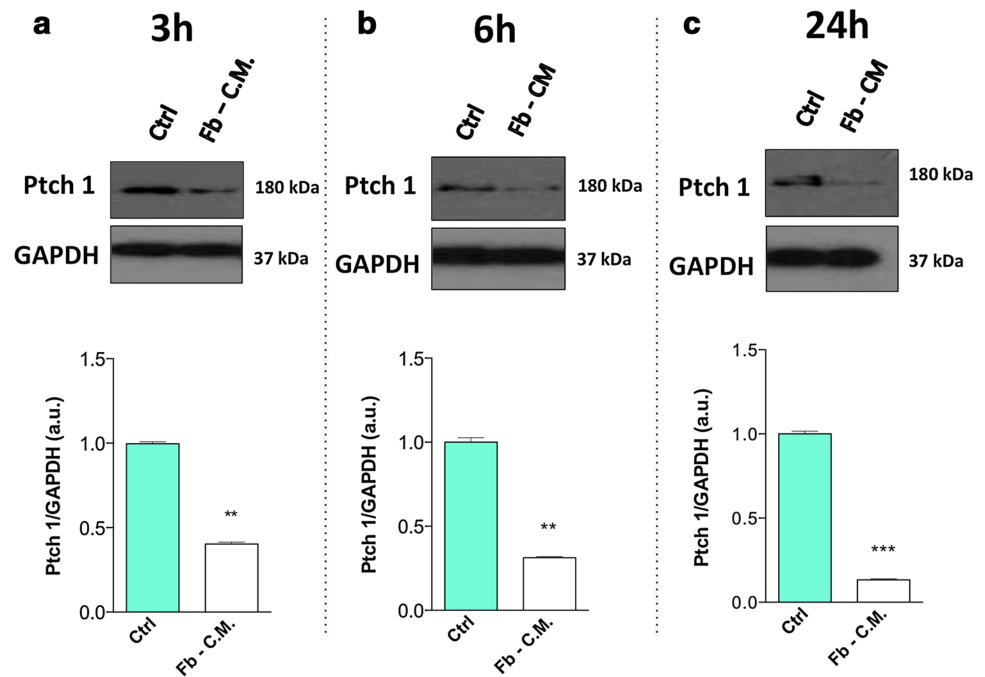

Fig. 3 Patched is significantly down-regulated in crosstalking with fibroblasts. Briefly, the cells were cultured respecting the details listed previously. Representative blotting are shown, and the graphs represent arbitrary values obtained by densitometric analysis of bands normalized by the average values of the respective GADPH bands (housekeeping control). The differences were considered significant when $^{***}p < 0.0002$ and $^{**}p < 0.0017$

understand osteoblast phenotype in response to external stimuli [16, 18–20].

As reported, FAK and Src are very interesting biomarkers being considered in the paradoxical response of osteoblasts to TNF-α and fibroblast-released factors. As Protein Tyrosine Kinases, both FAK and Src are modulated by eventual Reactive Oxygen Species (ROS) because Protein Tyrosin Phosphatases are very sensitive to them. We have showed previously that Low Molecular Weight-Protein Tyrosine Phosphatases (LMW-PTP) are able to modulate transient FAK and Src phosphorylations during pre-osteoblast adhesion [21]. In this regard, the modulation of upstream signaling transducers, such as FAK and Src, will certainly affect downstream molecules such as Rac-1, an important signaling transducer that directly guides cytoskeleton rearrangement-based pre-osteoblast adhesion by modulating cofilin phosphorylation.

Hence, we decided to also investigate the phosphorylation of cofilin (at serine 03), and thus, we found that during up to the first 24 h of seeding, cofilin phosphorylation was finely modulated, in agreement with the upstream signaling protein activation, such as Rac-1 and FAK. Thus, we believe FB starts modulating osteoblast phenotype by interfering with their viability and adhesion performance.

Thereafter, we investigated the influence of FB-released factors in contributing to osteoblastic phenotype. We showed that both alkaline phosphatase (ALP) and Alizarin red staining were up-modulated in response to FB-produced conditioned medium. In addition, we explored the dependency on canonical Sonic Hedgehog (Shh) signaling in this osteogenic phenotype acquisition by assessing Shh protein, Gli-1, and Patched. Our results showed that FB-released factors promoted osteoblast differentiation in a Shh signaling-independent manner, as we found a decrease of Gli-1 and Patched in osteoblast metabolism in crosstalk with fibroblast. In contrast, osteogenic metabolism promoted by the classical osteogenic medium (used here as an internal control) required both Patch and Gli-1. Thus, we believe that several mechanisms are capable of promoting osteoblast differentiation and later mineralizing bone matrix.

Another protein evaluated in osteogenic phenotype acquisition was MAPK-ERK. Our results showed MAPK-ERK was up-activated in response to osteogenic medium, while differentiated osteoblast in response to FB-released factors did not present the same profile. MAPK-ERK is a signaling protein involved with a wide number of signaling pathways such as survival, proliferation, and differentiation [22, 23]. During the specific osteoblast differentiation, its involvement presents controversial results [24–29]. We consider these findings relevant because we suggest that the osteogenesis may come from a synergism of external trophic factors that require specific intracellular pathways.

Altogether, our results showed for the first time a possible mechanism involved in the crosstalk between fibroblasts and osteoblasts, resulting in the osteogenic phenotype at a Shh-independent manner. This collaborates to our understanding about paracrine signaling molecules that participate in the crosstalk among bone-resident cells, and thus explains, at least partially, biological mechanisms responsible for bone tissue dynamism, opening new avenues for comprehending bone diseases etiologies.

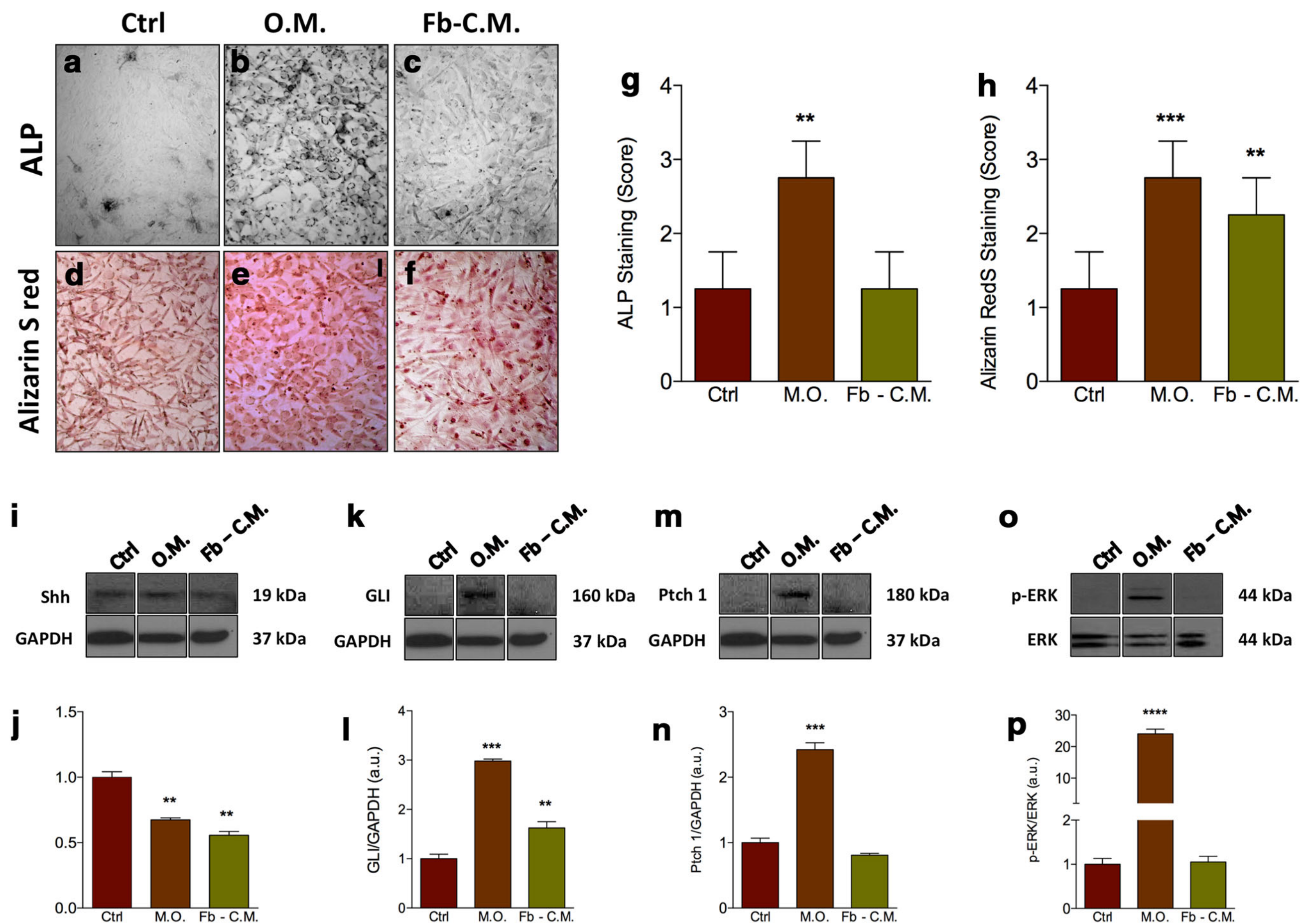

Fig. 4 Fibroblast paracrine effects promote osteoblast differentiation in a MAPK-ERK and Shh-independent manner. The pre-osteoblasts were cultured and in the semi-confluence were treated with conditioned medium from fibroblasts up to 10 days. Then the cells were subjected to osteoblast differentiation approaches: Alkaline phosphatase (**a**–**c**) and Alizarin red (**d**–**f**), which were scored, and the arbitrary values were attributed (**g** and **h**, ALP and alizarin red, respectively). For immunoblotting, the cells were cultured respecting the details listed previously. Representative blotting is shown (**i**, **k**, **m**, **o**), and the graphs (**j**, **l**, **n**, **o**) represent arbitrary values obtained by densitometric analysis of bands normalized by the average values of the respective GADPH bands (housekeeping control). The differences were considered significant when $^{***}p < 0.0002$ and $^{**}p < 0.0017$

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Compliance with ethical standards

Conflict of interest All the authors declare to have no conflict of interest with the materials used in the present study.

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