Original Research Article

Characterization of Notch signaling during osteogenic differentiation in human osteosarcoma cell line MG63†

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ABSTRACT

Osteogenic differentiation is a multi-step process controlled by a complex molecular framework. Notch is an evolutionarily conserved intercellular signaling pathway playing a prominent role in cell fate and differentiation, although the mechanisms by which this pathway regulates osteogenesis remain controversial.

This study aimed to investigate, *in vitro,* the involvement of Notch pathway during all the developmental stages of osteogenic differentiation in human osteosarcoma cell line MG63. Cells were cultured in basal condition (control) and in osteoinductive medium (OM). Notch inhibitors were also added in OM to block Notch pathway.

During osteogenic differentiation, early (alkaline phosphatase activity and collagen type I) and late osteogenic markers (osteocalcin levels and matrix mineralization), as well as the gene expression of the main osteogenic transcription factors (Runx2, Osterix and Dlx5) increased. Time dependent changes in the expression of specific Notch receptors were identified in OM versus control with a significant reduction in the expression of Notch1 and Notch3 receptors in the early phase of differentiation, and an increase of Notch2 and Notch4 receptors in the late phase. Among Notch nuclear target genes, Hey1 expression was significantly higher in OM than control, whilst Hes5 expression decreased. Osteogenic markers were reduced and Hey1 was significantly inhibited by Notch inhibitors, suggesting a role for Notch through the canonical pathway.

In conclusion, Notch pathway might be involved with a dual role in osteogenesis of MG63, through the activation of Notch2, Notch4 and Hey1, inducing osteoblast differentiation and the depression of Notch1, Notch3 and Hes5, maintaining an undifferentiated status.

INTRODUCTION

Osteoblast differentiation is a multi-step process where mesenchymal cells acquire the ability to deposit the mineralized extracellular matrix characteristic of bone tissue (Ducy et al., 2000). Significant progress has been made over the past decade in the understanding of the molecular framework that controls osteogenic differentiation. A large number of morphogens, signaling pathways, and transcriptional regulators have been implicated in regulating bone development, including Wnt/β-catenin, TGFβ/BMP, and Notch signaling pathways (Deng et al., 2008). Notch pathway is an evolutionarily conserved intercellular signaling mechanism that plays a prominent role in cell fate decision during development and maintenance of homeostasis in adults (Bray, 2006). The canonical Notch pathway is activated when Notch receptors (Notch-1, -2, -3, and -4) interact with ligands [Jagged-1 and -2 and Delta-like (Dll-1, -3, and -4)] on adjacent cells, triggering two proteolytic cleavages of the receptor, the last one being performed by the presenilin–γ-secretase complex (Bray, 2006). Notch receptor activation releases the Notch intracellular domain (NICD), which translocates to the nucleus and , through the interactions with DNA-binding CSL protein and the co-activator Mastermind (MAML), activates transcription of Hes (hairy/enhancer of split) and Hey (Hes-related proteins) gene family members. Hes and Hey proteins are basic helix-loop-helix transcriptional regulators including the isoforms of Hes (HES-1, -3, and -5) and Hey (HEY-1, -2, -3, -L) (Iso et al., 2003).

Among the currently identified signaling molecules that play an important role in osteogenic differentiation, it has been reported a role for Notch signaling, although the mechanisms by which this pathway regulates osteogenesis are poorly understood (Deng et al., 2008; Sciaudone et al., 2003; Tezuka et al., 2002). Osteogenic differentiation proceeds through different developmental stages which are characterized by specific markers including early markers such as alkaline phosphatase (ALP), collagen type I and late markers such as osteocalcin and mineralization. Further, several transcriptional factors have been identified as important regulators of osteogenic lineage commitment and terminal differentiation. These factors include Runt-related transcription

factor 2 (Runx2), a master regulator of osteoblast differentiation and chondrogenesis (Komori, 2010) and Osterix, a zinc-finger-containing transcription factor that is essential for bone development and appears to act downstream of Runx2 in osteogenic differentiation (Nakashima et al., 2002). It is known that also other transcription factors including Dlx5 are involved and regulate osteogenic differentiation (Komori, 2006).

It remains controversial whether Notch signaling acts as a positive or negative regulator of osteogenic differentiation in osteoblast progenitor cells. It has been reported that overexpression of Hes-1 or Hey-1 enhances osteogenic differentiation of mesenchymal stem cells (Sharff et al., 2009) in part through positive regulation of and cooperation with Runx2, suggesting that Notch signaling may play positive roles in bone formation. On the other hand, presenilin-2 null mice have greatly increased trabecular bone mass, and Hey1 was shown to inhibit Runx2 transcriptional activity in mouse MC3T3 and C2C12 cells, suggesting the negative role of Notch signaling in osteogenesis (Zamurovic et al., 2004). Also, very few studies have investigated the role of specific Notch receptor on osteogenic differentiation and there are controversial data about Notch1 receptor (Deregowski et al., 2006; Shimizu et al., 2009; Tezuka et al., 2002). Therefore, further examination of the role of Notch signaling in regulating osteogenesis and bone formation is required.

To date no study has characterized the Notch pathway during all the developmental stages of osteogenic differentiation including proliferation, extracellular matrix synthesis, and mineralization. The aim of this study was to investigate *in vitro* the involvement of Notch pathway during all the phases of osteogenic differentiation in human osteosarcoma cell line MG63. MG63 cells were chosen because they have been well characterized as immature osteoblasts and have been applied as a tool for studying differentiation processes (Kraus et al., 2012). In order to study the role of Notch signaling during osteogenic differentiation, MG63 were cultured in basal condition and in osteoinductive medium. 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 prevents the formation of NICD active form, was

also used in order to confirm the involvement of Notch pathway during osteogenic differentiation. Furthermore, SAHM1, a peptide mimetic of a dominant negative form of MAML1, that specifically inhibits canonical Notch transcription complex formation (Ashley et al., 2015; Kornilova et al., 2003; Moellering et al., 2009) was also used to block Notch pathway and investigate possible effects on osteogenic differentiation.

To our purpose, classical biochemical markers of osteogenic differentiation such as ALP activity, osteocalcin levels and mineralized bone nodules as well as the gene expression of the main osteogenic transcription factors (including Runx2, Osterix and Dlx5) and of the osteogenic gene collagen type I (Coll I), were analyzed to verify the behavior of the cells in the three experimental conditions tested (basal condition, osteoinductive medium in the absence and in the presence of DAPT or SAHM1). The characterization of Notch pathway during osteogenic differentiation was done by evaluating, in all the conditions, gene expression of Notch receptors (Notch1, Notch 2, Notch3, Notch4), their ligands (Jagged1, Dll1, Dll4) and nuclear target genes (Hey1, Hey2, HeyL, Hes1, Hes5) at different time points.

A better understanding of molecular mechanisms behind osteogenic differentiation would not only help us to identify pathogenic causes of bone and skeletal diseases but also lead to the development of targeted therapies for these diseases.

MATERIALS AND METHODS

Cell Cultures

Human osteosarcoma cell lines, MG63 were purchased from the ATCC (American Type Culture Collection, by LGC Standards S.r.l Sesto S.Giovanni (MI) Italy). Cells were grown in high glucose Dulbecco's modified Eagle's medium/Nutrient mixture F-12 (DMEM/F12) supplemented with 4 mM L-glutamine, 10% FBS, penicillin ($100Uml^{-1}$) and streptomycin ($100\ \mu gml^{-1}$) (complete DMEM/F12) (all purchased by GIBCO Life Technologies, Monza, Italy).

All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Osteogenic Differentiation

Cells were seeded in 4-well plate (for ALP activity, osteocalcin and Alizarin Red staining assays), 96-well plate (for cell proliferation assay) or 35 mm diameter cell culture dishes (for RealTime-PCR) at 2×10^3 cells/cm^2 in Osteogenic Differentiation Medium (OM) (Lonza, Walkersville, Maryland, USA) for 28 days (Ongaro et al., 2014). Control cells were cultured in basal condition (i.e. complete DMEM/F12). Medium was changed twice per week. Samples were harvested at days 1, 3, 7, 14, 21 and 28 for assays.

Cell Proliferation

Cell proliferation was assessed by Prestoblue Cell Viability Reagent (Invitrogen by Life Technologies, Monza, Italy). PrestoBlue Cell Viability Reagent solution was added to each well for all experimental conditions, at days 1, 3, 7, 14, 21 and 28 followed by incubation for 1 h. The cell absorbance values were measured at a wavelength of 570 nm with the correction at 620 nm using Sunrise microplate reader (TECAN, Cernusco sul Naviglio Milano, Italy).

Notch Inhibitors Treatment

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 at the final concentration of 10 μM (Ashley et al., 2015) to MG63 cultured in OM during the whole differentiation period. In the same experiments, we also evaluated the effect of DAPT by treating cells for a limited periods at the beginning (first week) and at the end (last week) of differentiation.

Alkaline Phosphatase (ALP) Activity

At the time points investigated, MG63 were washed with PBS and lysed in non-denaturant conditions by using 0.1% Triton X 100 (Sigma-Aldrich) in double-distilled H_2O followed by three times freezing and thawing of the membrane fractions at -20°C/25°C. ALP activity was determined by incubating cellular lysates at 37°C for 30 minute in the presence of 10 mM p-nitrophenylphosphate (Sigma-Aldrich) in alkaline buffer containing 100 mM diethanolamine and

0.5 mM MgCl2, pH 10.5 (Sigma-Aldrich). The reaction was stopped with 0.4 M NaOH and the absorbance of each sample was read at 405 nm with a Sunrise microplate reader. ALP activity was normalized to total protein quantity measured using the bicinchoninic acid assay, a protein assay reagent kit (QuantumProtein by Euroclone) according to the manufacturer's instructions. ALP activity was expressed as µMol/(min × g protein).

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

MG63 cells were washed three times with PBS. Then 0.5 M HCl solution was added to each well followed by incubation for 30 min at 37°C, and neutralized by 1 M NaOH. The extracts obtained from cell monolayer were analysed using commercial ELISA kit (Invitrogen by Life Technologies) which uses monoclonal antibodies directed against distinct epitopes of human osteocalcin. We evaluated the intracellular levels of osteocalcin in order to avoid possible interference of serum or other medium components with the measurement of osteocalcin released in the medium (Ongaro et al., 2014). Osteocalcin levels were expressed as ng osteocalcin/mg protein.

Alizarin Red S Staining

MG63 were washed in PBS and fixed in 4% formalin for 10 min. The formalin solution was removed and after washing with distilled water, an Alizarin Red S solution (1%) (Histo-Line Lab. S.r.l, Milano, Italy) was added for 5 min. 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 minutes, liquid was transferred into cuvettes and the quantity of dissolved Alizarin red was measured by the spectrophotometer (Jenway 6305, Vetrotecnica, Padova, Italy) at a wavelength of 450 nm.

Real-Time PCR

For RNA extraction, MG63 were washed three times with Earle's Solution and total RNA was extracted using commercially available kit (PureLink RNA minikit Invitrogen by Life Technologies). RNA concentration and purity were determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, Inc., MA, USA). 2 µg 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 32 ng of the cDNA mixture were 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 Notch1, Notch2, Notch3, Notch4, Jagged1, Dll4, Hey1, Hey2, Hes1, Hes5, Runx2, Dlx5, Osterix, Coll I 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). A concentration of 500 nM of primers (all from IDT Tema Ricerca, Bologna, Italy) was used. The sequences of primers used for Notch (Caliceti et al., 2013; Rizzo et al., 2008) and other genes 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 reference gene of control cells (grown in DMEM/F12) at day 1.

Western Blotting and Densitometric Analysis

Western blot analysis was carried out to detect Notch1, Notch2, Notch3, Notch4 and β-actin at day 21, by using the corresponding antibodies. Antibodies to Notch1 (C-20) and Notch4 (H-225) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody to Notch3 (ab23426) was from Abcam (Cambridge, UK). Antibody to cleaved Notch1, valine 1744 (4147) was from Cell Signaling Technology (Beverly, MA, USA). Notch2 antibody (clone C651.6DbHN) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Department of Biology, Iowa City, IA 52242). β -actin antibody was from Sigma Aldrich (St. Louis, MS, USA). Western blotting was performed as previously reported by Caliceti et al. (Caliceti et al., 2013). Protein immunoreactive bands were

analyzed and quantitated by using 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.

Statistical Analysis

All the experiments (n=5) were performed in triplicate. Data were expressed as means ± SEM. Statistical differences between the mean were determined by Student's t test. 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 and OM + DAPT versus OM, at the different time points investigated (1, 3, 7, 14, 21, 28 days), as reported in figure legends.

RESULTS

Biochemical results in MG63 cell line

The osteogenic biomarkers analyzed in MG63 during osteogenic differentiation indicated the capability of these cells to differentiate into mature osteoblasts as shown by the analysis of ALP activity, osteocalcin (OC) production and calcium salts deposition (Figure 1). Specifically, ALP activity significantly increased at day 7 and day 14 in OM versus control (Figure 1A). OC was detectable in our cultures in OM from day 14 and it increased significantly until day 28 when compared to control cells (Figure 1B). The matrix mineralization analyzed by Alizarin red staining confirmed a trend similar to OC, showing an increase of calcium deposits mainly at the end of the differentiation period (Figure 1C). To verify if the changes observed in OM were due to differences in cell number, we also tested cell proliferation both in control and in OM treated cells and we found no significant differences between the two groups of cells (Figure 1D).

Gene expression of osteogenic genes

The analysis of the expression of the osteogenic transcription factors showed that both Runx2 and Dlx5 significantly increased during the differentiation from day 7 to day 28 when their expression

in OM was compared to control cells (grown in DMEM/F12 at day 1) (Figure 2A-B). Differently, the expression of Osterix was significantly increased only at day 7 in OM with respect to control (2.25 fold, p=0.008) (Figure 2C). In addition to osteogenic transcription factors, we investigated also Coll I, a matrix gene typically expressed in osteoblasts. Coll I was more expressed in OM than in control at all time-points (Figure 2D) with significant differences from day 3 until day 28.

Gene expression of NOTCH pathway genes

The analysis of Notch related genes showed different results among Notch receptors, ligands and nuclear target genes. As far as concerns Notch receptor genes, a significant reduction in the expression of Notch1 and Notch3 at day 7 and 14 and an increase of Notch2 and Notch4 at day 21 and 28 were found, when OM was compared versus control (Figure 3A-D). Differently, no significant changes in the expression of Notch ligands (Jagged1, Dll1 and Dll4) were observed, comparing OM versus control (Figure 3E-G). Among the Notch nuclear target genes investigated, during osteogenic differentiation significant variations were obtained for Hey1 and Hes5, comparing OM versus control (Figure 3H-L). Specifically, Hey1 expression was significantly higher in OM compared with control from day 7 until day 28, with a maximum increase at day 21 (7.60 fold, $p<0.0001$). Hes5 expression decreased during differentiation in OM respect to control with significant differences at day 14 (0.37 fold, p=0.025) and 21 (0.33 fold, p=0.011). Differently, no significant change was observed for HeyL, Hey2 and Hes1.

Effects of Notch inhibitors

The presence of both Notch inhibitors in OM had significant effects on osteogenic biomarkers and genes. In particular, DAPT decreased ALP activity at all the time points with significant reduction from day 14 until day 28 (Figure 4A). At the same time points, DAPT determined also a significant decrease in OC levels with a complete inhibition of OC production at day 14 and 21 during differentiation (Figure 4B). A similar inhibition was induced also by SAHM1, both on ALP activity and OC with a further stronger reduction for OC, at day 28 (-84% SAHM1 vs OM; -36% DAPT vs

OM). Similar results were obtained by Alizarin red staining as calcium deposits were reduced in the presence of DAPT and of SAHM1 (Figure 4C). The reduction was more appreciable in the middle-late stage of differentiation as shown in Figure 4C, reporting also the spectrophotometric measure of the dissolved Alizarin red staining, with a stronger effect at day 28 due to SAHM1 (- 90% vs OM) respect to DAPT (-28% vs OM). However, the reduction of osteogenic biomarkers found in the presence of Notch inhibitors was independent from the cell number because neither DAPT nor SAHM1 significantly influenced cell proliferation at any time point investigated (Figure 4D).

In the presence of Notch signaling inhibition with either DAPT or SAMH1 changes in the specific osteogenic transcription factors were observed (Figure 5). A reduction of Runx2 was found at all time-points, but with significant value only at day 28 for both the inhibitors (Figure 5A). Differently, Dlx5 was influenced at the early stage of differentiation in a similar way by DAPT and SAHM1, decreasing significantly its expression at day 3 and day 7 (Figure 5B). The expression of Osterix was inhibited at the day 7 and 14 by both the inhibitors and also at day 21 by SAHM1 (Figure 5C). Also the expression of Coll I was reduced in the presence of DAPT and SAHM1 in OM compared with OM alone, with significant decrease from day 3 until day 28 (Figure 5D).

Furthermore, when Notch pathway was blocked by DAPT or SAHM1, a significant reduction in Hey1 expression was observed at all the differentiation times lowering the Hey1 levels as in control (Figure 6) indicating that the activation of Hey1 occurred mainly by the canonical Notch signaling. No effect of Notch inhibitors on the mRNA expression of the other nuclear target genes investigated neither on Notch receptors and ligands was observed.

When we investigated the effect of DAPT in limited periods, at the beginning (first week) and at the end (last week) of differentiation, we found that the use of DAPT for the first week did not provoke any significant inhibition neither on osteogenic markers and transcription factors, nor on Hey1 gene expression. Differently, the use of DAPT during the fourth week, significantly inhibited ALP activity (-39% vs OM), OC levels (-63% vs OM), Coll I expression (0.25 fold vs OM) and Hey1

(0.19 fold vs OM) at day 28. No significant inhibition was observed on Runx2, Dlx5 and Osterix expression when DAPT was added to the osteogenic medium only in the last week of differentiation (data not shown).

The effects of DAPT treatment on MG63 during osteogenic differentiation were verified also by Western blotting analysis of Notch receptors (Figure 7). The immunoblots reported in Figure 7 show the pattern of Notch receptors in lysates from MG63 cultured in control and in OM in the absence and in the presence of DAPT at day 21, the time-point at which greater differences were observed in gene expression, at least for Notch2 and Notch4.

The results confirmed the presence of bands corresponding to all Notch receptors (Figure 7). However, differences in the intensity of the bands, between cells treated or untreated with DAPT, were found in OM only for Notch 2 and Notch 4, whilst no changes were observed for Notch1 and Notch3. Western blot analysis utilizing an antibody (C-20) directed against the C-terminus of the Notch1 protein, showed that under the conditions tested, DAPT did not modified the intensity of a ~ 110 KDa band which represents the transmembrane form of Notch 1 (TM) receptor (Caliceti et al., 2013) neither in control, nor in OM (Figure 7). Further, the antibody specific for Notch1 cleaved at valine 1744, the active form of Notch1, did not detect any band (Figure 7), suggesting low steady-state levels of active Notch1 under our experimental conditions, or an alternative γ-secretase cleavage site.

Notch2 immunoblotting of cell lysates showed Notch2 trans-membrane and intracellular fragment (TMIC) of ~90 KDa (Figure 7), which intensity was significantly increased in OM respect to control, as confirmed by densitometric analysis. The presence of DAPT in MG63 resulted in an accumulation of faster migrating product, similar to Notch2 extracellular truncation (N2EXT) cleavage fragments previously described (Groot et al., 2014). Groot et al., in fact, reported that the activation of Notch2 receptor by ligands results in a reduction of TMIC fragment and in the appearance of a faster migrating product that accumulated when γ-secretase activity was blocked

using γ-secretase inhibitor. Accordingly, the comparison of our immunoblotting results in the presence and in the absence of DAPT suggests in OM, for Notch2, the accumulation of faster migrating product when cells were treated with DAPT suggesting an involvement for active Notch2 receptor during osteogenic differentiation.

Similarly to what observed for Notch1, we did not find differences in the immunoblotting pattern for Notch3 transmembrane form in the absence or presence of DAPT, neither in control nor in OM (Figure 7).

As far as concerns Notch4, the immunoblotting of cell lysates with an antibody against the C-terminus of Notch4 protein, permitted to detect a fragment of 64 KDa (Figure 7) corresponding to the active form of Notch4 (N4ICD), as previously reported (Caliceti et al., 2013). N4ICD significantly increased in OM respect to control, as confirmed by densitometric analysis. In OM, the treatment with DAPT significantly reduced the expression of Notch4 active form compared with the treatment with OM alone, suggesting an active role for this receptor during osteogenic differentiation of MG63.

DISCUSSION

The present study reveals several novel findings concerning the significant role of Notch signaling during osteogenic differentiation. In spite of previous *in vitro* and *in vivo* studies investigating the role of Notch pathway in osteogenic differentiation, however to date no definitive and clear conclusions can be driven (Chen et al., 2014). Studies reported that *in vivo* or *in vitro* overexpression of NICD suppresses osteoblastic differentiation and bone formation (Zanotti et al., 2008), however, some controversy on the effect of Notch pathway on osteoblastic maturation exists, and both inhibitory (Bai et al., 2008; Zamurovic et al., 2004) and stimulatory (Nobta et al., 2005; Tezuka et al., 2002) effects on osteoblast differentiation and function have been reported. On the other hand, most previous studies generally investigated the possible involvement of Notch pathway by experimentally inducing overexpression or loss of function in specific and single Notch

receptors or Notch related genes and generally the effects were evaluated only in early phases of differentiation process.

In this study, we aimed to characterize the Notch signaling pathway by analyzing the expression of all four Notch receptors, some ligands and the main nuclear target genes during the whole period of differentiation in MG63 cells, used as an *in vitro* cellular model of differentiation. During the osteoinductive treatment, MG63 showed the ability to differentiate, as shown by the increase in the production of early (ALP activity, Coll I) and later osteogenic markers (OC, matrix mineralization). Further, as expected, differentiation was associated to the increased expression of known specific osteogenic transcription factors (Runx2, Dlx5, Osterix).

Interestingly, the analysis of the expression of Notch related genes has shown changes in the expression of specific Notch receptors and nuclear target genes at different times during differentiation, confirming the involvement of this pathway in the process. Specifically, in the osteoinductive condition a significant increase of Notch2 and Notch4 receptors was observed respect to untreated control cells in the later stage (days 21-28) of differentiation, whilst an underexpression of Notch1 and Notch3 receptors was found in the middle stage (days 7-14). These results are partially confirmed in literature. In fact, a reduced expression of Notch1 and Notch3 was previously reported by Zamurovic et al. (Zamurovic et al., 2004) in the osteogenic differentiation of MC3T3, a non-transformed mouse calvarial cell-line, induced by bone morphogenetic protein (BMP)-2, although the investigation was only until day 3. Viale-Bouroncle (Viale-Bouroncle et al., 2014) reported that Notch1 signaling regulates the BMP2/Dlx3 directed osteogenic differentiation of dental follicle cells via a negative feed-back loop, with a consequent reduction of ALP activity and mineralized nodules formation. Further, it has been reported that Notch1 maintained bone marrow mesenchymal progenitors in a stem-like state by suppressing Runx2 activity *in vitro* and *in vivo* (Hilton et al., 2008) and that the activity of Notch1 was suppressed during the induction of osteogenic differentiation, as manifested by the decreased expression of cleaved Notch1 and Hes1 (Li et al., 2014).

Although only few studies have investigated Notch2 and Notch4 receptors during osteogenic differentiation and none in our cellular model, however data reported in human alveolar bone-derived osteoprogenitor cells (Chakravorty et al., 2014) are in line with the increase of Notch2 and Notch4 expression found in our results.

Our study, investigating all Notch receptors and Notch related genes during the whole period of osteogenic differentiation, appears to clarify and add new information indicating a complex involvement of Notch pathway in osteogenic differentiation with temporally dependent expression of specific genes. In particular, the increase in Notch 2 and Notch4 expression (at day 21 and 28) suggest that they are mainly involved in supporting differentiation, whilst the decrease in Notch1 and Notch3 expression seems to indicate a limited involvement of these two receptors in the progression of differentiation, confirming, at least partially, the role previously suggested for Notch1 in maintaining a stem-like state of the cells (Hilton et al., 2008). Our data showed that the differences in Notch receptors expression were also related to specific changes in Notch nuclear target genes, further supporting the involvement of Notch pathway. Among the nuclear target genes, we found the upregulation of Hey1 (from day 7 until day 28) and the downregulation of Hes5 (at day 14-21) during differentiation (Figure 8). Notably, the increase of Hey1 expression was temporally associated with the enhancement of both early (ALP, Coll1) and late (osteocalcin, matrix mineralization) osteogenic markers, as well as of the known osteogenic transcription factors (Runx2, Dlx5, Osterix) expression. Although with the limitations of this study, which is mainly based on observational data, this correlation suggests a significant role for Hey1 during osteogenesis. On the other hand, a similar role of Hey1 has been also shown in other studies performed on mesenchymal stem cells and C2C12 cell line under BMP2 or BMP9 stimulation (de Jong et al., 2004; Sharff et al., 2009). However, in those studies Hey1 was investigated as a target gene modulated by BMPs stimulation. Differently, in our study Hey1 was investigated within Notch pathway, in association with the analysis of Notch receptors and ligands and its expression resulted

increased during the whole differentiation process, also independently from the stimulation with BMPs.

Indeed, the correlation between Notch pathway and BMP signaling and the molecular mechanism behind the Hey1 functional role in osteogenesis remains to be defined, as controversial data have been previously reported (de Jong et al., 2004; Sharff et al., 2009; Zamurovic et al., 2004).

To our knowledge, no previous data have been reported about Hes5 expression during osteogenic differentiation, however, findings in agreement with the trend found in our results have been reported in chondrogenic differentiation, showing a higher expression of Hes5 in dedifferentiated chondrocytes and a lower expression in differentiated cells, indicating that Hes5 may function as a negative regulator of cartilage differentiation in humans (Karlsson et al., 2007). Further, other pathways besides Notch can also be involved in Hes5 regulation, as it has been shown for Hes1 (Curry et al., 2006).

As the γ-secretase-mediated cleavage is the rate-limiting step of initiating Notch signaling, γ-secretase inhibitors, such as DAPT, have been frequently used as the effective research tools in uncovering novel functions of the Notch signaling.

In order to better explore the involvement of Notch pathway during osteogenic differentiation, we used two different inhibitors which block Notch pathway in two different points: DAPT, which inhibits γ-secretase or SAHM1, which inhibits the NICD-MAML-CSL transcription complex formation. Both DAPT and SAHM1induced a similar decrease in the osteogenic markers investigated, as well as in Hey1 expression. These results seem to indicate a direct involvement of Notch transcriptional activity during osteogenic differentiation. The inhibition of Hey1 gene expression not only by DAPT but also by SAHM1, reinforced an interaction of Hey1, among Notch target genes, to drive osteogenesis. Although data obtained in the presence of DAPT and SAHM1 suggest that Notch signaling is directly involved in osteogenic differentiation, however, we cannot exclude cooperation between Notch and other pathways such as BMPs/Smads signaling.

Further, to evaluate a possible time-dependent involvement of Notch signaling during osteogenic differentiation, the inhibition of Notch pathway was analyzed also in specific stages (early and later) during differentiation. As DAPT and SAHM1 induced similar results, only DAPT was used as Notch inhibitor. The results showed no effect of DAPT if used only during first week of differentiation. Differently, when cells were treated with DAPT for the last week, a reduction of ALP activity, OC levels, Coll I and Hey1 expression was found, suggesting a role of Notch signaling particularly during the middle-late stage of differentiation.

Also, Western blotting analysis in DAPT confirmed the inhibition of the activation of Notch2 and Notch4, supporting the involvement of these receptors activation in mediating Hey1 expression. Accordingly, the activation of Hey1 downstream Notch2 and Notch4 has been previously reported in endothelial cells (Quillard et al., 2010, 2009).

Moreover, the absence of the active form of Notch1 and no change in the activated form of Notch3 in the absence and in the presence of DAPT, appear to suggest a minor role for these receptors. Anyway, we cannot exclude that these data could reflect the requirement for higher concentration of DAPT to inhibit the Notch receptors (1 and 3) involved in transcription of Hes5 (Chen et al., 2012). As with the progression of differentiation, we observed an underexpression of Notch1, Notch3 and Hes5, we suggest that these genes may be mainly involved in maintaining cells in an undifferentiated status.

In conclusion, the results obtained in this study confirm a role for Notch pathway in favoring osteogenic differentiation through the induction of Notch transcriptional activity. Data collected also suggest that Notch might be involved with a dual role in osteogenesis of MG63, with the induction of osteoblast differentiation through the activation of Notch2, Notch4 and the Notch target gene Hey1 and, on the other hand, with the maintaining of an undifferentiated status through the depression of Notch1, Notch3 and Hes5. Although further investigations are required to better clarify the molecular mechanism behind Notch pathway involvement, however, the increase of

knowledge in major signaling pathways which contribute to regulate osteogenesis is of great relevance. In clinics, the ability to manipulate osteogenesis has far-reaching clinical potential for pathological conditions such as osteoporosis, osteogenesis imperfecta, osteolytic lesions in metastatic cancers, primary bone tumors and bone regeneration.

Conflict of Interest

The Authors state that they have no conflicts of interest.

Authorship

AO, Study conception and design, data analysis and interpretation, writing and revising manuscript, approving final version and submission of the manuscript; AP, LB, CC, data collection, data analysis, revising manuscript content, approving final version of the manuscript; PR, data analysis and interpretation, revising manuscript content, approving final version of the manuscript; LM, revising manuscript content, approving final version of the manuscript; MDM, data interpretation, discussing and revising manuscript content, approving final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Biochemical markers during osteogenic differentiation of MG63 cells. Cells were cultured in DMEM/F12 (control, white bar) or in OM (grey bar) for 28 days. At different time points (1, 3, 7, 14, 21, 28) cells were tested for ALP activity (A), osteocalcin levels (B) and matrix mineralization by Alizarin red (C). The quantification of Alizarin red staining was done spectrophotometrically and reported in the graph under the panel. To verify if the changes observed in OM were due to differences in cell number, cell proliferation was evaluated both in control and in OM treated cells (D). *Statistical significance versus control, at the corresponding time point.

Figure 2. Expression of osteogenic transcription factors Runx2 (A), Dlx5 (B), Osterix (C) and expression of collagen type I (D) in MG 63 at different times during osteogenic differentiation. Cells were cultured in DMEM/F12 (control, white bar) or in OM (grey bar) for 28 days. *Statistical significance versus control, at the corresponding time point.

Figure 3. Expression of Notch receptors, ligands and nuclear target genes in MG63 at different times during osteogenic differentiation. Cells were cultured in DMEM/F12 (control, white bar) or in OM (grey bar) for 28 days. (A) Notch1 receptor; (B) Notch2 receptor; (C) Notch3 receptor; (D) Notch4 receptor; (E) Jagged1; (F) Dll1; (G) Dll4; (H) HeyL; (I) Hey1; (J) Hey2; (K) Hes1; (L) Hes5. *Statistical significance versus control, at the corresponding time point.

Figure 4. Effects of DAPT or SAHM1 treatments on osteogenic biochemical markers and cell proliferation in MG63 at different times during osteogenic differentiation. Cells were cultured in OM (light grey bar), in OM+DAPT (black bar) or in OM+SAHM1 (grey bar) for 28 days. The presence of DAPT or SAHM1 in OM reduced ALP activity (A), osteocalcin levels (B) and matrix mineralization by Alizarin red (C). The quantification of Alizarin red staining was done spectrophotometrically and reported in the graph under the panel. DAPT neither SAHM1 did not influence cell proliferation (D). °Statistical significance versus OM, at the corresponding time point.

Figure 5. Effects of DAPT or SAHM1 treatments on osteogenic transcription factors Runx2 (A), Dlx5 (B), Osterix (C) and on collagen type I (D) in MG63 at different times during osteogenic differentiation. Cells were cultured in OM (light grey bar), in OM+DAPT (black bar) or in OM+SAHM1 (grey bar) for 28 days. °Statistical significance versus OM, at the corresponding time point.

Figure 6. Effects of DAPT or SAHM1 treatments on Hey1 in MG63 at different times during osteogenic differentiation. Cells were cultured in OM (light grey bar), in OM+DAPT (black bar) or in OM+SAHM1 (grey bar) for 28 days. DAPT significantly inhibited Hey1 expression at all differentiation times investigated. °Statistical significance versus OM, at the corresponding time point.

Figure 7. Effects of DAPT treatment on Notch receptors in MG63 at day 21 in basal condition and during osteogenic differentiation. MG63 were cultured in DMEM/F12 without (C) and with DAPT (C+D) and in OM and in OM+DAPT (OM+D) until day 21. Cell lysates were electrophoresed and immunoblotted with antibody for Notch1, Notch2, Notch3, Notch4. β-actin antibody was used to ensure equal loading. The panel shows, for each Notch receptor, the transmembrane (TM) and/or intracellular (IC) fragment. Extracellular truncation cleavage fragments were present for Notch2 (N2EXT), and for Notch3 (N3EXT), as described in the Results section. Densitometric analysis of Western blot assay quantified protein levels. Results are expressed as mean ±SEM of three independent experiments. °Statistical significance versus OM. *Statistical significance versus C and C+D.

Figure 8. Notch pathway. The Notch cascade consists of Notch receptors and ligands, as well as intracellular proteins transmitting the Notch signal to the cell's nucleus. In mammalian cells, there are four different Notch receptors, referred to as Notch1, Notch2, Notch3, and Notch4. Notch receptors are large single pass Type I transmembrane proteins. The extracellular domain of all Notch proteins mediates interactions with ligand. Binding of the ligand on one cell to the Notch receptor, on another cell, results in two proteolytic cleavages of the receptor. The ADAM10 or

TACE (TNF-α converting enzyme) metalloprotease catalyses the S2 cleavage, generating a substrate for S3 cleavage by the γ-secretase complex. This proteolytic processing mediates release of the Notch intracellular domain (NICD), which 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 target genes is induced.

During osteogenic differentiation of MG63, an increase of gene expression of Notch2 and Notch4 and a reduction of Notch1 and Notch3 has been observed. Among the target genes, the expression of Hey1 is enhanced during osteogenic differentiation, whereas Hes5 is decreased.

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′-AGCCAGGCAAGCGACAA-3′
Notch3	5′-TGCGATCAGGACATCAATGAC-3′	5-CTCAGGCACTCATCCACATC-3′
Notch4	5′-CAACTGCCTCTGTCCTGATG-3′	5′-GCTCTGCCTCACACTCTG-3′
Jagged1	5′- GACTCATCAGCCGTGTCTCA-3′	5′-TGGGGAACACTCACACTCAA-3′
Dll1	5′-CAGCAAGCGTGACACCAAGT-3′	5′-TTCAGATGCTTCTCCACCCCTG-3′
Dll4	5′-GCGAGAAGAAAGTGGACAGG-3′	5′-ATTCTCCAGGTCATGGCAAG-3′
Hey1	5′-CCGAGATCCTGCAGATGACCGT-3′	5′-AACGCGCAACTTCTGCCAGG-3′
Hey2	5′-AAAAGGCGTCGGGATCG-3′	5′-AGCTTTTTCTAACTTTGCAGATCC-3′
HeyL	5′-AAGAGGGCCAGCTGAGCCAGA -3′	5′-GATGCGGTCTCGACGCCGTT-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′-TGAGGAGGAAGTTCACTATG-3′	5′-CATTAGTGCTTGTAAAGGGG-3′
Coll1	5′-GCTATGATGAGAAATCAACCG-3′	5′-TCATCTCCATTCTTTCCAGG-3′
GUSB	5′-CCCGCGGTCGTCATGTGGTC-3′	5′-GCCGGGAGGGGTCCAAGGAT-3′

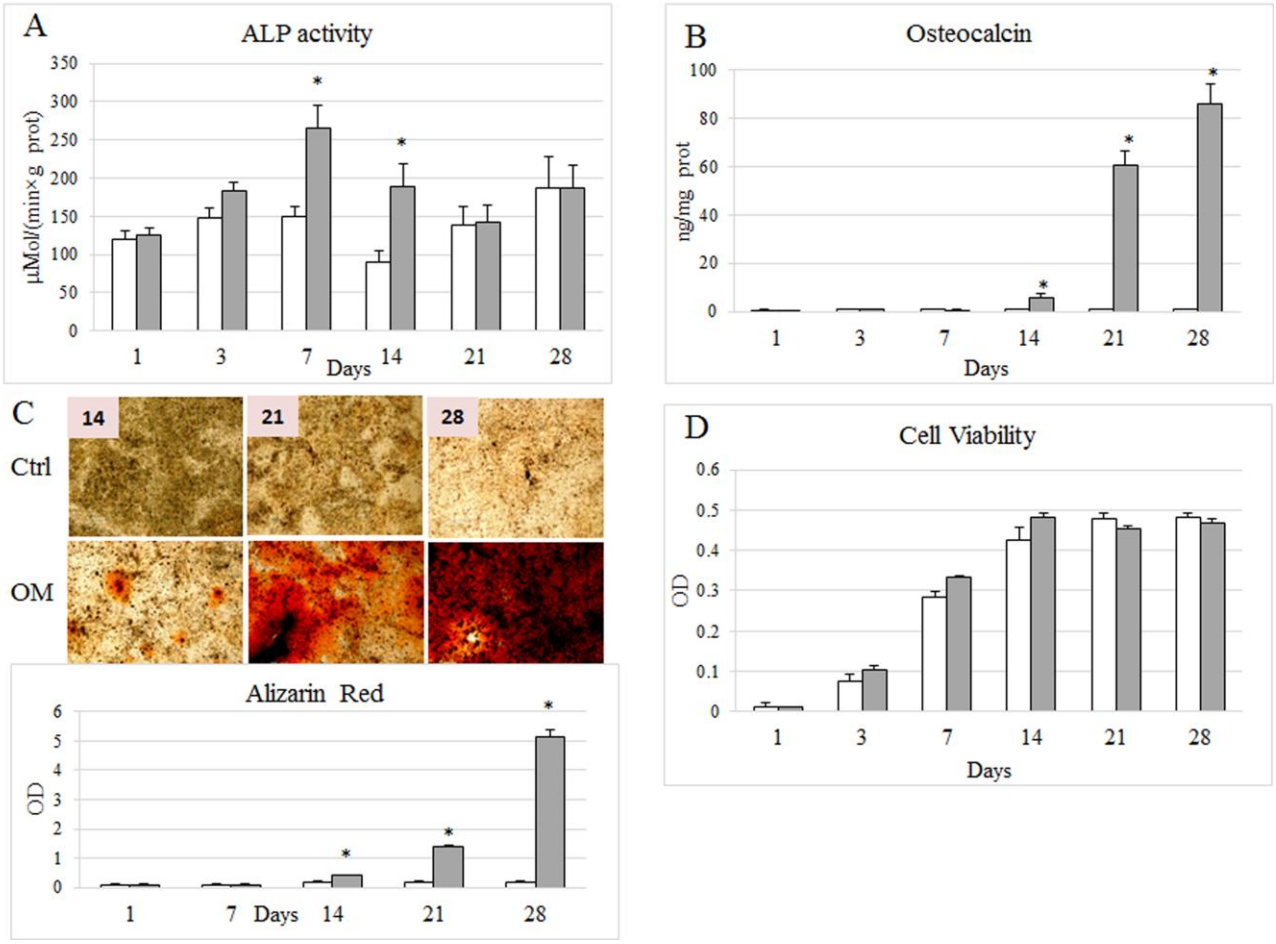


Figure 1

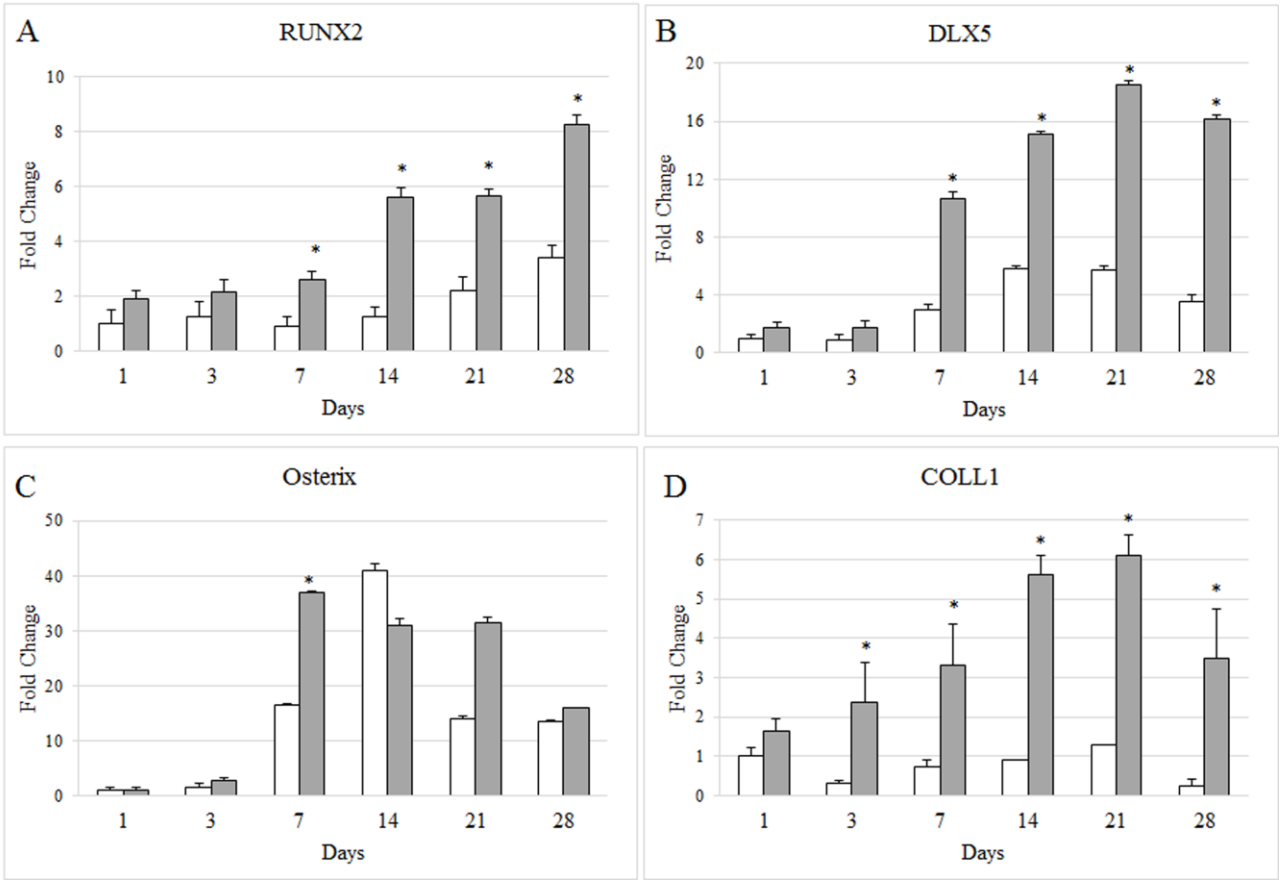


Figure 2

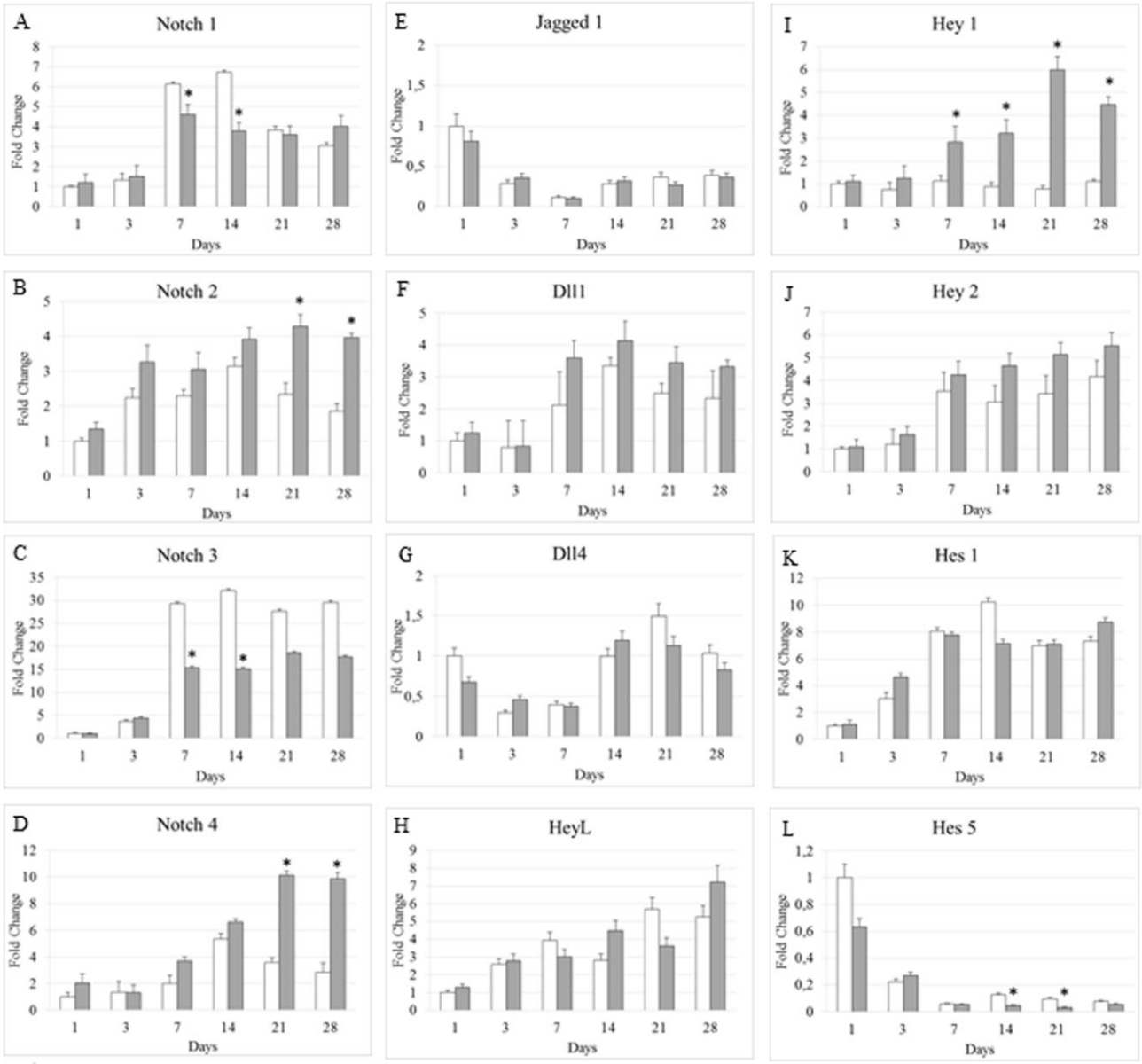


Figure 3

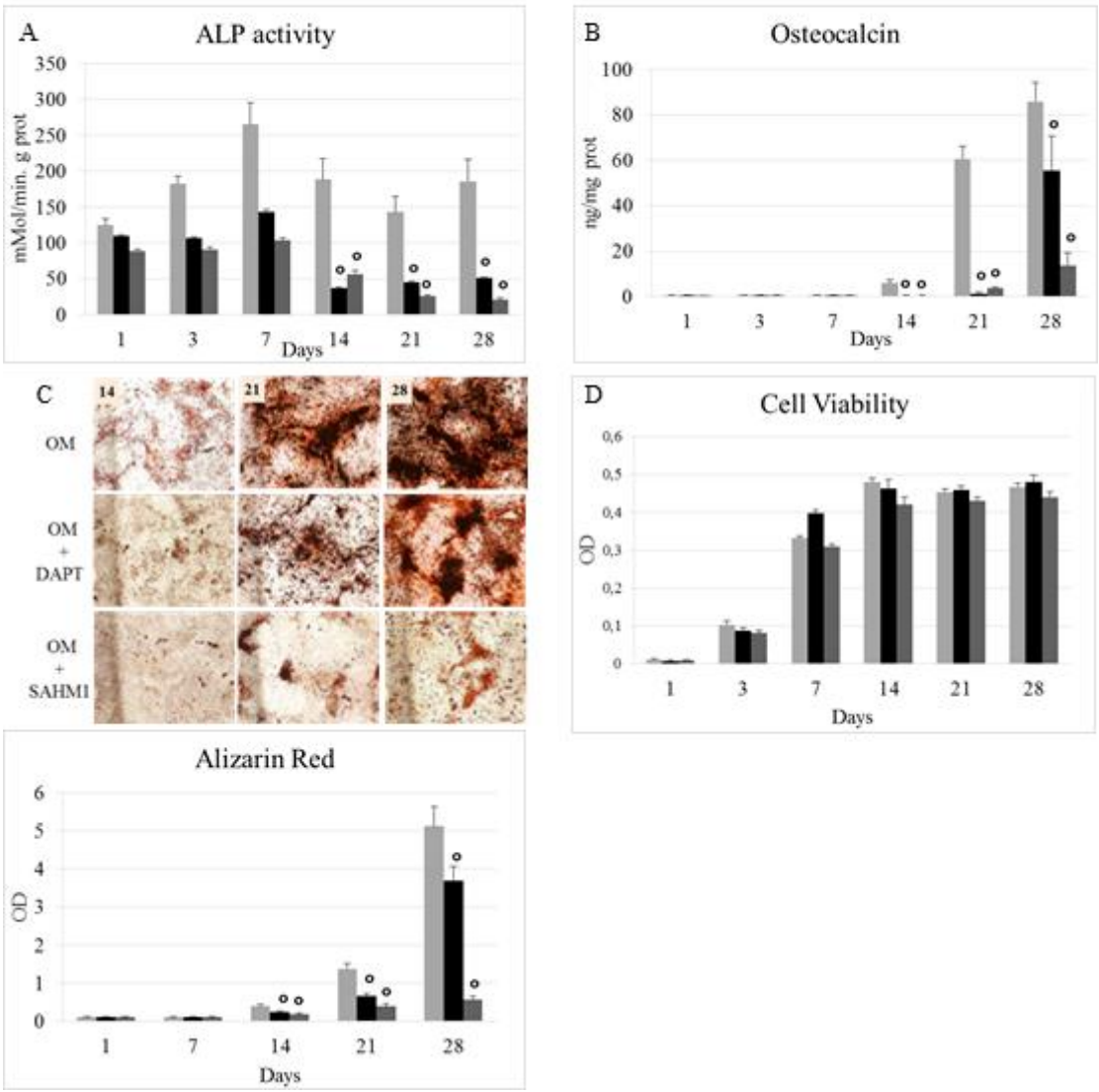


Figure 4

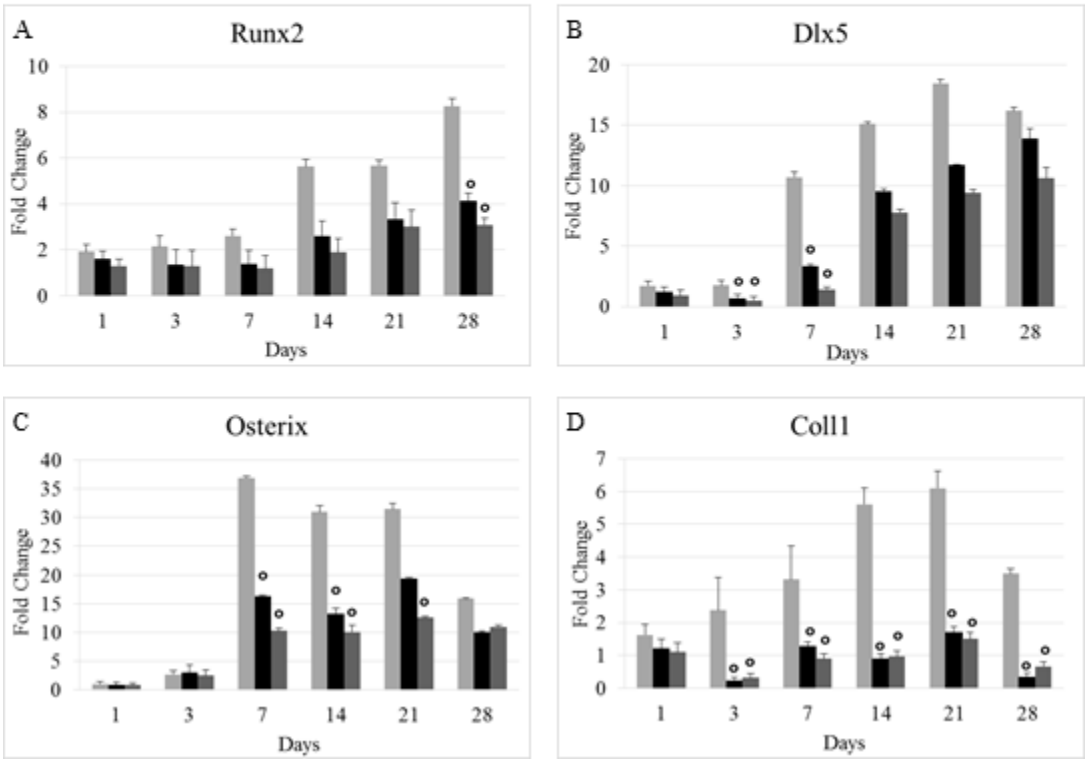
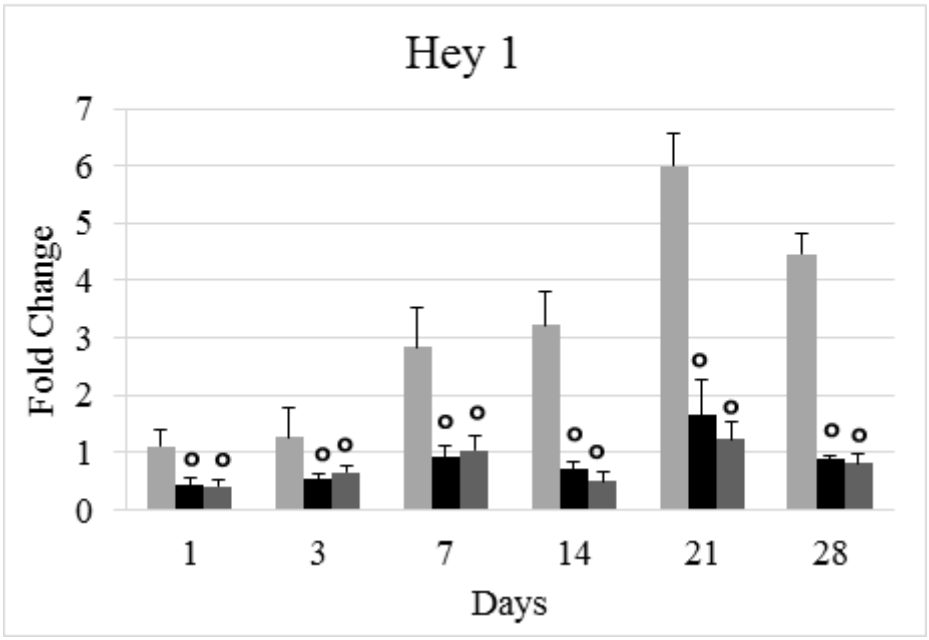


Figure 5



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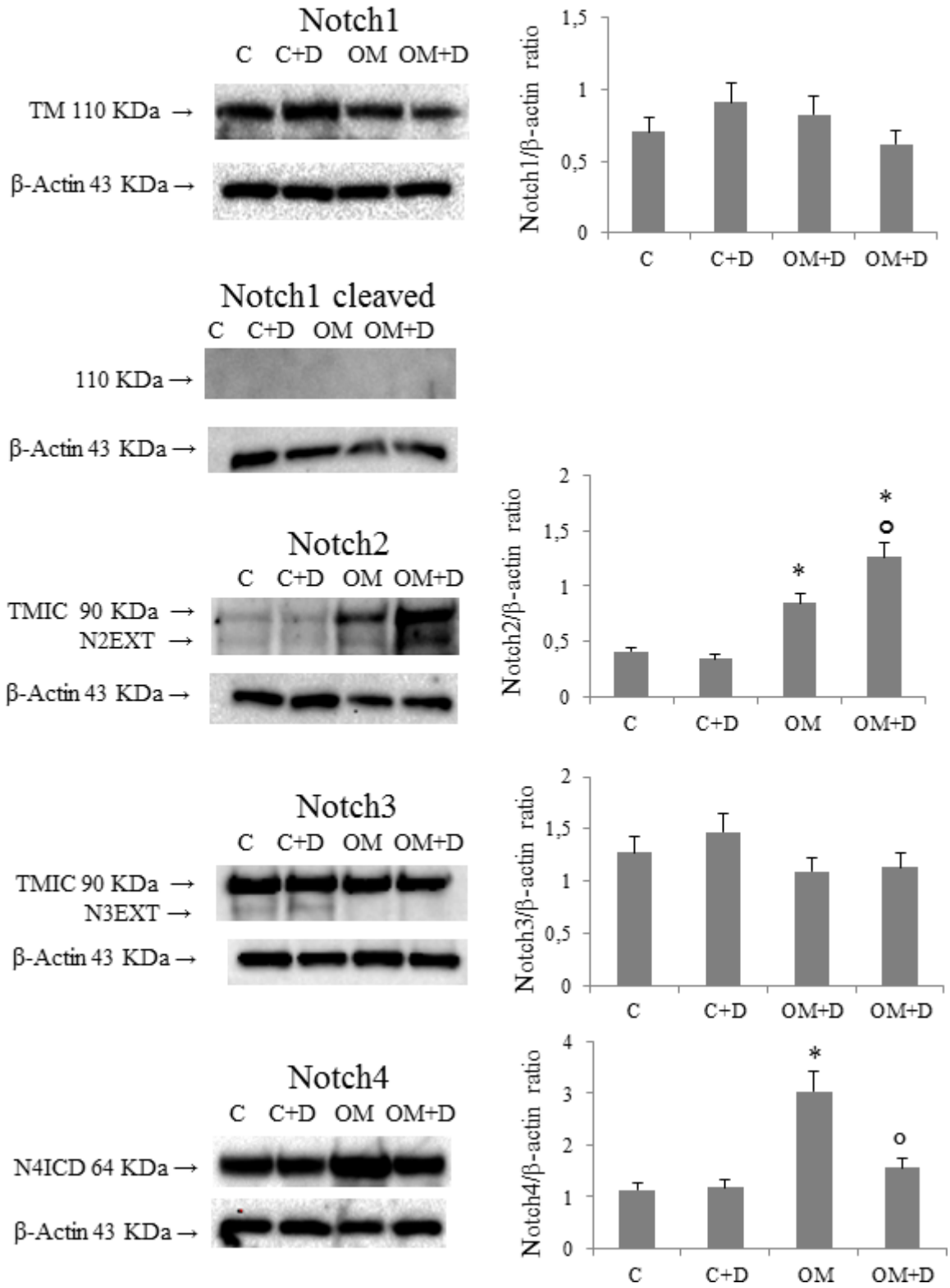

Figure 7

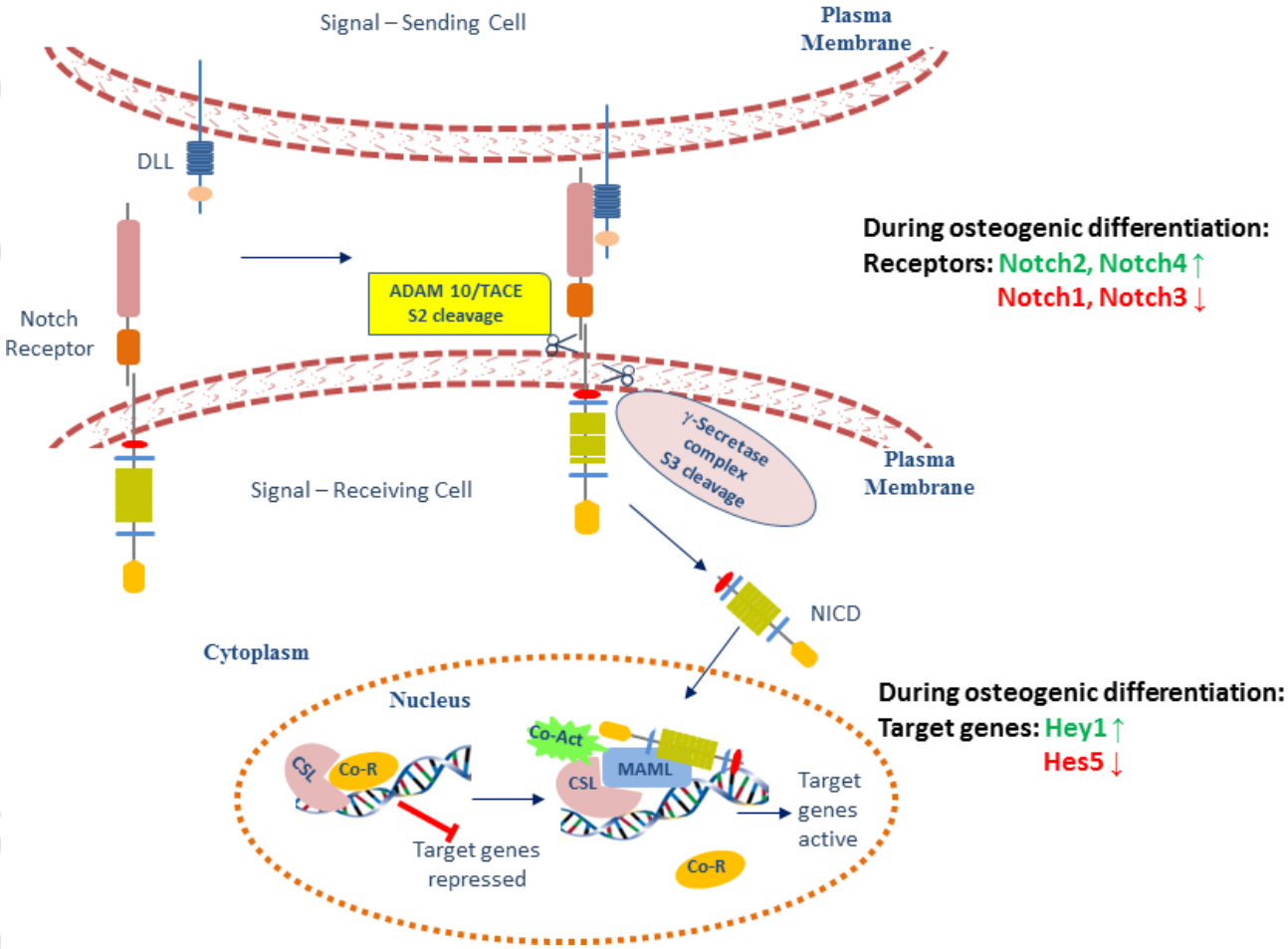

Figure 8