Fibroblast contributes to osteoblast differentiation in a sonic hedgehog signaling-independent manner

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Dear Editor

We are pleased to submit the attached manuscript entitled: **Fibroblast contributes to osteoblast differentiation in a sonic hedgehog signaling-independent manner**, by Fernandes et al.

Regarding bone tissue, it is very known the crosstalk between the two principle bone cells, bone forming osteoblasts and bone resorbing osteoclasts. However, the bone marrow stromal cells have a wide variety of cells playing each other but very few information supports the crosstalk among them, such as fibroblasts and osteoblasts. In this study, we examined our hypothesis that fibroblast could serve as resource of factors to activate osteoblasts and the possibility of Sonic hedgehog (Shh) plays a central role on this biological process, since hedgehog members are a physiological relevant factors to support tissue development and integrity. In order to address this topic, we showed that fibroblast released factors interfere on osteoblast metabolism and viability by up-regulating the both phosphorylation of FAK and Rac-1 proteins at the early stage (up to 24 hours) and later contributing with osteoblast differentiation by up-modulating alkaline phosphatase (ALP) and in vitro mineralization. In addition, all stages studied in this study happened in a Shh-independent manner. In this sense, we believe these results help us to understand the importance of the paracrine metabolism through bone resident cells for supporting bone dynamic biology, opening new avenues for comprehending bone diseases etiologies, as the case of osteoporosis.

Also, we wish to confirm that:

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

The manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the corresponding authors are the contact for the Editorial process (including Editorial Manager and direct communications with the office). They are responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

We look forward to hearing from you concerning this manuscript at your earliest opportunity.

Sincerely yours,

Willian F. Zambuzzi, on behalf of all authors

Fibroblast contributes to osteoblast differentiation in a sonic hedgehog signaling-independent manner

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ABSTRACT

We hypothesized that communication between osteoblast and fibroblasts (FB) contributes to bone dynamic tissue. We harvested cell-free supernatants from fibroblast to treat osteoblast for testing viability and differentiation mechanisms, mainly monitoring the involvement of Shh signaling in this paracrine mechanism. By exploring immunoblotting approach we have shown that FB-released factors interfere on osteoblast metabolism by up-regulating the phosphorylation of FAK and Rac-1 proteins at the early stage and later contributing with osteoblast differentiation by up-modulating alkaline phosphatase (ALP) and *in vitro* mineralization. During osteoblast differentiation process, we also showed that Shh signaling proteins weren't up-modulated in disagreement with the osteogenic medium (O.M.) response, where Shh, Patched and Gli-1 were up-regulated. Altogether, our results show for the first time a possible mechanism involved on the crosstalk between fibroblast and osteoblast, since it is possible to observe that factors released from fibroblasts interfere on osteoblast metabolism at a Shh-independent manner. In this sense, we believe these results help us to understand the importance of the paracrine metabolism through bone resident cells for supporting bone dynamic biology, opening new avenues for comprehending bone diseases etiologies, as the case of osteoporosis.

Key words: Bone; Fibroblast; Osteoblast; Crosstalk; Cell signaling.

INTRODUCTION

The perfect crosstalk among cells has been proved to be extremely relevant during bone development (Kusumbe et al., 2014; Ramasamy et al., 2014) and response to physiologic requirements (Brotto et al. 2015; Funck-Brentano et al. 2011). Regarding bone tissue, it is very known the crosstalk between the two principle bone cells, bone forming osteoblasts and bone resorbing osteoclasts (Gemini-Piperni et al., 2014). However, the bone marrow stromal cells have a wide variety of cells playing each other but very few information supports the crosstalk among them, such as fibroblasts and osteoblasts. As it is very known, osteoblasts are cells originated from mesenchymal tissue and they are nearly indistinguishable from fibroblasts (Lee et al., 1997; Ducy et al., 1996; Ducy et al. 1997). However, there is no evidence to date that bone matrix mineralization is orchestrated by genes selectively expressed in osteoblasts (Ducy et al 2000). Current understanding of the series of event, regulated by trophic factors, that contributes bone remodeling has been reviewed (Blumenthal et al., 2003; Zambuzzi et al. 2009; Milani et al., 2010; Aspenberg, 2013; Fernandes et al., 2014).

In this sense, morphogenetic proteins guarantee important roles during tissue development, remodeling and repairing process. Among others, bone morphogenetic protein (BMP) and Hedgehog (Hh) protein are extremely decisive for bone homeostasis (Carreira et al, 2014; Gurpreet et al, 2014; Krishnan et al, 2016; Jinkyu et al, 2017). Member of the Hh family, indian hedgehog (Ihh) was linked to be an important factor during mineralized tissue development. It has been proposed in chick and in mouse, the Ihh gene is expressed in pre-hypertrophic chondrocytes (Philip et al, 2001; Nikolas et al, 2015).

In this study, we examined our hypothesis that fibroblast could serve as resource of factors to activate osteoblasts and the possibility of Sonic hedgehog (Shh) plays a central role on this biological process, since hedgehog members are a physiological relevant factors to support tissue development and integrity. In order to address this topic, we showed that fibroblast released factors interfere on osteoblast metabolism and viability by up-regulating the both phosphorylation of FAK and Rac-1 proteins at the early stage (up to 24 hours) and later contributing with osteoblast differentiation by up-modulating alkaline phosphatase (ALP) and *in vitro* mineralization. In addition,

all stages studied in this study happened in a Shh-independent manner. In this sense, we believe these results help us to understand the importance of the paracrine metabolism through bone resident cells for supporting bone dynamic biology, opening new avenues for comprehending bone diseases etiologies, as the case of osteoporosis.

MATERIAL 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), 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), Anti-ERK ½ (phospho-Thr202/Tyr204) antibody (ab214362).

Cell culture: we used NIH-3T3-E1 fibroblasts and MC3T3-E1 osteoblasts (Pre-osteoblasts, subclone 4). During all experiments, the cells were maintained and cultured in Alpha-MEM medium containing antibiotics (100U/ml penicillin, 100mg/ml streptomycin) and Ribonucleosides and Deoxyribonucleosides. In the medium was added 10% Bovine Fetal Serum (Nutricell, Campinas, SP, Brazil). Cells were maintained at 37°C and at 5% CO2 and 95% humidity.

Cell viability: for the cell viability test the medium was conditioned for 3 days by the fibroblasts and then used to treat the Osteogenic cells, which were plated at the density of $5x10^4$ cells/ml. After 24 hours expositing to the conditioned medium, viability was assessed by the MTT test. The culture medium was removed, MTT solution (1mg/ml) was added and maintained in an incubator for an additional 3 hours. Thereafter, the medium was removed and 1 ml of DMSO was added for solubilizing of the dye formed by viable cells. After, the absorbance was measured at 570 nm using a microplate reader (SYNERGY-HTX multi-mode reader, Biotek, USA).

Immunoblot: after 3, 6 and 24 hours (adhesion) and 10 days (Differentiation - medium exchange every 3 days) 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]), The samples were sonicated (1 pulse per second) - SONICS Vibra-Cell. The protein extracts were centrifuged and the protein concentration determined by the Lowry method. To the extracts was added 1:1 sample buffer (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 e alkaline phosphatase staining. the pre-osteoblasts were plated ($5x10^4$ cells/ml) in 24-well plates and in the semi-confluence were treated with the conditioned medium of fibroblasts for 10 days. The medium was changed every 3 days to maintain the biological properties of the medium conditioning. The Sigma-Aldrich kit (SIGMAFAST BCIP/NBT tablet) was used for ALP staining. Previous, cells were fixed with 10% formalin for 1 minute, then washed with Wash Buffer (0.05% Tween in 20ml in PBS free of Ca2+ and Mg2+). Afterwards they were kept for 10 minutes in the dark in ALP solution, finally the cells were washed with PBS and photographed in Inverted Microscope. For Alizarin Red S staining, the cells were fixed with 10% formalin for 30 minutes at room temperature, after which time Alizarin-Red S 2% was added and the plate was incubated in the dark for 45 minutes. After, the wells were washed with PBS and the plate was photographed under 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 $p < .05$ considered statistically significant and $p < .001$ considered highly significant. In experiment where there were > 2 groups, we used one way ANOVA (non parametric) with post-test of Bonferroni, in order to 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

Fibroblast affects osteoblasts viability by modulating crucial signaling proteins. As detailed in the material and methods, fibroblasts (FB) were maintained under culture conditions up to 72 hours when the conditioned medium was collected to be used later. In this meantime the pre-osteoblasts (MC3T3-E1) were grown up at the semi-confluent stage when were treated with the FB-conditioned medium as mentioned previously. After 24h, the pre-osteoblasts were tripsinized, counted and re-plated, when the cells were lyzed and the biological samples collected after 3, 6 and 24 hours of seeding. These samples were used mainly to run SDS-gels for evaluating specific intracellular proteins at an immunoblotting protocol. We have investigated crucial proteins involved with cell viability such as Rac-1, FAK and Cofilin. Our results showed that FAK was progressively phosphorylated from 3h to 24 hours **(Fig. 1a-c)**, suggesting an involvement of FB on pre-osteoblast adhesion mechanism and viability; in addition, Rac-1 phosphorylation was found in this model, it being significant at 24 hours **(Fig. 1d-f)**. Interesting, we have also shown that cofilin presented a fluctuation on its phosphorylation profile where the cofilin phosphorylation was decreased at 3 hours **(Fig. 1g)** and significantly increased at 24 hours **(Fig. 1i)**. It is important to mention that cofilin suffers a transient phosphorylation during mechanism involved with cell adhesion by governing cytoskeleton rearrangement.

In addition, we have showed that there are a 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)**.

Fibroblast contributes to osteoblast differentiation in a Shh-independent manner. Later, in order to check the influence of FB on pre-osteoblast differentiation performance, we decided to maintain the pre-osteoblast under FB released factors up to 10 days. Thereafter, the osteoblast differentiation was evaluated by assaying 2 classical methodologies: Alkaline phosphatase **(Fig.4a-c)** and Alizarin red **(Fig.4d-f)** staining. The groups evaluated at this moment were: 1. negative control (conventional medium plus Fetal bovine serum); 2. positive control (conventional medium plus inductors as ascorbate and ß-glycerophosphate); 3. test group (conventional medium

conditioned by FB as detailed earlier). Thus, our results showed that FB conditioned medium was able to stimulate pre-osteoblast differentiation since those two parameters were up-regulated **(Fig.4g,h)**, when compared to other 2 groups (Ctrls).

In addition, in order the evaluate the sonic hedgehog signaling involvement on this biological model, osteoblasts were treated at the same condition and the biological samples were collected to run immunoblotting for identifying Shh, Patched (Ptch) and Gli-1. Curiously, our results revealed that the O.M. triggers intracellular signaling governing osteoblast differentiation requiring Shh signaling since Shh, Ptch and Gli-1 **(Fig.4i-n)**; on the other hand the differentiation promoted by the FB factors did not present the same profile, since all of the three proteins were down-regulated during osteoblast differentiation **(Fig.4i-n)**. Moreover, activation of mapk-erk was also investigated since mapk-erk has been reported in bone metabolism. In this regard, our results showed that erk displays a different profile in response to O.M. or FB released factors; while O.M. promoted an increase on mapk-erk phosphorylation, FB released factors promoted absence of the erk phosphorylation **(Fig.4o,p)**.

We considered these findings relevant since we have suggested that the osteoblast differentiation is a synergic event and can be stimulate by different intracellular signaling pathways dependent on the external stimulus.

DISCUSSION

Over the last decades we are interested to understand the trophic factors involved with osteoblast differentiation as well as the paracrine effects among cells into bone niche. In this way, we assayed how fibroblast can influence osteoblast performance. In order to develop this issue, first we collected the conditioned medium from fibroblasts cultured up to 3 days; thereafter we used this conditioned medium to incubate pre-osteoblast cells in order to investigate its influence at 2 important stages interfering osteoblast phenotype: adhesion (evaluated periods: 3, 6 and 24 hours after seeding) and differentiation (when cells were maintained along 10 days under incubation). First, at the first stage, we have shown that FB modulated osteoblast viability by up-modulating crucial signaling proteins such as FAK and Rac-1 activations. At this point, is very convenient to mention we have showed from previous works that during

an adequate osteoblast adhesion there is a well-orchestrated signaling governing osteoblast morphological changes recruiting mainly the phosphorylation of Rac-1 and later, modulating the phosphorylation of cofilin (Zambuzzi et al, 2009, 2011; Blandy et al, 2012; Chen et al, 2015); because on this statement, we decided to investigate the phosphorylation of cofilin (S03) and thus, we have showed that up to first 24 hours of seeding, cofilin phosphorylation was finely modulated, in agreement with the up-stream signaling protein activation, as Rac-1. Thus, we believe at this stage FB starts modulating osteoblast phenotype by interfering on their viability and adhesion performance.

Thereafter, we investigate the influence of FB released factors in contributing on osteoblast differentiation. Here, osteoblasts were maintained under FB conditioned medium up to 10 days when classical biomarkers were assayed. We showed that both alkaline phosphatase (ALP) and Alizarin red staining were up-modulated in response to conditioned medium. For this purpose, we have included osteogenic medium (O.M.) as an internal positive control group. In addition, we explored some of the osteoblast mechanisms involved with the FB released factors response and in order to address this issue we evaluated the dependency on canonical Sonic Hedgehog (Shh) signaling by assessing Shh protein, Gli-1 and Patched. Our results showed that FB released factors promoted osteoblast differentiation in a Shh signaling independent manner, since we showed a decrease of Gli-1 and Patched into osteoblast metabolism in crosstalk with fibroblast. In addition, at the internal positive control group (named O.M.), the cells over-expressed both Ptch and Gli-1. Thus, it's clear for us there are several mechanisms turned on able to promote osteoblast differentiation and later mineralized bone matrix. Another protein evaluated in this scenario was mapk-erk; our results showed erk up-activated in the osteoblast treated with 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 as survival, proliferation and differentiation (Megan et al, 2017).

Altogether, our results show for the first time a possible mechanism involved on the crosstalk between fibroblast and osteoblast, when it is possible to observe that factors released from fibroblasts interfere on osteoblast viability and differentiation at a Shh independent manner. In this sense, we believe these results help us to understand the

importance of the paracrine metabolism through bone resident cells for supporting bone dynamic biology, opening new avenues for comprehending bone diseases etiologies, as the case of osteoporosis.

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FIGURE CAPTIONS

Fig. 1. Fibroblast interferes on 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 hours the cells were tripsinized, counted and re-seeded. After 3, 6 and 24 hoursof seeding the cells were lysed using standard lysing buffer (described in M&M), 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 are shown and the graphs represents 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 **** $p < 0.0001$.

Fig. 2. Integrin-ß1 profile in response to fibroblast conditioned medium. Briefly, The cells were cultured respecting the details listed previously. Representative blotting are shown and the graphs represents 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 * $p < 0.01$.

Fig. 3. Patched is significantly down-regulated in cross-talking with fibroblast. Briefly, The cells were cultured respecting the details listed previously. Representative blotting are shown and the graphs represents 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 ** $p < 0.0017$.

Fig. 4. Fibroblast paracrine effects promote osteoblast differentiation in a Shh-independent manner. The pre-osteoblasts were cultured and in the semi-confluence were treated with conditioned medium from fibroblasts up to 10 days, when 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 are shown (**i,k,m,o**) and the graphs (**j,l,n,o**) represents 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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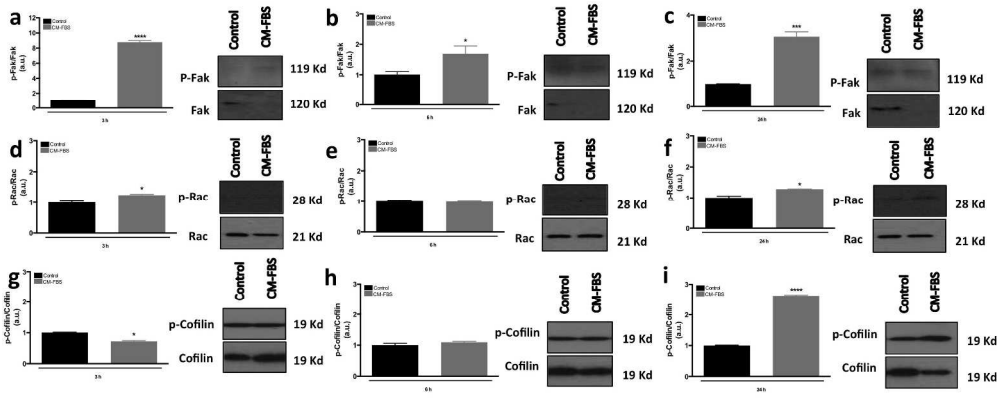
FIGURE CAPTIONS

Fig. 1. Fibroblast interferes on 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 hours the cells were tripsinized, counted and re-seeded. After 3, 6 and 24 hoursof seeding the cells were lysed using standard lysing buffer (described in M&M), 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 are shown and the graphs represents 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 **** $p < 0.0001$.

Fig. 2. Integrin-ß1 profile in response to fibroblast conditioned medium. Briefly, The cells were cultured respecting the details listed previously. Representative blotting are shown and the graphs represents 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 * $p < 0.01$.

Fig. 3. Patched is significantly down-regulated in cross-talking with fibroblast. Briefly, The cells were cultured respecting the details listed previously. Representative blotting are shown and the graphs represents 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 ** $p < 0.0017$.

Fig. 4. Fibroblast paracrine effects promote osteoblast differentiation in a Shh-independent manner. The pre-osteoblasts were cultured and in the semi-confluence were treated with conditioned medium from fibroblasts up to 10 days, when 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 are shown (**i,k,m,o**) and the graphs (**j,l,n,o**) represents 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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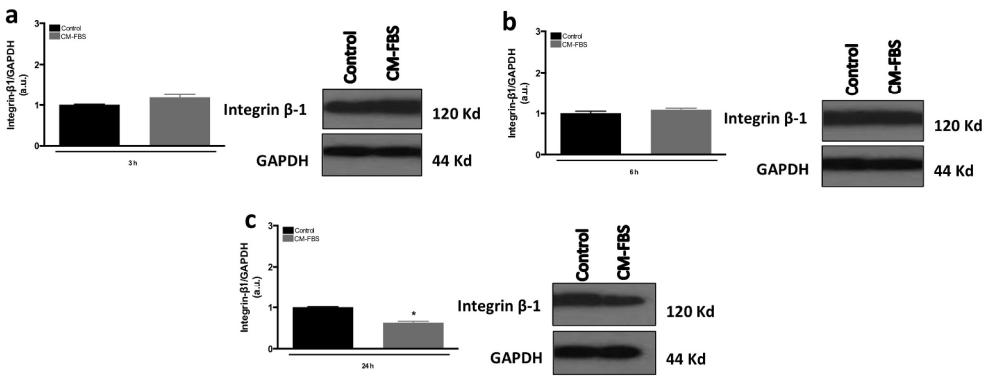

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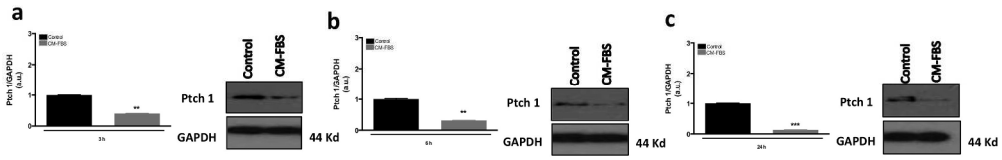

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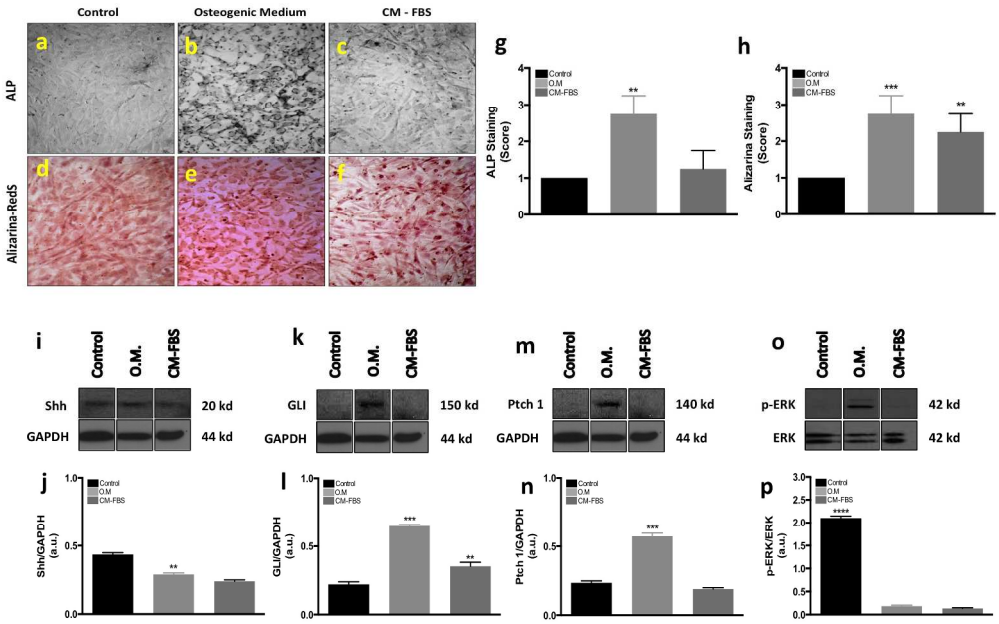

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