

Notch pathway is active during osteogenic differentiation of human bone marrow mesenchymal stem cells induced by pulsed electromagnetic fields

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operating in the sectors most affected by the earthquake in May 2012; Document n.109, 25th september 2013).

ABSTRACT

Pulsed electromagnetic fields (PEMFs) have been used to treat bone diseases, particularly non-union healing. Although it is known that PEMFs promote the osteogenic differentiation of human mesenchymal stem cells (hMSCs), to date PEMF molecular mechanisms remain not clearly elucidated. The Notch signaling is a highly conserved pathway which regulates cell fate decisions and skeletal development.

The aim of this study was to investigate if the known PEMF-induced osteogenic effects may involve the modulation of the Notch pathway. To this purpose, during in vitro osteogenic differentiation of bone marrow hMSCs in the absence and in the presence of PEMFs, osteogenic markers (alkaline phosphatase (ALP) activity, osteocalcin and matrix mineralization), the mRNA expression of osteogenic transcription factors (Runx2, Dlx5, Osterix) as well as of Notch receptors (Notch1-4), their ligands (Jagged1, Dll1 and Dll4) and nuclear target genes (Hes1, Hes5, Hey1, Hey2) were investigated.

PEMFs stimulated all osteogenic markers and increased the expression of Notch4, Dll4, Hey1, Hes1 and Hes5 in osteogenic medium compared to control. In the presence of DAPT and SAHM1, used as Notch pathway inhibitors, the expression of the osteogenic markers, including Runx2, Dlx5, Osterix, as well as Hes1 and Hes5 were significantly inhibited, both in unexposed and PEMF-exposed hMSCs. These results suggest that activation of Notch pathway is required for PEMFs-stimulated osteogenic differentiation. These new findings may be useful to improve autologous cell-based regeneration of bone defects in orthopedics.

Keywords: bone marrow mesenchymal stem cells, osteogenic differentiation, pulsed electromagnetic fields, Notch pathway, biophysical stimulation, bone repair.

1. INTRODUCTION

Biophysical stimulation with electromagnetic fields (EMFs) is a non-surgical, conservative, successfully used treatment in nonunions. For many years, EMFs have been applied with beneficial effects to promote bone ununited fracture healing in clinics (Griffin et al., 2008; Assiotis et al., 2012; Della Bella et al., 2015), although some controversial data are reported (Griffin et al., 2011; Hannemann et al., 2012).

The use of such biophysical stimuli in clinical setting is supported by several *in vivo* and *in vitro* studies, which have identified the effects of EMFs on the cells involved in the bone repair processes, particularly osteoblasts and mesenchymal stem cells (MSCs) (De Mattei *et al.*, 2005; Ongaro *et al.*, 2014).

Data from studies reporting the stimulatory effects of EMFs on the osteogenic differentiation of MSCs show that they support the different stages of differentiation by increasing cell proliferation and stimulating the production of bone matrix (Tsai *et al.*, 2009; Luo et al., 2012; Ceccarelli *et al.*, 2013; Liu *et al.*, 2013; Yong *et al.*, 2014; Lu *et al.*, 2015). Further, it has been shown that EMFs play a synergistic action with bone morphogenetic protein-2 (BMP-2), an essential growth factor for bone cells (Schwartz *et al.*, 2008). Most of these previous studies have been performed in order to investigate the biological response to EMFs in both animal and human mesenchymal stem cells (hMSCs) derived from different sources including bone marrow and fat (Tsai *et al.*, 2009; Ceccarelli *et al.*, 2013; Ongaro *et al.*, 2014; Yong *et al.*, 2014; Lu *et al.*, 2015). Moreover, these studies identified optimal exposure conditions for increased osteogenesis, showing that the physical characteristics of the signal and exposure length can influence the induced osteogenic effects (Luo *et al.*, 2012; Kang *et*

al., 2013; Lin *et al.*, 2015). However, few data are present in literature concerning the EMFs mechanism of action and the potential involvement of specific signal transduction pathways. It has been reported that EMFs increase the activity of certain kinases belonging to known intracellular signaling pathways such as the protein kinase A (PKA) and the MAPK ERK1/2 (Yong *et al.*, 2014; Song *et al.*, 2014) and modulate anti-inflammatory effects by increasing the adenosine receptor A_{2A} (Ongaro *et al.*, 2012). EMFs stimulation also upregulates BMP2 expression in association to increased differentiation in hMSCs (Kim *et al.*, 2015; Lin *et al.*, 2015).

Indeed, the osteogenic differentiation is a complex process which involves several signaling pathways which work cooperatively, including Wnt/ β -catenin, BMP and Notch signaling (Lin and Hankenson, 2011). In this study, we focused on Notch pathway, an evolutionarily conserved signaling pathway which regulates cell fate decisions and plays a significant role in bone development and repair (Dishowitz *et al.*, 2013; Zanotti and Canalis, 2016). Notch signaling (Figure 1) is initiated by the binding of a ligand (Jagged-1-2 and Delta-like [Dll]-1,-3,-4) to a cell surface Notch receptor (Notch-1, -2, -3, -4) on adjacent cells, resulting in a final cleavage of the receptor by the presenilin- γ -secretase complex and the release of the Notch intracellular domain (NICD) which translocates into the nucleus, and activates the transcription of nuclear Notch target genes, such as the Hes/Hey family genes (Ayaz and Osborne, 2014; Zanotti and Canalis, 2016).

Our aim was to assess the potential involvement of Notch signaling pathway in the osteogenic differentiation of hMSCs, the cells naturally involved in bone repair processes, under biophysical stimulation with pulsed electromagnetic field (PEMFs). To this purpose, we analyzed potential changes in the expression of Notch signaling pathway components, including receptors, ligands and nuclear target genes, and their correlation with osteogenic markers and transcription factors, during *in vitro* hMSCs osteogenic differentiation, in the

absence and in the presence of PEMF exposure. We used PEMFs with specific biophysical characteristics (1.5 mT, 75 Hz), successfully used in clinics (Massari et al., 2006; Zorzi et al., 2007) and *in vitro* to stimulate osteogenic differentiation of both bone marrow and adipose hMSCs (Ongaro *et al.*, 2014). Further, the involvement of Notch pathway on osteogenesis was studied also in the presence of Notch signaling inhibitors. N-[N-(3,5-Difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), which inhibits the γ -secretase responsible for intramembranous cleavage of Notch receptors and SAHM1, a peptide mimetic of a dominant negative form of MAML1, that specifically inhibits canonical Notch transcription complex formation (Ashley et al., 2015) were used to block the Notch pathway and to observe potential related effects on osteogenesis.

2. MATERIALS AND METHODS

2.1. Cell cultures

Human mesenchymal stem cells (hMSCs) from bone marrow were purchased from Lonza (Lonza, Walkersville, Maryland, USA) and guaranteed to express CD29, CD44, CD105, CD166, CD90 and CD73 and to not express CD14, CD34, and CD45, HLA-DR or CD19. Cells were grown in T75 tissue culture polystyrene flasks (Falcon BD, Franklin Lakes, NJ, USA) at the density of 5 000 cells/cm² in complete mesenchymal stem cell basal medium (MSCBM) (Lonza) according to the product specifications. The cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C, up to the third passage and then used for the differentiation experiments.

2.2. Osteogenic Differentiation

Cells were seeded in 4-well plates with a surface of 1.9 cm² per well (for ALP activity, osteocalcin and Alizarin Red staining assays), or in dishes with an area of 8.8 cm² (for RealTime-PCR and Western blot analysis), at an initial density of 4 000 cells/cm² in Osteogenic Differentiation Medium (OM) (Lonza), for 28 days (Ongaro *et al.*, 2014). The OM contained basal medium supplemented with dexamethasone, L-glutamine, ascorbate, penicillin/streptomycin, b-glycerophosphate, and mesenchymal cell growth supplement (all from Lonza). Control cells were cultured in complete MSCBM medium (Lonza). Medium was changed every three days and when Notch signaling inhibitors were used, they were freshly added to the culture medium from stock solutions, at each medium change. Samples were harvested at days 3, 7, 14, 21 and 28 for the assays of osteogenic markers and the evaluation of gene expression by Real-Time PCR.

2.3. Characteristics of PEMFs and exposure conditions

In each experiment, a parallel set of control and OM cell cultures were randomly assigned to PEMF exposure. The PEMF generator system was the same used in previous studies (De Mattei *et al.*, 2004, 2005, 2007, 2009, Ongaro *et al.*, 2011, 2012, 2014, 2015, 2016). It consisted of a pair of circular Helmholtz coils of copper wire placed opposite to each other and in a signal generator (IGEA S.p.A., Carpi, Italy). PEMF-exposed cultures were placed between the pair of Helmholtz coils. The power generator produced a pulsed signal with pulse duration of 1.3 ms and frequency of 75 Hz, yielding a duty cycle of 1/10. The intensity peak of the magnetic field was 1.5 mT and was detected between two coils from one side to the other, by the Hall probe of a Gaussmeter (Walker Scientific, Auburn Hills, MI, USA) with a reading sensitivity of 0.2%. A schematic representation of the device used to apply PEMFs is shown in Figure 2. In PEMF-exposed cells, the PEMF exposure was maintained for the whole differentiation time (28 days). Control cells were maintained in the same incubator,

placed at a distance from the coils where no difference from background magnetic field was observed when the PEMF generator was turned on.

2.4. Notch pathway inhibition

Both in unexposed and in PEMF-exposed cells cultured in OM, DAPT (LY-374973, Sigma Aldrich S.r.l., Milano, Italy), which inhibits γ -secretase, or SAHM1 (Calbiochem by MERCK S.p.a. Vimodrone Milano, Italy), which inhibits NICD-MAML-CSL transcription complex formation, were added to culture medium at the final concentration of 10 μ M (Ashley et al., 2015). Both inhibitors were dissolved in DMSO (Fisher Scientific, BP231-100) at 10mM, stored at -80°C and freshly diluted in culture medium at each medium change during the whole differentiation period.

2.5. Alkaline Phosphatase (ALP) Activity

At the time points investigated, ALP activity was spectrophotometrically evaluated in hMSCs as previously reported (Ongaro et al., 2014, 2016). In brief, cells were washed with PBS and lysed by using 0.1% Triton X 100 (Sigma–Aldrich) in double-distilled H_2O . Cellular lysates were incubated at 37°C for 30 min in the presence of 10mM p-nitrophenylphosphate (p-NP; Sigma–Aldrich) in alkaline buffer containing 100mM diethanolamine and 0.5mM MgCl_2 , pH 10.5 (Sigma–Aldrich). The reaction was stopped with 0.2M NaOH and absorbance was read at 405 nm with a Jenway 6305 spectrophotometer (Barloworld Scientific, Dunmow, Essex, UK). ALP activity was then normalized to total DNA content measured by the NucleoSpin Tissue XS Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and expressed as $\mu\text{M}/(\text{min} \times \mu\text{g DNA})$.

2.6. Detection of Osteocalcin Levels by Enzyme Linked Immunosorbent Assay (ELISA)

The temporal pattern of osteocalcin levels during the culture period, was evaluated as previously described (Ongaro *et al.*, 2014, 2016), by using a commercial ELISA kit which uses monoclonal antibodies directed against distinct epitopes of human osteocalcin (Invitrogen by Life Technologies, Monza, Italy). Osteocalcin levels were expressed as ng osteocalcin/ μ g DNA.

2.7. Matrix mineralization analysis

At the end of the differentiation period, matrix mineralization was visualized by staining cultures, fixed in 10% formalin, with 2% Alizarin red (Histo-Line Laboratories S.r.l, Milano, Italy) solution for 30 min at room temperature. Images were taken using a standard light microscope (Nikon Eclipse TE 2000-E microscope, Nikon Instruments Spa, Sesto Fiorentino (FI), Italy) equipped with a digital camera (DXM 1200F; Nikon Instruments Spa, Italy). Further, the mineralized substrates were quantified by using a solution of 20% methanol and 10% acetic acid (both from Sigma–Aldrich) in water. After 15 min, liquid was transferred into cuvettes and the quantity of Alizarin red dissolved was read by the Jenway 6305 spectrophotometer (Barloworld Scientific) at a wavelength of 450 nm (Ongaro *et al.*, 2014, 2016).

2.8. Reverse Transcription and Real-Time PCR

For RNA extraction, hMSCs were washed three times with Earle's Solution and total RNA was extracted using commercially available kit (PureLink RNA minikit Invitrogen by Life Technologies). Following treatment with DNase, performed according to PureLink RNA minikit (Invitrogen by Life Technologies) protocol, in order to limit DNA contamination, RNA concentration and purity were determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, Inc., MA, USA). 2 μ g of total RNA were reverse transcribed in a final

volume of 20 µl using the SuperScript™ III First-Strand Synthesis system for RT-PCR (Invitrogen by Life Technologies) and 50 ng of random hexamers. Then, cDNA mixture was amplified using PerfeCta SYBR Green SuperMix ROX kit (Quanta Biosciences by VWR, Milano, Italy) according to the manufacturer's protocol in a final volume of 20 µl. Real-time PCR was carried out for Runx2, Dlx5, Osterix, Notch1, Notch2, Notch3, Notch4, Dll1, Dll4, Jagged1, Hes1, Hes5, Hey1 and Hey2 in the total 40 cycles of amplification: 95 °C for 15 s and 60 °C for 1 min and examined on a 7500 Fast Real-Time PCR system (Applied Biosystems, Life Technologies). In order to assess the possible contribution of DNA contaminant, PCR for each target also included a no-reverse transcribed control sample. A concentration of 500 nM of primers (all from IDT Tema Ricerca, Bologna, Italy) was used. The sequences of primers used for Notch and other genes (Caliceti et al., 2013; Ongaro et al., 2016; Rizzo et al., 2008) have been reported in Table 1. GUSB was used as reference gene.

The data were calculated by the $2^{-\Delta\Delta Ct}$ formula and changes in gene expression levels were referred to the expression of the same genes in control cells, grown in basal medium (MSCBM) at day 3.

2.9. Western Blotting and Densitometric Analysis

Western blot analysis was carried out to detect Notch4 protein changes in all the tested experimental conditions at day 7. β -actin was used as control. Antibody to Notch4 (H-225) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibody to β -actin was from Sigma-Aldrich. Western blotting was performed as previously reported (Caliceti *et al.*, 2013; Ongaro *et al.*, 2016). Briefly, cells were lysed in RIPA Buffer (0.05% sodium-deoxycholate was freshly added) containing protease inhibitors. Protein samples were denatured by incubation at 70°C for 10 minutes in sample buffer (Life Technologies) containing 0.5 M DTT, separated on 7% NuPAGE gels (Life Technologies) and transferred

to PVDF membrane (Whatman) at 30 V for 150 min. PVDF membranes were incubated overnight at 4°C with the primary antibody, washed in TBS/Tween, and incubated for 60 minutes at room temperature with the secondary antibody peroxidase-conjugated in TBS/Tween containing 5% nonfat dry milk. Membranes were then developed using ECL Western Blotting Detection Reagents (Life Technologies). Protein immunoreactive bands were analyzed and quantitated by the Image Lab software 4.0 (Bio-Rad, CA, USA). Values were expressed as the ratio of each band density to the respective β -actin band density.

2.10. Statistical Analysis

Experiments (n=8) were performed using hMSCs from eight different donors. Within each experiment, every experimental condition was tested in triplicate. Data were expressed as means \pm SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's post hoc test used for multiple comparison. This analysis was carried out by using the GraphPad Prism version 6.01 Software (GraphPad Software, San Diego, CA). P values < 0.05 were considered statistically significant.

The analysis of gene expression during osteogenic differentiation was done by comparing the fold changes obtained in OM versus Control, OM+PEMF versus OM, OM+DAPT versus OM, OM+SAHM1 versus OM, OM+PEMF+DAPT versus OM+PEMF, OM+PEMF+SAHM1 versus OM+PEMF, OM+PEMF+DAPT versus OM+DAPT and OM+PEMF+SAHM1 versus OM+SAHM1 at the different time points investigated (3, 7, 14, 21, 28 days), as reported in figure legends.

3. RESULTS

3.1. Analysis of osteogenic markers and transcription factors during osteogenic differentiation in hMSCs and effects of PEMF exposure

The analysis of biochemical markers investigated during osteogenic differentiation showed an expected increase of ALP activity (from day 7) and of OC levels (from day 21 until day 28), in hMSCs grown in OM versus control (Figure 3). Analogously, Alizarin red staining, confirmed an increase in matrix mineralization in the late stage of differentiation (21 and 28 days) (Figure 3).

Gene expression analysis of osteogenic transcription factors showed an increase of Runx2 (4.5-fold \pm 0.3), Dlx5 (2.8-fold \pm 0.2) and Osterix (5.5-fold \pm 0.4) mRNAs during the early phase of differentiation (day 3), with a prolonged upregulation of the latter gene until day 14, in hMSCs grown in OM compared to control (Figure 4).

The PEMF exposure in hMSCs grown in control medium did not produce any significant change on osteogenic markers levels compared to unexposed control cells (data not shown). Differently, when hMSCs were grown in OM and exposed to PEMFs, an enhancement of osteogenic differentiation, with time-dependent variations of osteogenic markers was observed. Specifically, PEMFs stimulated ALP activity with a significant progressive increase at day 7 (1.9-fold \pm 0.1), 14 (2.0-fold \pm 0.1) and 21 (1.8-fold \pm 0.1) compared to OM (Figure 3). In addition, PEMFs determined a significant enhancement of OC production at day 21 (3.4-fold \pm 0.4) and 28 (3.9-fold \pm 0.5) respect to OM. Similarly, Alizarin red staining increased in PEMF-exposed hMSCs, confirming a greater matrix mineralization in the late stage of differentiation under PEMF exposure compared with OM alone (Figure 3).

PEMF exposure also significantly enhanced the gene expression of all the transcription factors compared to OM at day 3 (Runx2: 2.7-fold \pm 0.1; Dlx5: 2.6-fold \pm 0.2; Osterix: 2.9-

fold \pm 0.1) (Figure 4). In addition, at day 7, PEMFs upregulated Runx2 (2.6-fold \pm 0.1) and Osterix (3.6-fold \pm 0.2) whilst for Dlx5 maintained an increased expression compared to OM at all the time points investigated (2.5-fold \pm 0.4 at day 7; 2.65-fold \pm 0.2 at day 14; 2.8-fold \pm 0.2 at day 21; 5.4-fold \pm 0.4 at day 28).

3.2. Analysis of the expression of Notch genes during osteogenic differentiation in hMSCs and effects of PEMF exposure

In order to investigate the potential involvement of Notch pathway during osteogenic differentiation in hMSCs in the presence and in the absence of PEMF exposure, we analyzed the expression of several genes related to the Notch pathway at different times during the whole period of cell differentiation. The gene expression of the Notch nuclear target genes Hes1, Hes5, Hey1 and Hey2 was modified during osteogenic differentiation compared with the basal condition (Figure 5). Specifically, we found an early upregulation of Hes5 from day 7 prolonged until day 28, in OM compared to control. Further, during the late stage of osteogenic differentiation (day 21 and day 28), a significant increase of Hes1 (2.72-fold \pm 0.3 at day 21; 7.1-fold \pm 0.7 at day 28) and Hey1 (2.8-fold \pm 0.2 at day 21; 4.2-fold \pm 0.3 at day 28) was also observed in OM compared to control. No differences were observed in Hey2 expression when comparing OM to control during the whole differentiation period (Figure 5).

As the Notch pathway is triggered by the binding between specific ligands and receptors, we determined if their expression was modulated during osteogenic differentiation. Among the ligands investigated, Dll4 was significantly increased at the end of differentiation period (7.4-fold \pm 0.6 at day 21; 4.9-fold \pm 0.4 at day 28) (Figure 6), whilst for Dll1 and Jagged1 no changes were observed (data not shown). As far as Notch receptors are concerned, significant changes during osteogenic differentiation were found only for Notch4, with a transient early

upregulation at day 3 (3.9-fold \pm 0.2) in OM compared to control, followed by a decrease to the control level at later time points (Figure 6).

The exposure to PEMFs of hMSCs grown in control medium did not produce any change on Notch related genes in comparison to unexposed control cells (data not shown), whilst differences in the expression of the same genes which appeared modulated during hMSCs differentiation in OM, were observed when comparing PEMF-exposed and unexposed hMSCs (Figure 5). Specifically, at day 14 and 21, PEMFs significantly increased Hes1 (4.0-fold \pm 0.3 at day 14; 2.8-fold \pm 0.2 at day 21), Hes5 (2.7-fold \pm 0.2 at day 14; 2.6-fold \pm 0.2 at day 21) and Hey1 (2.9-fold, \pm 0.2 at day 14; 2.7-fold \pm 0.2 at day 21) expression compared to OM. The increase in Hes1 and Hey1 expression induced by PEMFs (day 14) was observed one week earlier than the increase observed in OM in the absence of PEMFs (day 21). No effects of PEMFs were observed on Hey2 expression.

Notably, a significant enhancement of Dll4 (8.8-fold \pm 0.5) expression compared to OM was also observed in hMSCs exposed to PEMFs during the middle phase of differentiation (day 14) (Figure 6). This increase was maintained also at day 21 (3.3-fold \pm 0.2 in OM+PEMF vs OM). No effect of PEMFs was observed on Jagged1 and Dll1 expression (data not shown).

Furthermore, PEMF exposure led to higher Notch4 expression at day 7 (2.8-fold \pm 0.1) compared to OM alone, prolonging its increased expression observed in the early phase of differentiation (at day3) (Figure 6). No significant effect of PEMFs was observed on Notch1, Notch 2 and Notch3 receptors (data not shown).

3.3. Effects of Notch inhibitors on osteogenic differentiation and Notch pathway

As gene expression analysis suggested the involvement of Notch signaling during osteogenic differentiation and the modulation of this pathway by PEMFs, we treated hMSCs with the γ -secretase inhibitor DAPT, and with SAHM1, an inhibitor of the Notch transcription complex formation, in order to block Notch signaling and to observe potential related effects on osteogenesis.

In preliminary experiments we verified that, at the concentration used, DAPT and SAHM1 were not toxic for the cells (data not shown).

In the absence of PEMFs, both Notch inhibitors induced a significant decrease of ALP activity at day 14 (DAPT: $-59\% \pm 5\%$; SAHM1: $-65\% \pm 5\%$;) and at day 21 (DAPT: $-60\% \pm 5\%$; SAHM1: $-63\% \pm 5\%$) and OC levels at day 21 (DAPT: $-52\% \pm 6\%$; SAHM1: $-70\% \pm 4\%$) and 28 (DAPT: $-62\% \pm 5\%$; SAHM1: $-78\% \pm 3\%$) compared to OM, observed also in the reduction of Alizarin red staining (Figure 3). In line with these data, both Notch inhibitors reduced the gene expression of the osteogenic transcription factors (Figure 4), with a significant inhibition from day 7 until day 21, compared to OM.

In the presence of PEMFs, the reduction provoked by Notch inhibitors on osteogenic markers and transcription factors was similar to what observed in unexposed hMSCs. Specifically, in PEMF-exposed cells, Notch inhibitors decreased ALP activity at day 14 (DAPT: $-41\% \pm 5\%$; SAHM1: $-56\% \pm 5\%$) and at day 21 (DAPT: $-64\% \pm 3\%$; SAHM1: $-67\% \pm 4\%$), compared to OM+PEMFs. However, despite the presence of Notch inhibitors, at day 14 PEMF could still increase ALP activity (OM+DAPT+PEMF: $+197\% \pm 8\%$ vs OM+DAPT; OM+SAHM1+PEMF: $+163\% \pm 12\%$ vs OM+SAHM1). A reduction induced by Notch inhibitors was also observed for OC at day 21 (DAPT: $-76\% \pm 3\%$; SAHM1: $-79\% \pm 3\%$) and at day 28 (DAPT: $-71\% \pm 4\%$; SAHM1: $-77\% \pm 3\%$) compared to OM+PEMFs (Figure

3). Also, under PEMF exposure, a significant inhibition due to DAPT or SAHM1 treatments was obtained for Runx2, Dlx5 and Osterix, comparing OM+DAPT+PEMF to OM+PEMF and OM+SAHM1+PEMF to OM+PEMF (Figure 4).

On Notch target genes expression, Notch inhibitors provoked a prolonged inhibition in the expression of Hes5 (from day 7 until day 21) and a shorter inhibition of Hes1 (day 21 and 28) compared to OM, whilst they did not reduce the expression of Hey1 (Figure 5). The results obtained for Hes5 and Hes1 with DAPT or SAHM1 were similar to those obtained also in the presence of PEMFs (comparing OM+DAPT+PEMF to OM+PEMF or OM+SAHM1+PEMF to OM+PEMF), with a decrease of Hes5 from day 7 until 21 and Hes1 at day 14 and 21. No reduction induced by DAPT or SAHM1 was found on Hey1 expression, both in the absence or in the presence of PEMFs. Furthermore, at least at day 14, the expression of Hey1 in the presence of PEMFs was maintained also in the presence of DAPT (OM+DAPT+PEMF) or SAHM1 (OM+SAHM1+PEMF).

Similarly, no effect of DAPT or SAHM1 treatments was observed on Notch ligands and receptors when comparing Notch inhibitors in the presence of PEMFs vs OM+PEMF (Figures 6). However, at day 14 and 21, the expression of Dll4 in cells exposed to PEMFs was maintained also in the presence of Notch inhibitors.

3.4. Immunoblotting

As the Notch pathway is activated by the release of a NICD, we verified by Western blot analysis the activation of Notch4, which represented the only Notch receptor differently expressed during osteogenic differentiation in OM compared with control. For this analysis, we used an antibody against the C-terminus of Notch4 protein that detected a fragment of 64 KDa, corresponding to the active form of Notch4 (N4ICD), as previously reported (Caliceti *et al.*, 2013; Ongaro *et al.*, 2016). The immunoblots reported in Figure 7 show the pattern of

N4ICD in lysates from hMSCs cultured in all our experimental conditions at day 7 of differentiation, a critical time-point at which PEMF exposure induced the overexpression of Notch4 compared to OM. The Western blot results showed a significant increase of N4ICD in OM compared to control. This increase was further enhanced in the presence of PEMFs. The treatment with DAPT provoked a reduction in the activation of N4ICD compared to OM, and this reduction was observed also in the presence of PEMF exposure.

4. DISCUSSION

The present study reveals several novel findings concerning the events involved in the stimulation of hMSCs osteogenic differentiation induced by PEMFs. In fact, a significant role of Notch signaling has been identified during differentiation driven by PEMF stimulation in osteogenic microenvironment. Notch signaling has been implicated in bone development during embryogenesis (Zanotti and Canalis, 2016) as well as in bone fracture healing in adults (Dishowitz *et al.*, 2013). In particular, although some conflicting results are present in literature, previous studies have shown that the activation of Notch signaling pathway may favor osteogenic differentiation (Nobta *et al.*, 2005; Ugarte *et al.*, 2009; Zhu *et al.*, 2013; Ongaro *et al.*, 2016). As it is known that PEMFs stimulate osteogenic differentiation, in this study, we hypothesized the involvement of Notch pathway in the PEMF stimulatory effects on hMSCs osteogenic differentiation. To investigate our hypothesis we analyzed possible changes in Notch related genes expression during the whole period of hMSC differentiation and evaluated the possible correlations with the expression of the typical osteogenic markers, both in the absence and in the presence of PEMFs.

In the absence of PEMFs, hMSCs, cultured in osteogenic medium, differentiated towards the osteoblast phenotype, as indicated by the increase in the production of early (ALP activity) and later osteogenic markers (OC, matrix mineralization). These changes, as expected, were associated to the early increase (3 days) in the expression of the known specific osteogenic transcription factors (Runx2, Dlx5, Osterix), with an increased expression of Osterix prolonged in time. In agreement with previous studies (Schwartz *et al.*, 2008; Tsai *et al.*, 2009; Luo *et al.*, 2012; Ceccarelli *et al.*, 2013; Kang *et al.*, 2013; Liu *et al.*, 2013; Ongaro *et al.*, 2014; Yong *et al.*, 2014; Lin *et al.*, 2015; Lu *et al.*, 2015), stimulation with PEMFs induced a significant increase in all the osteogenic markers and transcription factors investigated, showing cooperation between PEMFs and osteogenic medium on cell differentiation.

When we investigated the expression of Notch related genes during hMSCs differentiation in the absence of PEMFs, we observed an increased expression in most of the investigated Notch target genes, in comparison to undifferentiated control cells, with an early (day 7) increase for Hes5, maintained until the end of the differentiation period and a later induction (days 21 and 28) for Hes1 and Hey1 genes. Interestingly, PEMFs induced the upregulation of the same Notch target genes Hes5, Hes1 and Hey1 during the middle-late times of differentiation (days 14 and 21). Furthermore, a significant increase in the expression of the ligand Dll4 (at day 21 and 28) and the Notch4 receptor (at day 3) was identified in cells maintained in osteogenic medium compared to control. PEMFs modulated the expression of the same molecules, enhancing Dll4 expression at day 14 and 21 and maintaining a higher expression for Notch4 receptor at day 7, in PEMF-exposed cells compared to unexposed cells cultured in osteogenic medium. Altogether, data on gene expression suggested the activation of the canonical Notch pathway (Ayaz and Osborne, 2014), during osteogenic differentiation of hMSCs, confirming previous data of literature (Ongaro *et al.*, 2016; Ugarte *et al.*, 2009;

Zhu *et al.*, 2013). In particular, our results are essentially in line with those obtained in our previous study in human osteosarcoma cell line MG63 (Ongaro *et al.*, 2016), although differences in the time dependent expression of specific Notch receptors and target genes have been identified. On the other hand, differences might be expected as it has been reported that Notch signaling is dysregulated in many human malignancies including osteosarcoma (Engin *et al.*, 2009; Liu *et al.*, 2016).

Interestingly, in hMSCs we observed an increased expression of the ligand Dll4, which may play a role in mediating the activation of Notch pathway, in agreement with previous studies showing the involvement of the ligand Jagged1 in the stimulation of osteogenesis (Osathanon *et al.*, 2013; Zhu *et al.*, 2013). Notably, our results showed that PEMFs increased the expression of the same Notch related genes which increased during differentiation in osteogenic medium in the absence of PEMFs. This suggests some cooperation between PEMFs and the osteogenic microenvironment, through the activation of Notch pathway, in favoring osteogenesis.

A way to clarify the involvement of a signaling pathway is based on the use of selective inhibitors. Therefore, during hMSCs differentiation we investigated the effects of Notch signaling inhibitors including DAPT, which prevents the formation of NICD, a rate-limiting step of initiating Notch signaling and SAHM1 that specifically inhibits canonical Notch transcription complex formation (Ashley *et al.*, 2015). In the absence of PEMFs, treatment with DAPT or SAHM1 inhibited osteogenic differentiation, as shown by the decrease in osteogenic markers and transcription factors expression, as well as the expression of the Notch target genes Hes5 and Hes1, from day 7. These results appear to confirm that the activation of Notch pathway was involved in hMSCs osteogenic differentiation at least in this differentiative phase (Nobta *et al.*, 2005; Ugarte *et al.*, 2009; Zhu *et al.*, 2013; Ongaro *et al.*, 2016). Also in PEMF-exposed cells, Notch inhibitors induced similar effects on osteogenic

markers, transcription factors and Notch target genes, suggesting that Notch pathway inhibition may negatively influence the differentiative effects induced by PEMFs.

However, both in PEMF-exposed or unexposed cells, at day 3, no inhibition on osteogenic transcription factor and Notch target gene expression was induced by DAPT or SAHM1, suggesting that osteogenic transcription factors may be influenced by alternative pathways in the early induction of osteogenesis.

Although, to our knowledge, no previous data have been reported about Hes5 expression during osteogenic differentiation, the increased expression of Hes1 during osteogenesis has been previously shown (Nobta *et al.*, 2005; Ugarte *et al.*, 2009; Osathanon *et al.*, 2013; Zhu *et al.*, 2013). Further, it has been reported that Hes1 may augment the protein stability and transcriptional activity of Runx2, which may result in the stimulation of osteoblast differentiation (Lee *et al.*, 2006; Suh *et al.*, 2008). Differently from what observed for Hes1 and Hes5, both in the PEMF unexposed or exposed cells, DAPT or SAHM1 had no effect on Hey1 expression. This may indicate that, during hMSCs differentiation, the increase in Hey1 expression is not directly related to the Notch pathway, but may be due to alternative signaling pathways. Indeed, increases in Hey1 expression have been associated to increased osteogenic differentiation (de Jong *et al.*, 2004; Sharff *et al.*, 2009; Ugarte *et al.*, 2009; Osathanon *et al.*, 2013), in line with a functional role for this gene in osteogenesis, which however remains to be defined, as controversial data have been previously reported (de Jong *et al.*, 2004; Zamurovic *et al.*, 2004; Sharff *et al.*, 2009). Interestingly, in some previous studies the increased expression in Hey1 has been described in association to BMP signaling activation (de Jong *et al.*, 2004; Sharff *et al.*, 2009) and, at least in endothelial cells, it has been shown that Hey1 expression may be induced by BMP signaling independently from Notch pathway (Wöltje *et al.*, 2015). Further, although mechanisms have not been elucidated, the PEMF effects have been previously associated to the BMP pathway (Schwartz *et al.*,

2008; Lin *et al.*, 2015; Kim *et al.*, 2015). Consequently, this might suggest the likely involvement of BMP pathway in the modulation of Hey1 expression and in mediating the PEMF-induced effects on osteogenesis. This is in line with the possible involvement of other signaling pathways suggested by the lack of inhibition induced by DAPT and SAHM1 on the expression of osteogenic transcription factors in the early phase of osteogenesis, described above.

Finally, as the Notch signaling is triggered by the activation of a Notch receptor, through the release of a Notch intracellular domain, to further investigate the involvement of Notch pathway during PEMF-mediated osteogenesis, we also investigated the presence of the Notch4 active form by Western blotting, under each experimental condition tested. As Notch4 gene expression increased early during differentiation (at day 3) and its expression was induced by PEMFs (at day 7), this Notch receptor appears to be a good candidate involved in mediating the effects of Notch pathway on cell differentiation. Western blotting analysis confirmed that the receptor was activated early during differentiation in osteogenic medium and that its activation could be increased by PEMFs. Further, DAPT inhibited the activation of the receptor both in the absence and in the presence of PEMFs. These results are in line with a previous study showing increased expression of Notch4 during osteogenic differentiation in human alveolar bone-derived osteoprogenitor cells (Chakravorty *et al.*, 2014).

In conclusion, the results of this study add new information concerning osteogenic differentiation of hMSCs and the events involved during PEMF-induced osteogenic differentiation. Our data show that Notch canonical pathway contributes to osteogenic differentiation and identify Hes1 and Hes5 as the Notch target genes involved. Furthermore, in this study, for the first time, we show that PEMFs can modulate the same Notch genes involved in osteogenesis, suggesting cooperation between PEMFs and osteogenic

microenvironment through Notch pathway. Thus, PEMFs effects can be associated to the modulation of a highly conserved pathway which plays essential roles in cell fate and differentiation. On the other hand, we have also found that PEMF exposure can increase Hey1 expression. This gene, involved in osteogenic differentiation, represents a target also for pathways different from the canonical Notch pathway such as BMP pathway. Further studies may elucidate the complex relationship among these pathways during PEMF-induced osteogenesis.

The requirement for Notch signaling in skeletal progenitors during fracture repair has been recently demonstrated (Wang *et al.*, 2016). Since PEMFs are efficacious in facilitating ununited fracture repair understanding the mechanisms by which PEMFs can stimulate osteogenesis may be of interest from a clinical point of view, for the evaluation of new strategies for bone tissue engineering optimization and application.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Table 1. Primer sequences of the genes investigated.

Gene	Primer Forward	Primer Reverse
Notch1	5'-GTCAACGCCGTAGATGACC-3'	5'-TTGTTAGCCCCGTTCTTCAG-3'
Notch2	5'-CAGGCACTCGGGGCCTACTCT-3',	5'-AGCCAGGCAAGCAGCGACAA-3'
Notch3	5'-TGCGATCAGGACATCAATGAC-3'	5'-CTCAGGCACTCATCCACATC-3'
Notch4	5'-CAACTGCCTCTGTCTGATG-3'	5'-GCTCTGCCTCACACTCTG-3'
Jagged1	5'-GACTCATCAGCCGTGTCTCA-3'	5'-TGGGGAACACTCACACTCAA-3'
Dll1	5'-AGCAAGCGTGACACCAAGTG-3'	5'-TTTCAGATGCTTCTCCACCCCTG-3'
Dll4	5'-GCGAGAAGAAAGTGGACAGG-3'	5'-ATTCTCCAGGTCATGGCAAG-3'
Hey1	5'-CCGAGATCCTGCAGATGACCGT-3'	5'-AACGCGCAACTTCTGCCAGG-3'
Hey2	5'-AAAAGGCGTCGGGATCG-3'	5'-AGCTTTTTTCTAACTTTGCAGATCC-3'
Hes1	5'-CGGACATTCTGGAAATGACA-3'	5'-CATTGATCTGGGTCATGCAG-3'
Hes5	5'-AAGCACAGCAAAGCCTTCGT-3'	5'-TGGAGCGTCAGGAACTGCAC-3'
Runx2	5'-AAGCTTGATGACTCTAAACC-3'	5'-TCTGTAATCTGACTCTGTCC-3'
Dlx5	5'-GCATTACAGAGAAGGTTTCAG-3'	5'-TTTTCACCTGTGTTTGTGTC-3'
Osterix	5'-TGAGGAGGAAGTTCATATG-3'	5'-CATTAGTGCTTGTAAGGGG-3'
GUSB	5'-CCCGCGGTCGTGATGTGGTC-3'	5'-GCCGGGAGGGGTCCAAGGAT-3'

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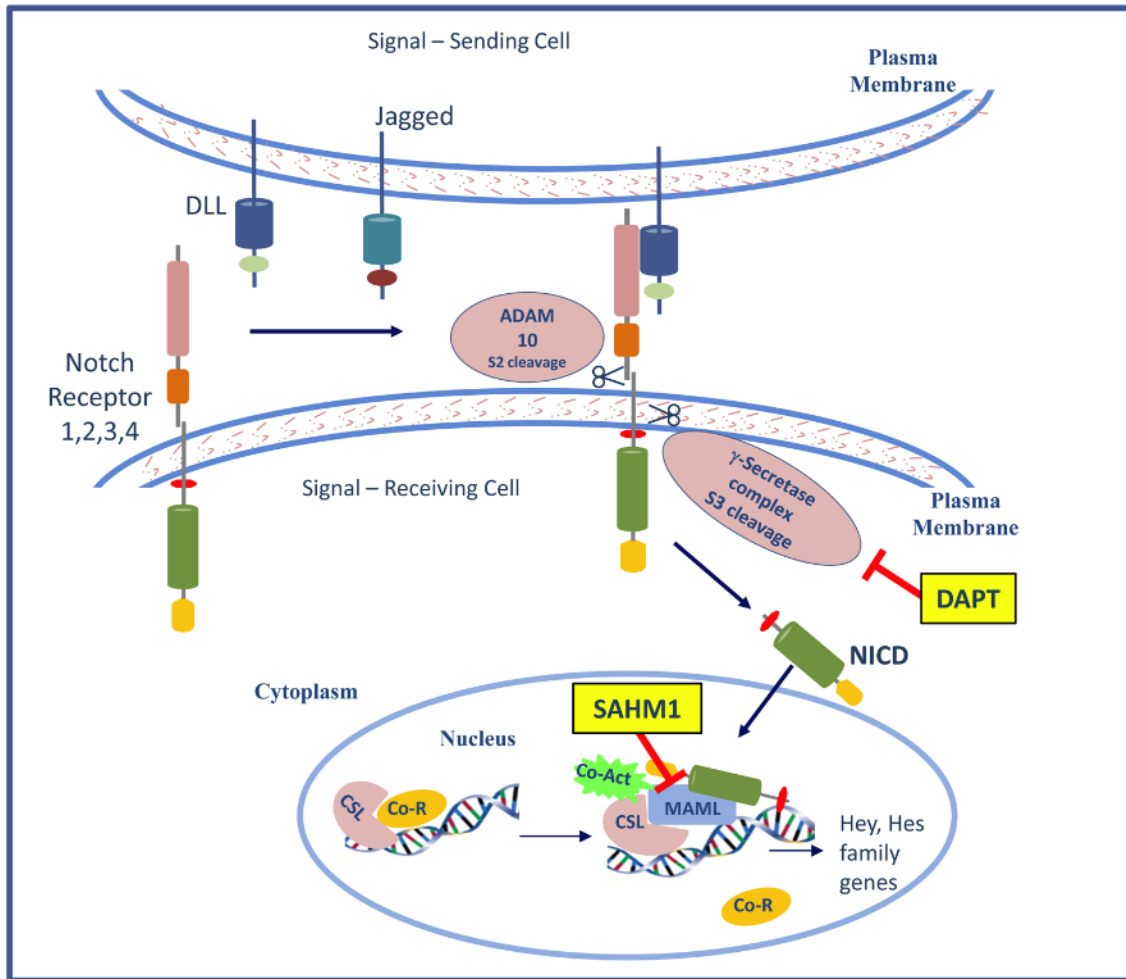


Figure 1. Notch pathway. Notch signaling is initiated by the interaction between adjacent cells. Mammalian Notch receptors (Notch-1, -2, -3, -4 in humans) are single-pass transmembrane proteins that transmit juxtacrine signals initiated by ligands of the Delta (DLL) and Jagged family. Ligand binding promotes two proteolytic cleavage events in the Notch receptor. The first cleavage is catalysed by ADAM-family metalloproteases, whereas the second is mediated by γ -secretase complex and releases the Notch intracellular domain (NICD). NICD translocates to the nucleus and interacts with the DNA-binding CSL protein. The co-activator Mastermind (MAML) and other transcription factors (Co-Act) are recruited to the CSL complex, whereas co-repressors (Co-R) are released and the transcription of Hes and Hey family genes is induced.

Notch pathway can be blocked by Notch inhibitors such as DAPT, which inhibits the γ -secretase and prevents the formation of NICD, and SAHM1, a peptide mimetic of a dominant negative form of MAML, that specifically inhibits NICD-MAML-CSL transcription complex formation.

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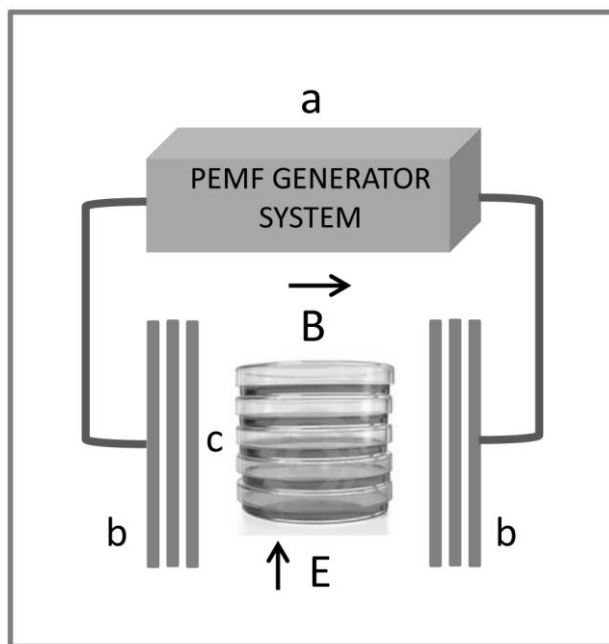


Figure 2. Schematic representation of the device used to apply PEMFs. (a) PEMF generator system, (b) coil generating magnetic field, (c) cell culture plates, (B) magnetic field, (E) electric field.

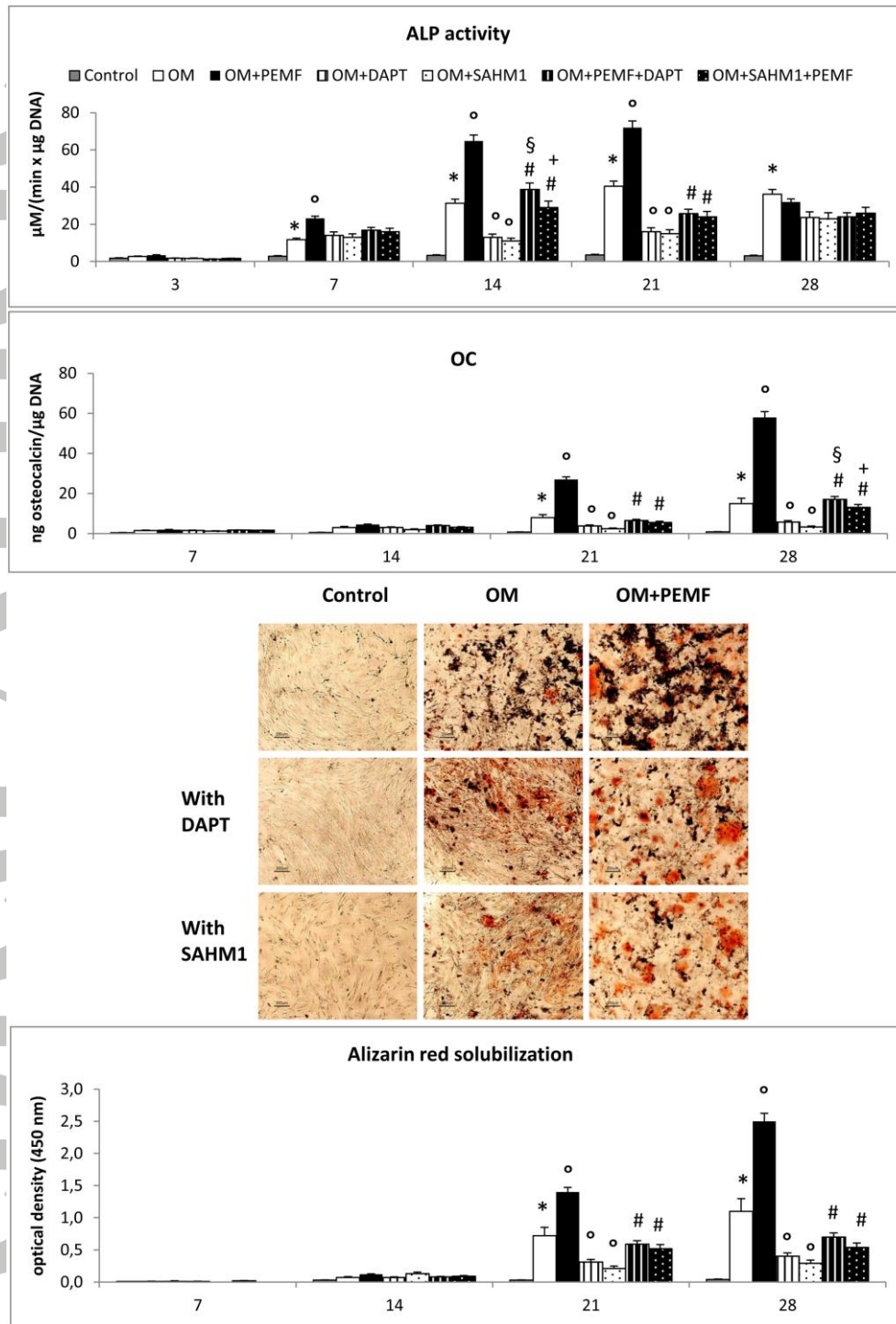


Figure 3. Biochemical and histological markers in hMSCs during osteogenic differentiation. At different time points (3, 7, 14, 21, 28 days) cells were tested for alkaline phosphatase (ALP) activity and osteocalcin (OC) levels. Matrix mineralization was evaluated by Alizarin red staining and its quantification was done spectrophotometrically and shown in the graph below the panel. In the panel is shown Alizarin red staining at day 28, in all the experimental

conditions tested. Symbols indicate statistical significance versus control (*), versus OM (°), versus OM+PEMF (#), versus OM+DAPT (§), versus OM+SAHM1 (+), at the corresponding time point.

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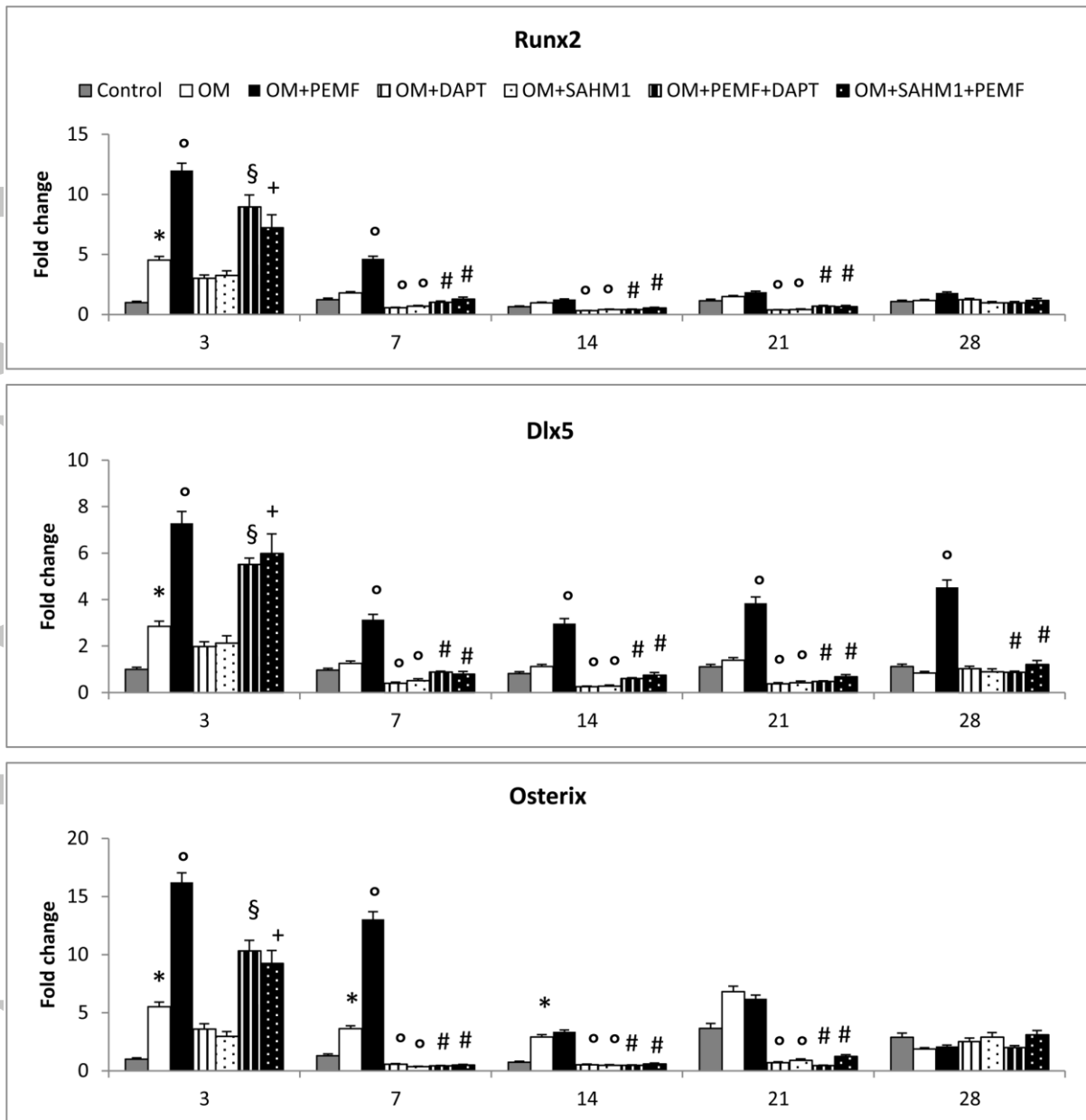


Figure 4. Gene expression of osteogenic transcription factors Runx2, Dlx5, Osterix in hMSCs at different times points (3, 7, 14, 21, 28 days) during osteogenic differentiation. Symbols indicate statistical significance versus control (*), versus OM (°), versus OM+PEMF (#), versus OM+DAPT (§), versus OM+SAHM1 (+), at the corresponding time point.

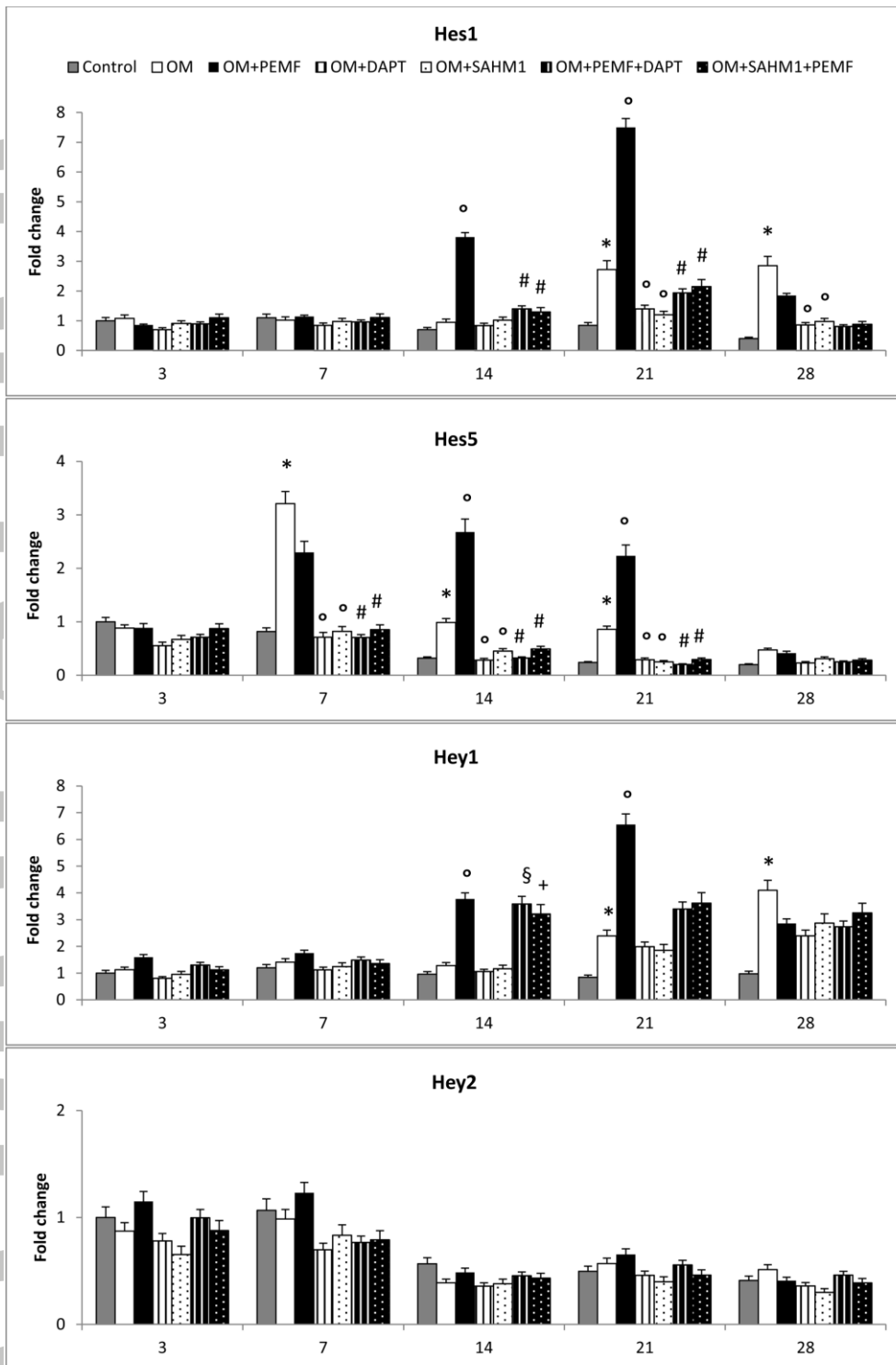


Figure 5. Expression of Notch nuclear target genes Hes1, Hes5, Hey1, Hey2 in hMSCs at different times points (3, 7, 14, 21, 28 days) during osteogenic differentiation. Symbols indicate statistical significance versus control (*), versus OM (°), versus OM+PEMF (#), versus OM+DAPT (§), versus OM+SAHM1 (+), at the corresponding time point.

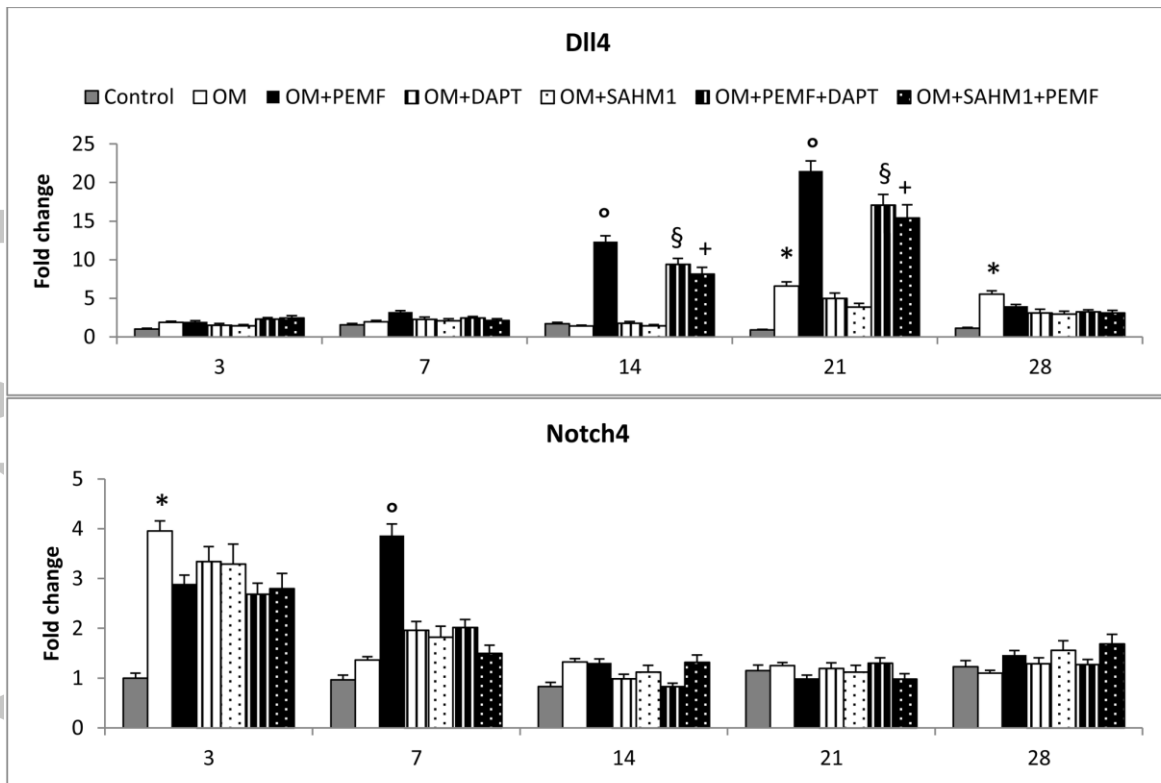


Figure 6. Gene expression of Notch ligand Dll4 and receptor Notch4 in hMSCs at different times points (3, 7, 14, 21, 28 days) during osteogenic differentiation. Symbols indicate statistical significance versus control (*), versus OM (°), versus OM+PEMF (#), versus OM+DAPT (§), versus OM+SAHM1 (+), at the corresponding time point.

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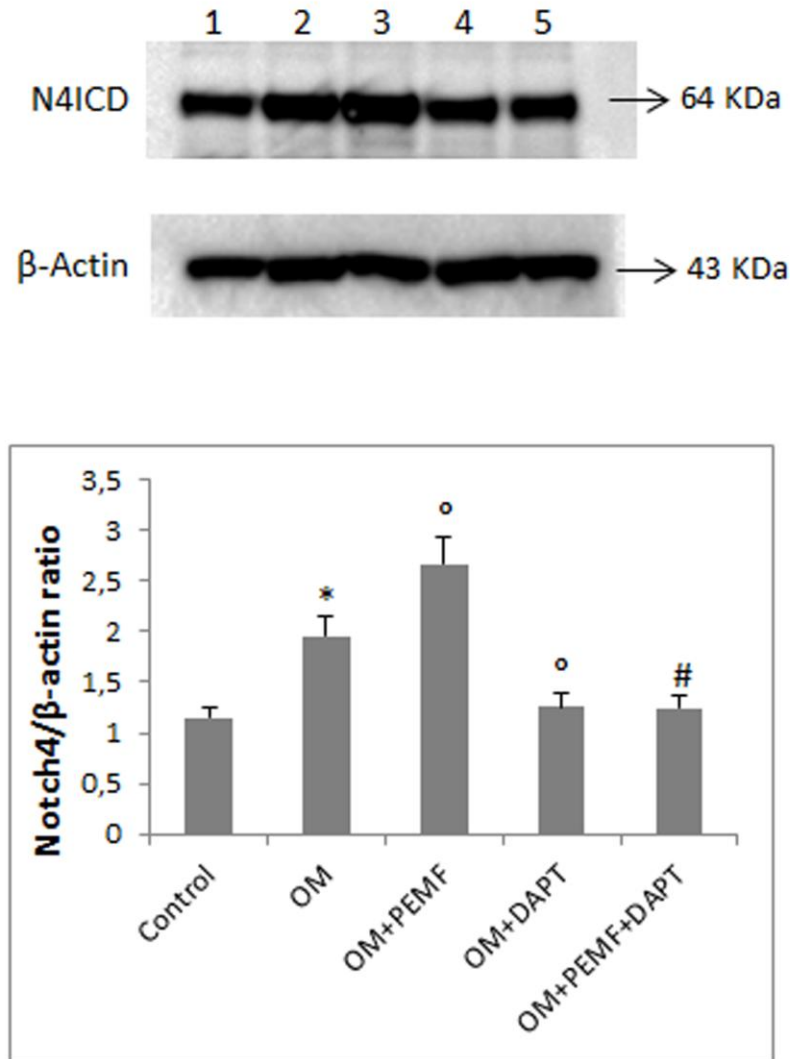


Figure 7. Expression of Notch4 receptor in hMSCs at day 7 in all the experimental conditions: control (1), OM (2), OM+PEMF (3), OM+DAPT (4), OM+PEMF+DAPT (5). Cell lysates were electrophoresed and immunoblotted with antibody for Notch4. β -actin antibody was used to ensure equal loading. The panel (upper) shows the fragment corresponding to the Notch4 intracellular domain (N4ICD). The graph (below) shows densitometric analysis of Western blot assay, to quantify protein levels. Results are expressed as mean \pm SEM of three independent experiments. Symbols indicate statistical significance versus control (*), versus OM (°) and versus OM+PEMF (#).