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The Notch pathway in osteogenic differentiation: an In vitro study

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3. Abbreviation list

β-glycerophosphate	β-Gly
Adams–Oliver syndrome	AOS
Alagille syndrome	AGS
Alkaline phosphatase	ALP
Ascorbic acid	Asc
Bone gamma-carboxyglutamic acid (Gla) protein	BGP
Bone marrow mesenchymal cells	BMSCs
Bone morphogenetic proteins	BMPs
Bone sialoprotein	BSP
C Promoter Binding Factor 1	CBF1
Collagen type I	Col I
Collagen type I alpha 1 (Col1α1)	Col1α1
Cysteine-rich Lin12-NOTCH repeats	LNR
Delta-like	DLL
Dexamethasone	Dex
Distal-less homeobox protein	DLX
Dulbecco's modified Eagle's medium	DMEM
Enzyme-linked immuno-sorbent assay	ELISA
Epidermal growth factor	EGF
Extracellular matrix	ECM
Extracellular signal-regulated kinases	ERK
Fibroblast growth factor	FGF
γ-secretase inhibitor	GSI
Hairy Enhancer of Split	HES
Hajdu-Cheney Syndrome	HCS
Hedgehog	HH
HES-related with YRPW motif	Hey
Insulin-like growth factors	IGFs
Interleukin-1 beta	IL-1β
Macrophage colony-stimulating factor	M-CSF
Mastermind-like	MAML
Mesenchymal cell growth supplement	MCGS
Mesenchymal stem cells	MSCs
Mitogen-activated protein kinases	MAPK
N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester	DAPT
Notch extracellular domain	NECD
Notch extracellular truncation	NEXT
Notch intracellular domain	NICD

Notch transmembrane and intracellular domain	NTMICD
Nuclear factor-kappa B	NF-kb
Nuclear regulatory domain	NRR
Osteocalcin	OC
Osteogenic medium	OM
Osteonectin	ON
Osteopontin	OPN
Osteoprogeterin	OPG
Osterix	Osx
Parathyroid hormone	PTH
Phenylmethanesulfonyl fluoride	PMSF
Protein kinase A	PKA
Pulsed electromagnetic fields	PEMFs
Real Time - Polymerase Chain Reaction	RT-PCR
Receptor activator of nuclear factor kappa-B ligand	RANKL
Recombination Signal-Binding Protein for immunoglobulin kappa J region	RBPjk
Runt-related transcription factor 2	Runx2
Spondylocostal dysostosis	SCDO
T cell acute lymphoblastic leukemia	T-ALL
Tartrate resistant acid phosphatase	TRAP
Transcription factors	TFs
Transforming growth factor beta	TGF-β
Transmembrane	TM
Tumor Necrosis Factor-α	TNF-α
Wingless-int/beta-catenin	Wnt/β-catenin

4. Abstract

Although most of bone defects can heal spontaneously with minimal treatment, repair of the bone defects in non-union fractures and excessive loss of bone as a result of trauma or malignant tumors resection is still a major challenge to orthopaedic surgeons. Cell therapy methods in which mesenchymal stem cells (MSCs) are the cellular key players, are used to repair bone defects by their stimulation to osteogenic lineage through osteogenic differentiation process. Osteogenic differentiation is a complex process which involves several signaling pathways such as Notch signaling, an evolutionarily conserved signaling pathway that seems to be essential for proper skeletal development and bone renewal. In spite of the several previous in vivo and in vitro studies on Notch signaling pathway during bone formation and osteogenic differentiation, still several conflicting results are present in literature, as both inhibitory and stimulatory effects have been reported. Moreover, most previous in vitro studies have investigated the role of Notch pathway by using cell manipulation protocols leading to the overexpression or inactivation of specific Notch components or only during a short period of the whole differentiation period. From this background, we set out to clarify the role of Notch signaling during the spontaneous in vitro differentiation of human MSCs without any genetic cell manipulation. To this aim, we induced the osteogenic differentiation of MSCs and verified the differentiation process by the analysis of typical early and late osteogenic markers (ALP, Osteocalcin and mineralization), as well as the gene expression of the main osteogenic transcription factors (Runx2, Osterix and Dlx5). Potential changes in the expression of Notch signaling pathway components, including receptors, ligands and Notch nuclear genes, were analyzed and correlated to the differentiation events during the whole period of hMSCs differentiation in culture (28 days). Moreover, we evaluated the effects of Notch signaling inhibition on the differentiation process in order to confirm the potential involvement of the pathway. Results have shown that Notch pathway is involved in driving osteogenic differentiation and identified Hes5 and Hes1 as the Notch target genes involved, following the activation of the pathway by the Notch4 receptor. On the other hand, we have also identified, at specific times during differentiation, a significant increase in the expression of Hey1, which appears as another important gene involved in osteogenic differentiation, although by a signaling pathway different from the canonical Notch pathway. In fact, in agreement with the complexity of the signal transduction pathways involved in osteogenic differentiation, our results

confirm that other signaling pathways are involved, as suggested by the temporal shift in the increase of osteogenic transcription factors expression and Notch nuclear target genes, as well as by the Notch pathway independent increase in Hey1, demonstrated by the use of Notch signaling inhibitors.

Further, for the first time in this study we show that the activities of pulsed electromagnetic fields (PEMFs), a well-known biophysical stimuli in favoring osteogenic differentiation and bone healing, can be associated to the modulation of Notch pathway as well as to the increased expression of the nuclear gene Hey1. These results add important knowledge concerning the molecular mechanisms by which PEMFs can modulate osteogenesis. Taken together, the data of this study show that Notch signaling pathway can favor the osteogenic differentiation of hMSCs. These results may be relevant also for the optimization of therapeutic treatments as well as bone tissue engineering approaches in clinics.

5. Riassunto

Sebbene la maggior parte dei difetti ossei possa guarire spontaneamente, tuttavia in clinica i ritardi di consolidazione ossea, così come le condizioni in cui la perdita di tessuto osseo è eccessiva in seguito a traumi o tumori maligni rappresentano ancora una sfida importante per i chirurghi ortopedici. Metodi terapeutici basati sull'utilizzo di cellule staminali mesenchimali (MSC) e la stimolazione del loro differenziamento verso la linea osteoblastica sono ampiamente studiati per la riparazione delle lesioni ossee in tali condizioni cliniche. Il differenziamento osteogenico è un processo complesso che coinvolge diverse vie di trasduzione del segnale, che comprendono la via di Notch, una via altamente conservata che sembra essere essenziale per il corretto sviluppo scheletrico e il rinnovamento delle ossa. Nonostante i numerosi studi in vivo e in vitro relativi alla via di Notch durante la formazione delle ossa ed il differenziamento osteogenico, ancora diversi risultati contrastanti sono presenti in letteratura, poiché sono stati riportati sia effetti inibitori e stimolatori. Inoltre, la maggior parte degli studi precedenti in vitro hanno studiato il ruolo della via di Notch, attraverso l'induzione della overespressione o della inattivazione di specifici componenti appartenenti alla via di trasduzione o analizzando il suo potenziale coinvolgimento solo durante un breve periodo rispetto all'intero periodo di differenziamento. In base a quanto esposto, lo scopo di questo studio è stato chiarire il ruolo del segnale di Notch durante il differenziamento di cellule staminali mesenchimali umane indotto in vitro, in assenza di manipolazione genetica delle cellule.

A tale scopo, abbiamo indotto il differenziamento osteogenico di cellule staminali mesenchimali e verificato il processo differenziativo tramite l'analisi dei tipici marcatori osteogenici precoci e tardivi (ALP, Osteocalcina e mineralizzazione), e della espressione genica dei principali fattori di trascrizione osteogenici (Runx2, Osterix e Dlx5). Possibili variazioni nella espressione di componenti appartenenti alla via di segnalazione Notch comprendenti i recettori, i ligandi ed i geni nucleari, sono state analizzate e correlate ad i principali eventi differenziativi durante tutto il periodo di differenziamento cellulare in coltura (28 giorni). Inoltre, abbiamo valutato gli effetti della inibizione della via di Notch sul differenziamento per confermare il potenziale coinvolgimento della via. I risultati ottenuti hanno dimostrato che la via di Notch è coinvolta nel favorire il processo di differenziamento osteogenico ed hanno suggerito che i geni nucleari target della via di Notch, Hes5 e Hes1 siano coinvolti, a seguito dell'attivazione dal recettore Notch4.

Inoltre è stato osservato un significativo aumento dell'espressione di Hey1, un altro gene bersaglio della via di Notch, in tempi specifici del differenziamento, che appare quindi come un altro importante gene coinvolto nel differenziamento osteogenico, sebbene i dati suggeriscano che la sua espressione sia regolata da un'altra via di segnalazione cellulare. Infatti, in accordo con la complessità delle vie di trasduzione del segnale implicate nel differenziamento osteogenico, i nostri risultati confermano che altre vie di segnalazione sono coinvolte, come suggerito dallo shift temporale nell'aumento di espressione dei tipici fattori di trascrizione osteogenica e dei geni nucleari della via di Notch, così come dall'aumentata espressione di Hey1, indipendentemente dalla via di Notch, come dimostrato dall'utilizzo di inibitori specifici della via di segnlazione.

Inoltre, per la prima volta in questo studio dimostriamo che gli effetti dei campi elettromagnetici pulsati (CEMP), che rappresentano un noto stimolo biofisico coinvolto nel favorire il differenziamento osteogenico e la guarigione ossea, possono essere associati alla modulazione della via di Notch e all'aumento della espressione del gene nucleare Hey1. Questi risultati forniscono importanti nuove informazioni riguardanti i meccanismi molecolari attraverso i quali i CEMP possono modulare l'osteogenesi. Complessivamente i risultati di questo studio chiariscono il ruolo della via di Notch nel differenziamento osteogenico indicando che tale via è in grado di stimolare tale processo differenziativo di cellule staminali mesenchimali umane. Tali risultati possono essere rilevanti anche dal punto di vista clinico per la ottimizzazione dei trattamenti terapeutici e degli approcci di ingegneria tissutale per la riparazione ossea.

1. Introduction

1.1. Bone tissue: biology, structure, and function

Bone is a vital, active and dense connective tissue, defined by its mineralized dense structure and capable of regenerating continuously and renewing due to demands for internal hormonal regulation and external mechanical changes during life. Bone acts as a supportive and protective tissue to body organs. It produces blood cells, stores growth factors, minerals and fat, and assists body movement (Bourne, 1971).

Bone specialized cells, bone matrix and bone marrow are the three main elements of the bone as an organ. Bone cells are embedded in an extensive matrix composed of both organic and inorganic elements. They are responsible for regulation of minerals, regeneration and maintenance of bone structure and development. Bone matrix is responsible for rigidity of bones due to minerals or inorganic materials (calcium phosphate, carbonates, sodium, magnesium and fluoride salts) and flexibility by organic materials (collagen fibers, lipids, peptides, proteins, glycoproteins polysaccharides and citrates), as well as storing body's mineral for releasing them when needed. Bone marrow is a soft and highly vascularized part responsible for production of blood cells and generating and storing stem cells (Bourne, 1971).

Different bones have different structure and components ratio that depends on their age and the site which they are located in, resulting in several kinds of bone with various mechanical and functional characteristics.

1.1.1. Bone structure

Calcification of bones occurs in several ways depends on their shape, size, location and consistency. Morphologically, mature bone is classified in two discrete types of tissue, compact or cortical bone and trabecular or cancellous bone. Bone surfaces consist of cortical bone, while cancellous bone is found in the interior of bones, such as within the femoral head, and vertebra (Martin, Burr, & Sharkey, 1998).

Compact (cortical) bone forms the dense outer layer of most bones especially flat bones or shaft portion of long bones. It functions as a cortex for other sections of the bone. It is composed by

densely packed collagen fibers that form concentric lamellae. The structural units of the cortical bone are designated by multiple microscopic columns or osteons which arranged in parallel to the long axis of the bone. Each osteon is multiple layers of lamellae (4-20) around a central canal called the Haversian canal. At right angles the osteons connect together by Volkmann's canals. Haversian and Volkmann's canals contain blood vessels, connective tissues, nerve fibers and lymphatic vessels. About 80% of the total bone mass of the adult skeleton has been composed of compact bone and is comprised of 80-90% calcified tissue. Compact bone has a few gaps and spaces with the porosity of 5 to 30% and thus plays a crucial role in mechanical and protective functions (Buckwalter, Glimcher, Cooper, & Recker, 1996).

In the other hand, trabecular or cancellous bone consists of thin calcified trabeculae, highly vascular, less dense, softer and weaker than compact bone. It has porous networks and a disordered spongy structure, but a higher surface area than compact bone and up to 20% of the total bone mass, with the porosity ranges from 50 to 90%, and 15-25% calcified tissue. Cancellous bone consists of trabecular networks separated by interconnecting spaces containing bone marrow. The trabeculae in cancellous bone are made up of irregular osteon fragments, contain osteocytes and are lined by a single layer of osteoblasts. Generally this type of bone is not penetrated by large blood vessels. The voids are filled with hematopoietic marrow in continuity with the medullar cavity of the diaphysis. The external surface (the periosteum) and the internal surface (the endosteum) of bone which comes in contact with soft tissues are lined with osteogenic cells organized in layers (Buckwalter et al., 1996; Colnot, 2009; Martin et al., 1998).

1.1.2. Bone Matrix

Biomechanically, bone is a material composite made of two phases: the organic and the mineral phase. The organic phase is essentially type I collagen fibers with bone matrix cells. The mineral phase is mostly composed by hydroxyapatite crystals deposited in the collagen fibers.

The organic portion of bone matrix is composed of type I collagen, non-collagenous proteins, glycoproteins, proteoglycans, peptides, carbohydrates, and lipids (Prolo, 1990). The un-mineralized organic matrix or osteoid which is secreted by osteoblasts, consists of 90% Collagen type I and 10% other ground organic compounds such as non-collagenous proteins, proteoglycans,

glycoproteins, peptides, carbohydrates and lipids. Osteocalcin, osteopontin and bone sialoprotein are some of non-collagenous proteins which play important roles in regulating the bone formation. The inorganic or calcified portion of bone matrix mainly consists of calcium phosphate which forms primarily hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) rod-shaped crystals. Bone matrix also contains low amounts of carbonate, magnesium, fluoride, and sodium salts. Hydroxyappetite crystals precipitate around and in parallel of the organized collagen fibers of the osteoid which together provide bone rigidity and strength. During two stages of mineralization process in bone, osteoblasts initiate the calcification process by releasing extracellular matrix vesicles containing phosphatases such as alkaline phosphatase (ALP) to cleave phosphate groups, and calcium binding molecules (e.g. annexin I and phosphatidyl serine). In the next mineralization step, hydroxyappetite crystals release through the matrix vesicle membrane to extracellular fluid and become as nuclei for the formation of new homologues crystals from calcium and phosphate ions exist in the extracellular fluid (Anderson, 2003).

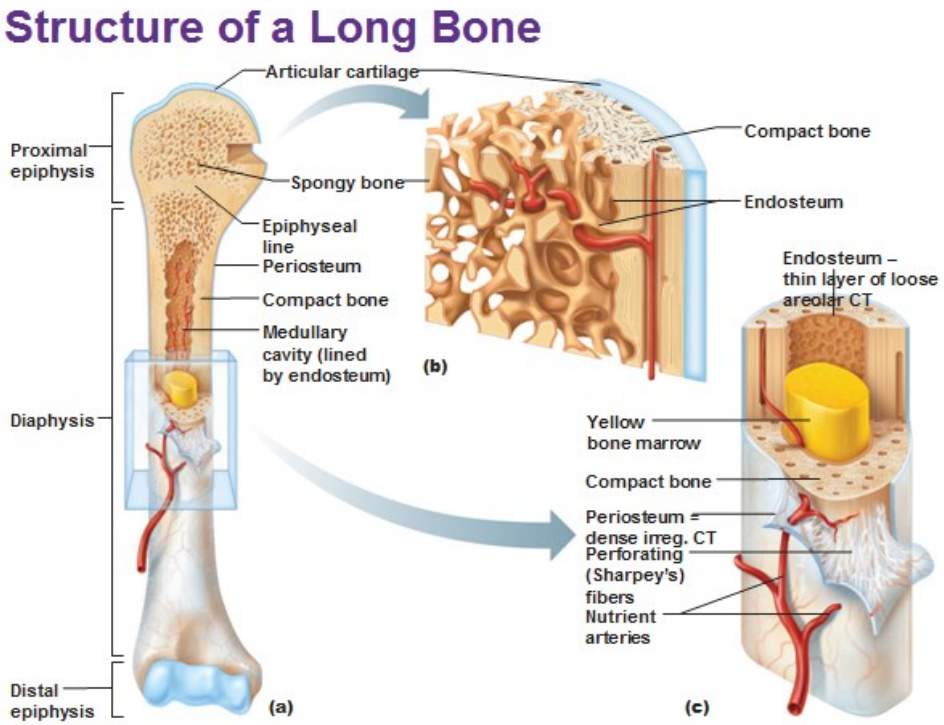
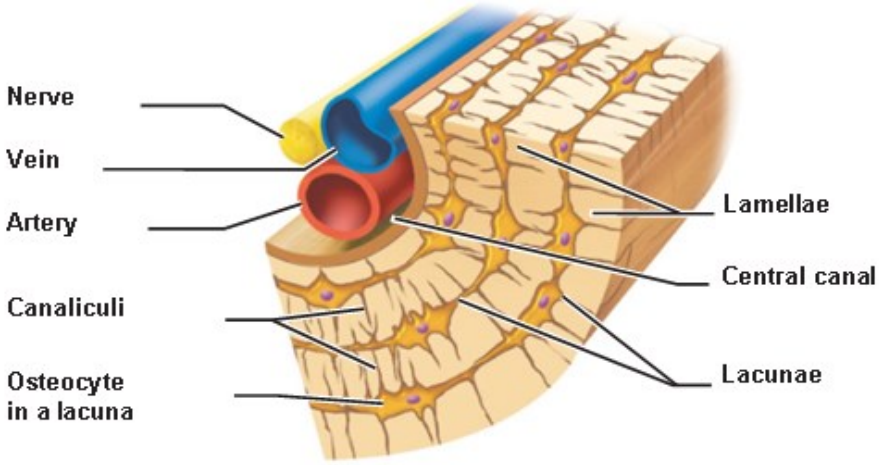


Figure 1-1: Bone structure: Long bone. Adapted from http://antranik.org/cartilage-and-bones/



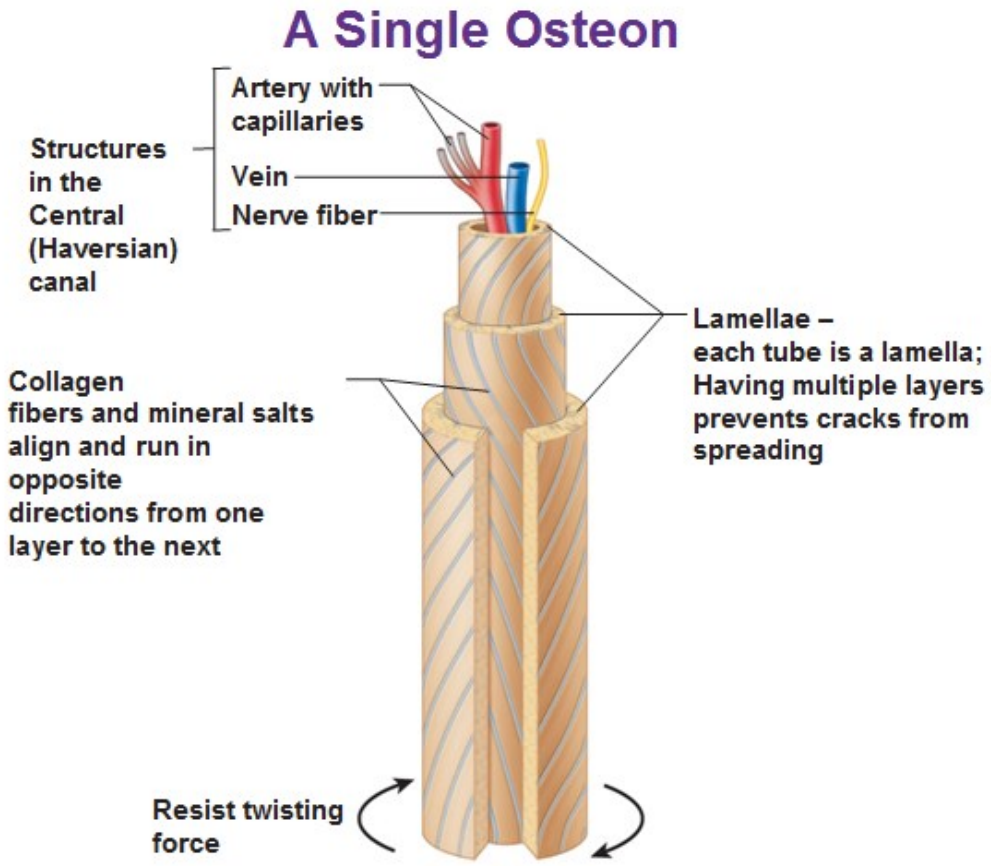


Figure 1-2: Bone structure: osteon. Adapted from http://antranik.org/cartilage-and-bones/

1.1.3. Bone cells

Bone has several specific cells that serve different actions to maintain bone in a normal condition. They include osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts.

Osteoprogenitor cells are bone immature cells and are originated from stem cells in endosteom, inner layer of periosteom and marrow, between trabeculae and along vascular channels. They are the only bone cells which can divide and differentiate to osteoblasts by the effects of stimuli specific for bone formation (Kalfas, 2001).

Osteoblasts are originated from osteoprogenitor cells which differentiate by growth factors such as fibroblast growth factor (FGF) and bone morphogenetic proteins (BMPs). They are responsible for the production of osteoid or organic part of bone matrix such as collagen type I, osteopontin, osteonectin, osteocalcin (OC), and bone sialoprotein (BSP). Also they are responsible for mineralization by producing of matrix vesicles containing organic and inorganic ground components such as alkaline phosphatase, calcium and phosphate ions. Osteoblasts are generally considered as immature bone cells which have no ability to proliferate. Osteoblasts trap in the bone matrix and become mature cells named osteocytes (Owen, 1978).

Osteocytes are the mature and the most abundant cells found in bone, embedded within mineralized matrix in the cavities called lacuna. They are specific star-shaped cells and connect to other osteocytes and osteoblasts by their long cytoplasmic extensions. They are involved in nutrition, control of extracellular concentration of calcium and phosphorus, and also maintenance of bone matrix by transducing messages to cells on the bone surface to initiate resorption or formation processes (Kalfas, 2001).

Osteoclasts are multinucleated large cells which are located in the special parts of cell surfaces; resorptive sites. They are originally derived from hematopoietic stem cells which turned into monocytes and macrophages (Marks & Walker, 1981; Roodman, Ibbotson, MacDonald, Kuehl, & Mundy, 1985; Udagawa et al., 1990; Walker, 1975). Under specific cell signaling, secretion of some specific factors and cytokines such as parathyroid hormone, prostaglandins, macrophage colony-stimulating factor (M-CSF), Interleukin-1 beta (IL-1β) and nuclear factor-kappaB (NF-kb) initiates the differentiation hematopoietic stem cells to monocytes/macrophages. Expression of Receptor activator of nuclear factor kappa-B ligand (RANKL) by osteoblasts induces immigration

of monocytes to resorption sites or Howship lacunae (Clarke, 2008) on the bone surfaces. Fusion of monocytes together or to macrophages form a multinucleated cell which expresses RANK receptor which directed to a specific aggressive resorptive cell with ruffled borders called osteoclast. Osteoclasts demineralize bone by providing an acidic environment in extracellular matrix, subsequently, lysosomal protease Cathepsin K digests the organic matrix in the later phase (Teitelbaum, 2000). At the end of resorption process, osteoclasts undergo apoptosis.

Osteoclasts are distinguished by their high expression of tartrate resistant acid phosphatase (TRAP), cathepsin K and calcitonin receptors.

1.2. Bone activities

1.2.1. Bone remodeling

Bone remodeling is a natural phenomenon in bone which involves bone resorption by osteoclasts, followed by bone formation (or ossification) by osteoblasts. It occurs when bone faces small fractures under stress forces or mechanical strain. Bone remodeling also is needed to adjust bone architecture at the end of bone healing process. Moreover, bone remodeling plays an important role in maintaining plasma calcium homeostasis by activating osteoclasts (solubilizing the mineral part) or osteoblasts (deposition of bone mineral) (Hadjidakis & Androulakis, 2006).Therefore, bone remodeling is necessary to maintain the structural integrity of skeleton as well as perform its metabolic functions properly.

During bone remodeling, osteoclasts become active under the effects of signals from osteocytes. Osteoclasts start to break down the mineral contents of the bone by secreting acidic components which are enzymes like cathepsin K. In the next step, osteoprogenitor cells from the lining cells started to differentiate into osteoblasts and migrate to the site of resorpted bone and secret organic components such as collagen type I followed by mineralization of the site (Lieberman & Friedlaender, 2005; Pogoda, Priemel, Rueger, & Amling, 2005).

1.2.2. Bone fracture healing

During fracture healing process, the damaged bone is restored to its original properties in the terms of shape and functions and new bone is regenerated without scar formation (Lieberman, Friedlaender, & SpringerLink (Online service), 2005; Marsell & Einhorn, 2011a).

The basic biology of healing process which was proposed for the first time by John Hunter (1728-1793) consists of three stages including: inflammation, callus formation, and remodeling. During fracture healing process, several growth and differentiation factors, extracellular matrix, cytokines and hormones together with osteoprogenitor cells play in a highly complex series of biological events to develop bone regeneration process.

Immediately following the fracture, initial inflammatory response begins and a blood clot named hematoma forms in between and around the fracture site. As a result of ruptured blood vessels, platelet aggregation, blood coagulation and clot formation occur in the fracture site within the first 12-14 hours of damage. Hematoma, which provides a matrix for migration of inflammatory cells, is rich of both peripheral and intramedullary blood cells as well as bone marrow cells and pro-inflammatory cytokines that are necessary for bone regeneration. Inflammatory response peaks within the 24h and ends after 7 days during which the inflammatory cells such as macrophages initiate the healing process with phagocytizing of necrotic tissues and other debris, and by releasing a range of specific growth factors and cytokines (Schmidt-Bleek et al., 2012). Secretion of growth factors and cytokines not only stimulates migration of osteoprogenitor cells from surrounding soft tissues such as periosteum and bone marrow to the injury site, but also induced their differentiation to chondroblasts and osteoblasts. Therefore, essential role in bone formation is played by osteoprogenitors such as bone marrow mesenchymal cells.

In the reparative phase and following the formation of hematoma, recruited cells set to build a fibrin-rich granulation tissue. Within this tissue, a non-stable endochondral formation occurs that later forms the soft callus to give the fracture a more stable structure. Formation of soft callus occurs within 7-9 days post fracture. Callus at the distal and proximal ends of fracture becomes hard callus through the intramembranous ossification. Bone formation in this area occurs by the differentiation of osteoblasts directly from precursor cells in periosteum, without the formation of cartilage as an intermediate step. Hard callus has a semi-rigid structure which is stable to bear mechanical stress.

While intramembranous ossification is taking place, mesenchymal stem cells in the internal part of granular tissue begins to undergo condrogenesis and an avascular tissue that is known as soft callus generates. In these region chondrocytes proliferate and synthesize the extracellular matrix

proteins specially collagen materials and glycosaminoglycans (Gerstenfeld et al., 2006; Marsell & Einhorn, 2011b; Marsh & Li, 1999).

In the next step, the extracellular matrix becomes calcified and undergoes hypertrophy through endochondral ossification. Following the endochondral ossification chondrocyte apoptosis, degradation of extracelluar matrix (ECM) and angiogenesis begins. The calcification mechanism started by accumulation of calcium granules in mitochondria which then elaborate to the extracellular matrix to precipitate with phosphate and initiate formation of mineral deposition sites. Calcification of callus peaks within day 14 as usually identifies by measuring of extracellular matrix markers such as osteocalcin and osteonectin or by histomorphometry of mineralized tissue. The final phase of fracture healing is remodeling phase in which the excess of callus resorbs by osteoclasts and woven bone replaces by lamellar bone through osteoblasts deposition. Modification of the fracture region under the influence of mechanical forces leads bone remodeling gradually to an optimal stability, in which the bone cortex obtains similar architecture of its original shape (Einhorn, 1998; Frost, 1989; Marsell & Einhorn, 2011a; Oryan, Monazzah, & Bigham-Sadegh, 2015).

1.2.3. Bone fracture healing therapy methods

Although most of bone defects can heal spontaneously with minimal treatment, further intervention of new and effective treatment strategies are needed in some cases such as non-union fractures, excessive loss of bone as a result of trauma or malignant tumors resection and metabolic diseases.

Therefore, repair of the bone defects is still a major challenge to orthopaedic surgeons. However, during the last several decades, several bone healing strategies including use of implanted tissue (such as bone autograft or allograft) or synthetic materials such as bioceramics as well as stimulation of local cell differentiation by biophysical stimuli such as pulsed electromagnetic fields (PEMFs) or by potential molecules or bio-stimuli such as BMP2 or BMP7 has been accepted as the most standard clinical treatment methods (Gomez-Barrena et al., 2015). Moreover, development of bone tissue engineering has been another promising strategy of bone regeneration during the last decade. Bone tissue engineering aims generating functional and viable tissue by culturing human stem cells within bio-degradable scaffolds and stimulating them into osteogenic lineage which results formation of bone. Therefore, in addition to a source of bone forming cells,

optimization of human mesenchymal stem cells (hMSCs) differentiation by identification of cellular events and biologically active signaling molecules would help us to discover more effective therapies in bone regeneration.

1.3. Osteogenic differentiation

Over the last few years, stem cells have been largely studied as the key players of bone tissue engineering both in *in vitro* bone formation and in *in vivo* bone regeneration. Mesenchymal stem cells (MSCs) are multipotent and nonhematopoietic stromal cells capable of multi-lineage mesenchymal differentiation and function as precursors to several cell types including osteocyte, chondrocyte, adipocyte and myocyte. In studies concerning differentiation, MSCs are generally derived from bone marrow, but other tissue sources such as adipose tissue, muscle and periosteum also have multipotent mesenchymal stem cell-like which are reported as alternative sources for clinical uses (De Bari et al., 2006; Qu-Petersen et al., 2002; Zuk et al., 2001). During bone repair *In vivo,* osteogenic differentiation of stem cells is initiated through the production of biochemical signals by osteoblasts and osteocytes in response to their external environmental changes. These biochemical signals stimulate migration of stem cells from bone marrow or periosteum to the fracture site. MSCs differentiation towards osteogenic lineage is a complex process consisting of several overlapped stages including lineage commitment, proliferation of mesenchymal precursors, differentiation and maturation to osteoblast-like cells. These stages are associated with a cascade of signals which are initiated by the secretion of several mediators and tissue specific factors including growth factors, transcriptional regulators, cytokines and hormones leading to the successive activation of specific genes. While non-tissue-specific mediators such as transforming growth factor beta (TGF-b), insulin-like growth factors (IGF), fibroblast growth factor (FGF) are involved in the early phase of bone formation and cell proliferation, specific osteogenic factors are required during differentiation and maturation stages to control the transcriptional regulation of specific genes which affect the fate of cells (Javed, Chen, & Ghori, 2010). Figure 3-1 shows Sequence and stages of the osteoblast lineage from stem cell to osteocyte. The first step of developing osteoblast phenotype is lineage commitment which leads to osteoprogenitor formation. This step involves the upregulation of lineage specific transcription factors (TFs) along with inhibiting factors that maintain lineage plasticity (Figure 1-3). Runt-related transcription factor 2

(Runx2)/Cbfa1 is one of the osteogenic TFs in commitment of MSCs toward osteoprogenitors and is believed to be a potential focal point for signaling integration (James, 2013; Lin & Hankenson, 2011). In fact, multipotent mesenchymal stem cells become bi-potential progenitors when they start to express Runx2; and those bipotential progenitors are able to differentiate into osteocytes or chondrocytes depend on the molecular events and activated signaling pathways. Other key transcription factors involve in MSCs commitment are Osterix, Sox9, and morphogens TGFβ/BMPs, FGFs. The second stage of osteoblastic development is extensive proliferation of progenitor cells through upregulation of growth related genes such as Cyclin D, Cyclin E, c-fos histones and c-myc along with some matrix related genes such as collagen type I and fibronectin. In the third stage of development, osteoblast clustering as well as production and maturation of extracellular matrix begin. Expression of Alkaline phosphatase and accumulation of collagen type I initiate. Cell-cell interaction-related signaling cascades are activated leading to expression of osteoblasts related-genes. The committed progenitors express osterix which is a marker of osteoblast maturation, and osterix-expressing cells are restrained toward chondrogenesis. This stage is continued with expression of non-collagenous extracellular matrix (ECM) proteins such as osteopontin, osteonectin, bone sialoprotein, and osteocalcin. The final stage of osteoblast development corresponds to maximizing the expression level of genes related to osteoblast maturation (such as osteopontin, osteonectin, bone sialoprotein, and osteocalcin) and mineral deposition which leads to formation of hydroxyapatite crystals. In this stage majority of cells undergo apoptosis and partially become osteocytes (James, 2013; Javed et al., 2010).

The main characteristics of MSCs are their self-renewal and proliferation potential after isolation from bone marrow. Moreover, their isolation is convenient although they are insufficient in number. They not only are able to be expanded *in vitro* in several passages with retaining their growth potential but also they are capable of differentiation to bone, cartilage, tendon, ligament, and muscle lineage. Therefore, they have been used as the potential therapeutic source for cell based therapy in bone tissue engineering (Schaefer, Klemt, Zhang, & Stark, 2000). To promote osteogenic differentiation *in vitro*, several protocols have been utilized in which differentiation factors such as bone morphogenetic proteins or a cocktail of dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-Gly) are the most common among them. It has been reported that Dex can promote upregulation of TAZ (a transcriptional co-activator) and also MKP-1 (a

component of the MAPK signaling pathway) which in turn activates Runx2 gene expression and promotes its activity. Ascorbic acid induces secretion of Collagen type I and β-Gly serves as the phosphate source for hydroxyapatite formation and also influences intracellular signaling molecules (Langenbach & Handschel, 2013).

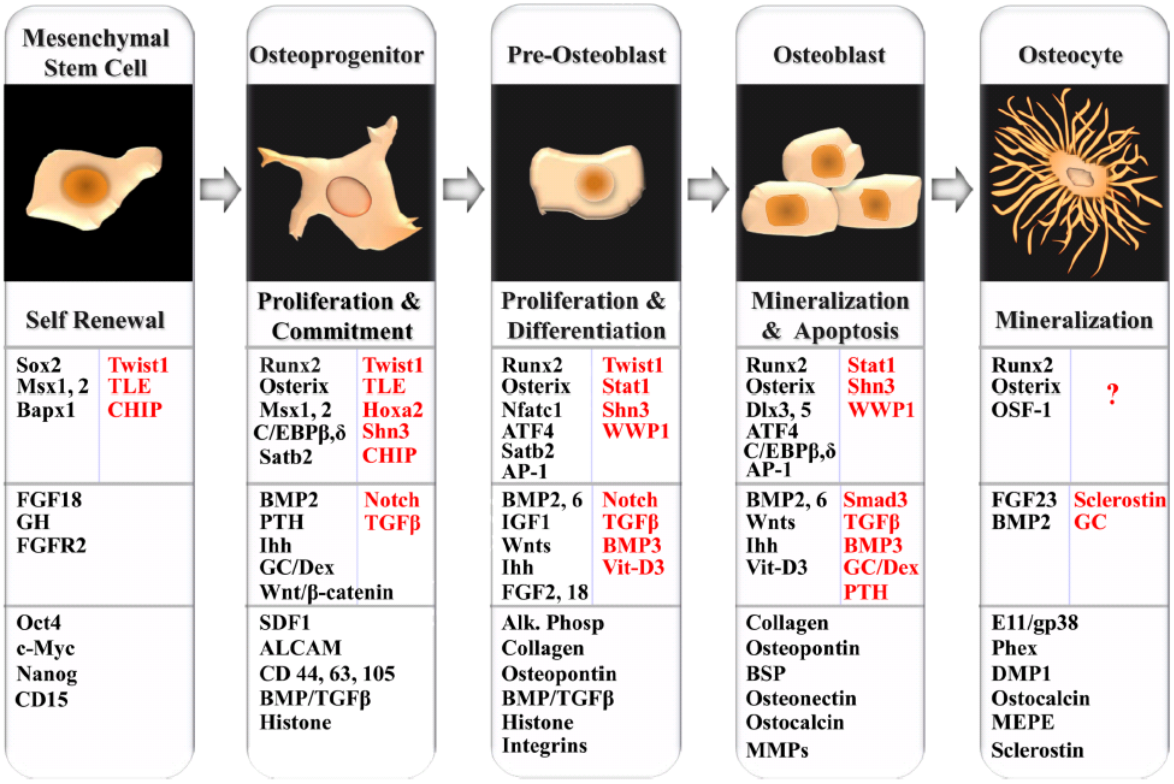


Figure 1-3: Sequence and stages of MSCs osteogenic differentiation.

Sequence and stages of the osteoblast lineage MSCs to terminally differentiated osteocyte is diagrammatically illustrated. Factors that negatively regulate Runx2 activity and osteoblast differentiation or inhibit osteoblast maturation are indicated in red. (Adapted from (Javed et al., 2010)).

All these illustrations have shown that the whole osteogenesis process is a complex network of interactions among molecules involved in several signal pathways leading to migration, proliferation, differentiation and maturation of cells. On the other hand, all data collected have begun to identify the signaling pathways involved in the osteogenic differentiation. The most known signaling pathways which drive osteogenesis involve molecules belonging to the bone morphogenetic proteins (BMPs), Wingless-int/beta-catenin (Wnt/β-catenin), Hedgehog (HH), and Notch pathways with a high degree of cross-talk among them (Lamplot et al., 2013; Lavery, Swain, Falb, & Alaoui-Ismaili, 2008; G. Liu et al., 2009; Marcellini, Henriquez, & Bertin, 2012; Plaisant et al., 2009; Zanotti et al., 2008).

1.4. Osteogenic transcription factors and markers

Over the past years of studying osteogenic differentiation process, various transcription factors (TFs) and regulatory molecules have been identified in different stages of osteogenic differentiation. (Marom, Shur, Solomon, & Benayahu, 2005). The role of some of TFs, but not limited, are listed below

1.4.1. Runx2

Runt-related transcription factor 2 (Runx2) a bone-related transcription factor homologous to the Drosophila protein, Runt, known as Cbfa1/PEBP2A/AML3 plays an essential role in osteoblast differentiation by binding to specific DNA sequences of target genes and influencing their transcription (Shui, Spelsberg, Riggs, & Khosla, 2003). In vivo, Runx2 is crucial for both intramembranous and endochondral ossification and regulates the expression of several osteoblast marker genes in osteoblasts and induces expression of osteoblast marker genes such as OC, Col1α1, BSP, and OPN in non-osteoblastic cells (Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997; Kern, Shen, Starbuck, & Karsenty, 2001; Selvamurugan, Chou, Pearman, Pulumati, & Partridge, 1998). The expression of Runx2 is observed in the early stage of skeletal development when mesenchymal condensation occurs and is maintained in the cells which are giving rise to osteoblasts but not to chondroblasts (Ducy, 1997). Runx2 can be induced by either BMP2/4/7 or TGF-β, fibroblast growth factor-2 or Insulin-like growth factor-1 through P38 Mitogen-activated protein kinases (MAPK) and Smad or kinase/ Extracellular signal-regulated kinases (ERK)-

dependent and Akt-independent pathways (K. S. Lee, Hong, & Bae, 2002; K. S. Lee et al., 2000; Qiao, Shapiro, Kumar, & Passaniti, 2004; Tsuji, Ito, & Noda, 1998; Xiao, Jiang, Gopalakrishnan, & Franceschi, 2002). M. H. Lee et al., reported that overexpression of DLX5 or BMP2 treatment stimulate Runx2 expression (M. H. Lee et al., 2005). Prince et al showed that while glucocorticoid dexamethasone induced both protein level and DNA binding activity of Runx2, it made no change in the Runx2 mRNA level in human osteoblast cell lines (Prince et al., 2001).

Runx2 plays crucial role in osteogenic differentiation and bone development. In fact, over-expression of dominant-negative Runx2 completely abolished the expression of the main bone matrix protein genes in postnatal bone development (Ducy et al., 1999) and homozygous Runx2 mutant mouse died just after birth and showed complete absence of bone formation with nearly normal cartilage development (Komori et al., 1997; Otto et al., 1997). Taken together, all these findings revealed that Runx2 is a master transcription factor of osteoblast differentiation.

1.4.2. DLX5

Distal-less homeobox protein 5 (DLX5) is a homeodomain-containing transcription factor that is specifically expressed in osteogenic cells. DLX5 is believed not only as an initiator of osteogenesis but also it regulates later stage of differentiation (Samee 2008, Holevile 2007, Ryoo 1997). DLX5 expression is induced by BMP2 or BMP4 or a mixture of ascorbic acid and b-glycerophosphate (Holleville, Mateos, Bontoux, Bollerot, & Monsoro-Burq, 2007; M. H. Lee, Kim, et al., 2003). Induction of DLX5 with BMP2 or BMP4 treatment or through overexpression of BMP receptor type IA and type IB (BMPRIA and BMPRIB) indicated that DLX5 is a specific target of BMP signaling. DLX5 expression by BMP signaling occurs even in the absence of endogenous Runx2 that means DLX5 may be upstream of Runx2 (Lee 2003, Lee 2005). Moreover, several in vitro studies showed that overexpression of DLX5 induced ALP, OC, mineralized nodules (Kim, Lee, Wozney, Cho, & Ryoo, 2004; M. H. Lee, Kwon, Park, Wozney, & Ryoo, 2003), and also anticipation in the expression time of Col1a1, osteopontin, alkaline phosphatase, and osteocalcin mRNA along with increase in their levels (Tadic et al., 2002). Some studies demonstrate that DLX5 might be involved in the maturation stage of bone cell differentiation as such, OC and BSP, two markers of differentiated osteoblasts implicated in the process of mineralization, have been reported to be under the direct transcriptional control of DLX5 (Holleville et al., 2007; Kiyoshima et al., 2002; Ryoo et al., 1997; Samee et al., 2008). Moreover, some studies have tried to elucidate the

correlation between DLX5 and other osteogenic TFs such as Runx2. In particular, it has been shown that $DLX5^{-/-}$ cells exhibited not only lower levels of Runx2, Osterix, OC and BSP expression but also decrease in ALP activity, osteocalcin and mineralized nodules both in vitro and in vivo (M. H. Lee, Kwon, et al., 2003; Samee et al., 2008). However, DLX5 can induce ALP and OC in Runx2-null cells which indicates Runx2-independent mechanism for inducing osteogenic markers (Hassan et al., 2006), therefore, It is believed that DLX5 might be only a modulator of Runx2 and Osterix (M. H. Lee, Kwon, et al., 2003).

1.4.3. Osterix

Osterix (Osx), a zinc finger-containing transcription factor that is specifically expressed in all developing bones was discovered by Nakashima et al., which induced C2C12 cells by BMP2. Human homologues of Osx is referred to Sp7 because of the high level of identity with zinc finger DNA-binding domains of Sp1, Sp3 and Sp4 transcription factors. Osx is only expressed in the bone matrix cells and inner and outer layers of bone surfaces, endosteum and preosteum. Osx exists at lower level in pre- and hypertrophic chondrocytes and also is highly expressed in secondary ossification centers and trabeculae bones after birth. Osx expression was also identified in a variety of bone cells such as osteosarcoma cell lines HOS and MG63, foetal and craniofacial osteoblasts, chondrocytes, while its expression is under the detection limit in adult osteoblasts (Milona, Gough, & Edgar, 2003). Osx has been reported to be induced by BMP2 and BMP9 (Celil & Campbell, 2005; Nakashima et al., 2002; Yagi et al., 2003).Osx is believed to be a downstream gene of Runx2 because Osx is not expressed in the skeletal parts of Runx2- null embryos, whereas Runx2/Cbfa1 is expressed in Osx null mice (Nakashima et al., 2002). Some studies indicate that Osx is a master regulator of osteogenic differentiation while negatively regulates osteoblast proliferation. Moreover, osx controls downstream target genes of bone formation and is involved in the maturation stage of osteogenic differentiation. In fact in osterix-null embryos, late markers of osteogenesis such as OC, OPN and BSP are not expressed and mineralization is blocked (Nakashima et al., 2002).

1.4.4. Alkaline phosphatase

Alkaline phosphatases (ALP) are membrane bound ectoenzymes occurs which exists in all human tissues but is particularly concentrated in bone, liver, kidney, bile duct, intestinal mucosa, placental. These isozymes are usually classified to four groups encoded by separate genes corresponding to intestinal, placental, placental-like and tissue non-specific (liver/bone/kidney (TNAP) known as ALPL). ALP catalyzes the hydrolysis of phosphomonoesters and also a transphosphorylation reaction in the presence of large concentrations of phosphate acceptors. ALP plays a crucial role in bone formation by increasing the level of inorganic phosphate as a necessary component in calcification and mineral deposition. Therefore, ALP can be considered as a marker of osteoblastic differentiation. Its activity increases in the early phase of differentiation and ALP mRNA has been reported to be expressed at day 2 of osteogenic differentiation (Qi et al., 2003). In vivo overexpression of ALP elevates skeletal mineralization (Golub, Harrison, Taylor, Camper, & Shapiro, 1992; Narisawa, Yadav, & Millan, 2013; Orimo, 2010). Although the mechanisms are not completely know, It has been reported that ALP expression is regulated by BMP, cAMP-PKA and Wnt/β-catenin signaling pathways through Runx2 and osterix, and parathyroid hormon actions (Rawadi, Vayssiere, Dunn, Baron, & Roman-Roman, 2003; Rey, Manen, Rizzoli, Ferrari, & Caverzasio, 2007).

1.4.5. Collagen type I

Collagens are a family of proteins present in the extracellular matrix of connective tissues and during bone formation their molecules assemble into fibrils which are mineralized at the later stage of osteogenic differentiation through formation of hydroxyapatite crystals (Nair, Gautieri, Chang, & Buehler, 2013). Collagen has a triple- helix structure consisting three polypeptide chains. Although there are more than 27 types of collagen, approximately 95% of the bone entire collagen is type I collagen [[a1(I)]2a2(I)] which exists widely in almost all connective tissues except hyaline cartilage and comprises about 80% of the total bone proteins. Bone also has small amounts of collagen type III, V and XII .The role of collagen in connective tissues is providing elasticity and structure for the component tissues and making a backbone for bone mineral deposition. Collagen I gene is upregulated from the early phase of osteogenic differentiation by a number of osteogenic stimuli such as BMP2, or osteogenic cocktails in vitro (Viguet-Carrin, Garnero, & Delmas, 2006).

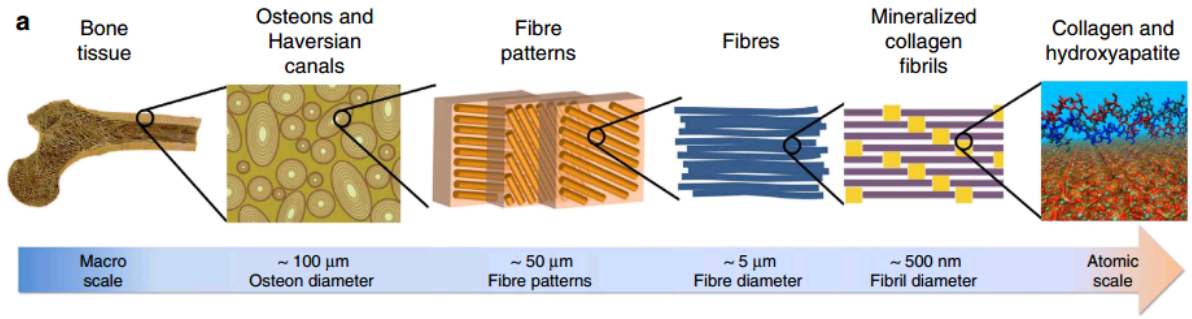


Figure 1-4: Hierarchical structure of bone.

Hierarchical structure of bone ranging from the macroscale skeleton to nanoscale collagen (Nair et al., 2013)

1.4.6. Osteocalcin

Osteocalcin (OC), the most abundant noncollagenous protein of bone matrix is also called "bone gamma-carboxyglutamic acid (Gla) protein or BGP. Osteocalcin is a highly specialized calcium-binding amino acid that plays an important role in matrix mineralization by binding to calcium in the bone tissue (Lian & Stein, 2003). OC is synthesized by osteoblasts and in the presence of calcium not only facilitates its binding to hydroxyapatite to deposit mineral in bone, but also regulates hydroxyapatite size and shape through its vitamin-K-dependent, gamma-carboxylated form (Booth, Centi, Smith, & Gundberg, 2013; Razzaque, 2011). Serum levels of osteocalcin is related to osteoblast activity and bone formation, as such higher levels of OC in serum reflect increased bone turnover and is corresponded with entire levels of parathyroid hormone (PTH) and ALP (Price, Parthemore, & Deftos, 1980).

1.4.7. Bone sialoprotein

Bone sialoprotein (BSP) is one of the major non-collagenous protein found in bone and is considered as a mineralized tissue-specific protein which is found in newly formed osteoblasts. BSP has ability to bind hydroxyapatite and cell-surface integrins through polyglutamic acid and arginine-glycine-aspartate (RGD) motifs, therefore, it plays important role in initiating bone mineralization and as well as in cell attachment (Ganss, Kim, & Sodek, 1999; N. L. Harris et al.,

2000). BSP−/− mice exhibits shorter long bones and reduced cortical thickness (Malaval et al., 2008). It has been suggested that tyrosine kinase, mitogen-activated protein kinase and cAMP-dependent pathways are responsible for BSP regulation (Ogata, 2008).

1.4.8. Osteopontin

Osteopontin (OPN) also known as bone sialoprotein I (BSP-1) is another non-collagenous and a highly phosphorylated sialoprotein that participates in regulation of bone mineralization and in cell-matrix and matrix-matrix/mineral adhesion (Sodek, Ganss, & McKee, 2000). Both osteoblasts and osteoclasts express OPN, therefore OPN has important role during bone remodeling. OPN also mediates mechanical stress signals to osteoblasts during bone formation (Morinobu et al., 2003) and OPN deficiency increases bone fragility (Thurner et al., 2010). Both BSP and OPN are regulated by Runx2 and Osx as such, they have been expressed by forced expression of Runx2 (Ducy et al., 1997) and are undetectable in Osx null mice embryos (Nakashima et al., 2002).

1.5. Notch signaling pathway

Near to one century ago in 1917, Thomas Hunt Morgan (Morgan, 1928) identified alleles of the gene that caused a notch in the fly wings which had been noticed in 1914 by John S. Dexter. Since that time, Notch function and its mechanisms have been under numerous studies revealing that Notch signaling is a highly conserved signaling pathway and one of the major signaling cascades during development and adulthood life regulating formation and maintenance of several cell types. It is activated by cell-cell contact of neighboring cells and affects cell fate decision by controlling a broad range of cellular processes such as cell proliferation, differentiation, survival and apoptosis (Artavanis-Tsakonas, Rand, & Lake, 1999). Deregulation of Notch signaling leads to various diseases (related to bone, heart, eye and brain) such as Alagille syndrome (Li et al., 1997; McDaniell et al., 2006), Hajdu-Cheney syndrome (Isidor et al., 2011; M. A. Simpson et al., 2011), spondylocostal dysostosis (Turnpenny et al., 2003), tetralogy of fallot (Donovan, Kordylewska, Jan, & Utset, 2002; van den Akker et al., 2007), aortic valve disease (Garg, 2006), a stroke and dementia syndrome known as CADASIL (Joutel et al., 2000), Multiple Sclerosis (MS) (John et al., 2002), several cancer types such as T cell acute lymphoblastic leukemia (T-ALL) (Weng et al.,

2003), osteosarcoma (Engin et al., 2008) and multiple myeloma (Jundt et al., 2004) (reviewed by Andersson, E. R. et al. (Andersson, Sandberg, & Lendahl, 2011)).

Identification of Notch molecules revealed that Notch pathway consists of four Notch transmembrane receptors (Notch-1 to Notch-4) and five type I transmembrane ligands including two Serrate-like proteins (Jagged-1 and Jagged-2) and three Delta-like proteins (DLL1,3 and 4). Both Notch receptors and ligands are single-pass transmembrane proteins in which Notch receptors receive signals from Notch ligands located in the neighboring cells. Figure 5-1 shows the structures of Notch receptors and ligands.

Notch receptors are synthesized in endoplasmic reticulum which then fucosylated on certain EGF repeats by the GDP fucose protein O-fucosyltransferase, a process that determine which ligands can activate receptors. Next, in the Golgi apparatus, Notch receptors are cleaved at S1 site by furin-like convertases into two domains including Notch extracellular domain and transmembrane and intracellular domain (NECD-NTMICD) which then translocates to the cell membrane. Overall, Notch receptor is composed of epidermal growth factor (EGF) repeats and a nuclear regulatory domain (NRR) composed of three cysteine-rich Lin12-NOTCH repeats (LNR) followed by a juxtamembrane region with specific proteinase cleavage sites, a transmembrane region including a cleavage site for γ-secretase, and a cytoplasmic region composed of four domains including RBPj association module (RAM), ankyrin repeats (ANK), the transcriptional activator domain (TAD), a C-terminal proline, glutamic acid, serine, threonine-rich (PEST) domain and two nuclear localization sequences (NLS) which are located before and after the ankyrin repeats.

Notch ligands are composed of three motifs including N-terminal DSL motif, specialized tandem EGF repeats and DELTA and OSM11-like proteins called the DOS domain. Both the DSL and DOS domains are involved in receptor binding (Yavropoulou & Yovos, 2014). Unlike Delta-like ligands, jagged ligands have a cysteine rich domain near to the cell transmembrane (D'Souza, Miyamoto, & Weinmaster, 2008; Kopan & Ilagan, 2009).

In the canonical Notch pathway, as it is shown in figure 1-6, binding of the DSL ligands (Delta, Serrate) to the extracellular part of the Notch receptors (NECD) triggers the Notch signaling. Upon the Notch ligand binding, a two steps proteolytic cleavage process begins within the juxtamembrane region and transmembrane domain of Notch receptor. The first proteolytic cleavage is mediated by ADAM/TACE (a TNF-a converting enzyme) metalloproteases at S2 site resulting Notch extracellular truncation (NEXT) molecule, a substrate for the next cleavage by the

γ-secretase complex. The second cleavage at S3 and S4 sites, is served by γ-secretase (including Presenilin1 and 2 in mammals) leading to cytoplasmic release of intracellular domain of Notch receptor (NICD). Therefore an inhibitor of γ-secretase such as DAPT can inhibit releasing of NICD (Leong & Karsan, 2006; Mumm & Kopan, 2000). Moreover, activation of Notch signaling is dependent on endocytosis of ligand-NECD into the signal-sending cell which provides necessary conformational change to receptor for ADAM's action (Yamamoto, Charng, & Bellen, 2010). Upon the last cleavage, NICD then translocates to the nucleus to interact with DNA-binding transcription factor CSL (C Promoter Binding Factor 1:CBF1/Recombination Signal-Binding Protein for immunoglobulin kappa J region: RBPjk in vertebrates, Suppressor of Hairless (Su(H)) in Drosophila, Lag-1 in C. elegans). NICD engagement with CSL provides a binding surface for co-activator proteins of mastermind-like (Mastermind in Drosophila, MAML1 in mammals, Lag-3 in C. elegans) family by displacing the co-repressor complex of SMRT/NcoR and SHARP/MINT/SPEN and change from transcriptional repressor into a transcriptional activator (Andersen, Uosaki, Shenje, & Kwon, 2012; Petcherski & Kimble, 2000; Wu et al., 2000). The resulting NICD-CSL-MAML1 ternary complex then recruits the core transcription machinery, affecting activation of Notch-dependent target genes such as Hairy Enhancer of Split (Hes) 1, 5, 6 and 7 and HES-related with YRPW motif (Hey)1, 2, HeyL (Iso, Kedes, & Hamamori, 2003).

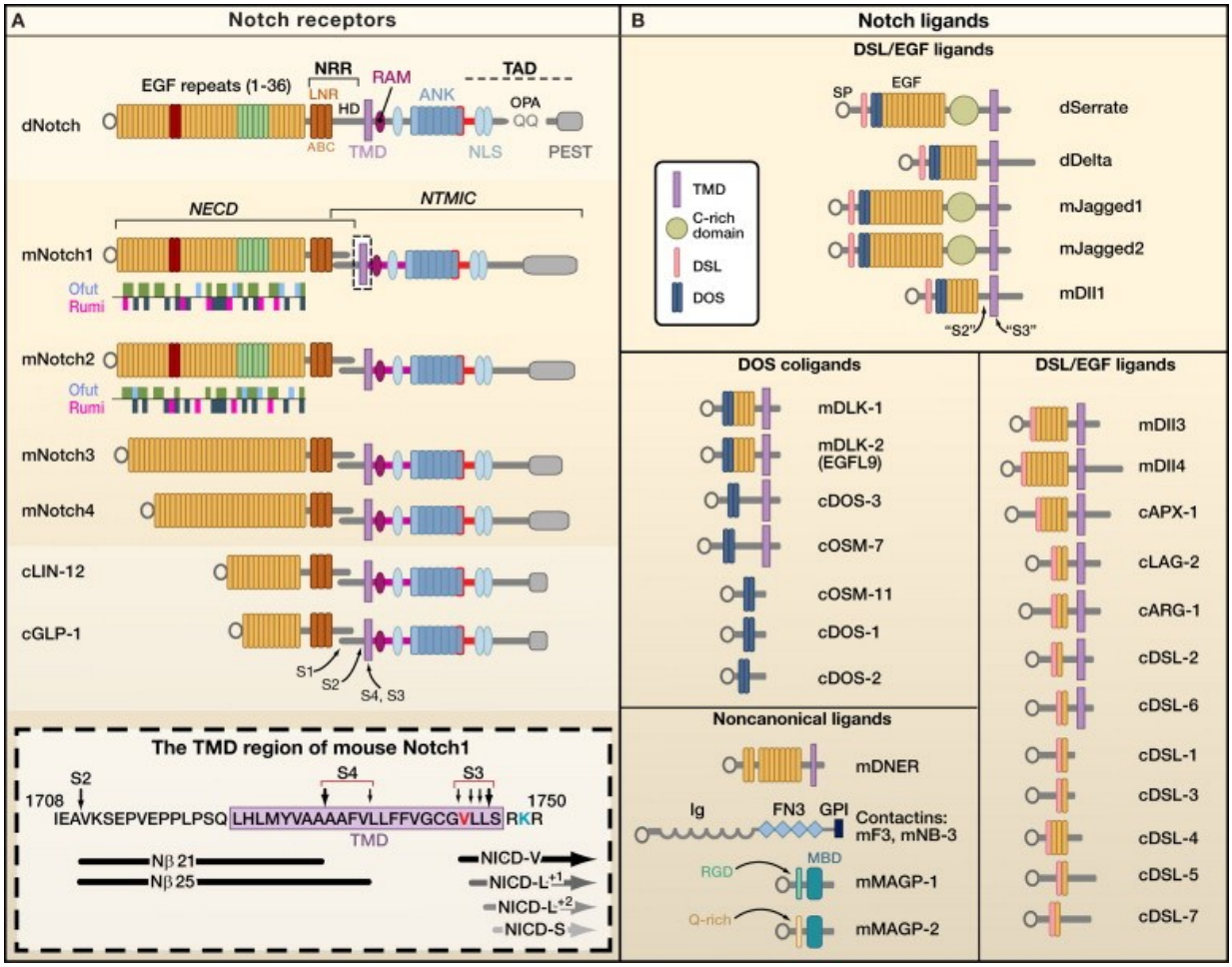


Figure 1-5: Notch receptors and ligands structures. (Adapted from (Kopan & Ilagan, 2009))

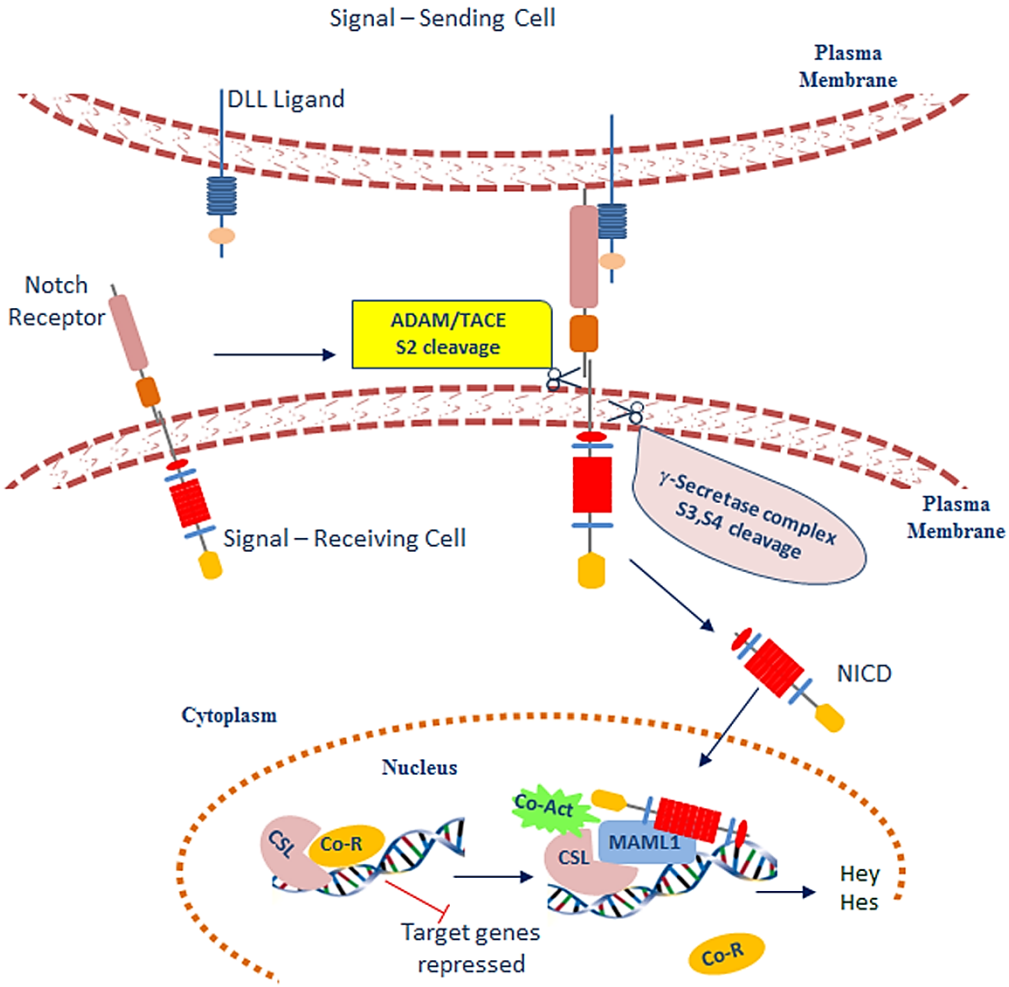


Figure 1-6: Canonical Notch signaling pathway.

Upon the Notch ligand binding, a two steps proteolytic cleavage process mediated by ADAM/TACE (a TNF-a converting enzyme) metalloproteases at S2 site and by γ-secretase at S3 and S4 sites, leading to cytoplasmic release of intracellular domain of Notch receptor (NICD). NICD then translocates to the nucleus to interact with DNA-binding transcription factor CSL. NICD engagement with CSL provides a binding surface for co-activator proteins of mastermind-like MAML1 family resulting NICD-CSL-MAML1 ternary complex which recruits the core transcription machinery, affecting activation of Notch-dependent target genes such as Hairy Enhancer of Split (Hes) 1, 5, 6 and 7 and HES-related with YRPW motif (Hey)1, 2, HeyL.

In non-canonical Notch signaling which can be dependent or independent of ligand binding, NICD activates other pathways by binding to their cytoplasmic molecules or interacts with non-CSL transcription factors in the nucleus. However, the exact mediators of non-canonical pathway are not well understood and proposed mechanisms appear to be context dependent. Regulation of WNT/β-CATENIN, JNK, NF-κB, BMP, TGFβ pathways or activation of GTPase R-Ras, or Deltex/Dtx are some examples of non-canonical Notch pathway (Acosta, Lopez, Revinski, & Carrasco, 2011; Berechid et al., 2002; Blokzijl et al., 2003; Dahlqvist et al., 2003; Endo, Osumi, & Wakamatsu, 2003; Hodkinson et al., 2007; Kwon et al., 2011; Shin et al., 2006; Zecchini, Brennan, & Martinez-Arias, 1999).

1.5.1. Notch regulation in skeleton

To date, function of Notch signaling in skeletal development and bone renewal has been examined extensively, and its role as an essential signaling pathway for proper skeletal formation during development and for postnatal skeletal homeostasis has been proven. Several diseases such as Alagille syndrome (AGS), spondylocostal dysostosis (SCDO), Adams–Oliver syndrome (AOS), Hajdu–Cheney syndrome (HCS) and osteoporosis are results of deregulation in Notch signaling functions. For instance, mutations in Notch1 cause Adams-Oliver syndrome (Stittrich et al., 2014), in Notch2 or Jagged1 cause Alagille syndrome (McCright, Lozier, & Gridley, 2002), in DLL3 and HES7 cause Spondylocostal dysostosis (Bonafe, Giunta, Gassner, Steinmann, & Superti-Furga, 2003). Hajdu-Cheney Syndrome, a rare disease characterized by acroosteolysis and severe osteoporosis is result of Notch2 gain of function mutations (Canalis & Zanotti, 2014). Moreover, mutations in other Notch signaling related components such as RBPJ, Presenilin1 and Presenilin2 result in skeletal defects (Conlon, Reaume, & Rossant, 1995; Oka et al., 1995; Shen et al., 1997).

1.5.2. Notch regulation in osteogenesis

Role of Notch signaling pathway in skeletal development and bone renewal has been examined both in vivo and in vitro studies. Results from previous studies show the potential role of Notch signaling in osteogenesis, although with conflicting results.

In *in vivo* studies, role of Notch signaling has been investigated mainly by cell manipulation leading to loss or gain of Notch function in mice models. The *in vivo* gain of Notch function by using NICD1 overexpression impaired terminal differentiation of committed osteoblasts

evidenced by downregulation of late osteogenic markers such as Osteocalcin. In this study, it was suggested that NICD1 directly binds Runx2 and represses its transactivation function on OC, resulting the inhibition of terminal osteoblastic differentiation and maturation. Moreover, NICD1 overexpression increased proliferation of committed osteoblasts, by increasing the levels of Cyclin D, Cyclin E and Osx led to the formation of woven bone and resulted in osteosclerosis through Rbpjk-dependent pathway (Engin et al., 2008; Tao et al., 2010). Further, NICD1 overexpression, directed by the Prx1 enhancer, induced proliferation and suppressed the differentiation of mesenchymal precursor cells in the adult mouse. Also it has been reported that inactivation of CSL/Rbpjκ restores normal osteoblastic differentiation, dsuggesting that canonical Notch signaling suppresses the commitment of mesenchymal cells to the osteoblastic lineage (Dong et al., 2010; Hilton et al., 2008). In contrast, other in vivo results obtained in transgenic mice overexpressing NICD1 caused osteopenia, suggesting Notch activation effects are dependent on cell maturation states (Zanotti et al., 2008). In fact, Canalis et al, established four mouse models in which NICD1 was overexpressed in four different phases of maturation. Activation of Notch in undifferentiated or differentiated cells of the osteoblastic lineage caused an initial decrease in trabecular bone volume, secondary to a decrease in differentiated osteoblastic function. In contrast, activation of Notch in osteoblasts or osteocytes caused an initial increase in trabecular bone volume due to the decrease in bone resorption. Overal, activation of Notch in cells of the osteoblastic lineage are dependent on the stage of cell maturation. Notch expression in undifferentiated cells or immature osteoblasts impairs osteoblast cell differentiation and causes osteopenia, while Notch expression in osteocytes causes an initial suppression of bone resorption and an increase in trabecular bone mass (Canalis, Parker, Feng, & Zanotti, 2013; Zanotti et al., 2008). In a recent study by Canalis et al., it has been shown that canonical Notch signaling activation in osteocytes causes osteopetrosis by suppressing bone resorption and increase bone volume (Canalis, Bridgewater, Schilling, & Zanotti, 2015). Moreover, in other in vivo studies, overexpression of Notch target genes Hes1 or Hey2 impaired osteoblast differentiation and function and caused osteopenia although with less pronounced effect than NICD1 (Zanotti & Canalis, 2013; Zanotti, Smerdel-Ramoya, & Canalis, 2011).

On the other hand, the loss of Notch function by deletion of precenilin1 and precenilin2 (which mediate the γ-secretase cleavage of Notch receptors) in committed osteoblasts did not show any skeletophenotype, however mice in this situation showed age-related osteoporosis which indicated

an enhancement of osteoclastogenesis as a result of reduction in OPG (Engin et al., 2008). In another study by Hilton et al., it has been shown that loss of Notch function in osteoblast progenitors by deletion of Presenilin1 and Presenilin2 via Prx1-Cre (PPS mice) or deletion of Notch1 and Notch2 via Prx1-Cre (PNN mice) led a high bone mass phenotype at 2 months of age. However, a significant bone loss progressed in PPS and PNN mice as they aged due to reduced osteoblast numbers and increased bone resorption suggesting that Notch signaling in bone marrow acts to maintain a pool of mesenchymal progenitors by suppressing osteoblast differentiation. Notch pathway functions via Jagged1-Notch2-Hes1 signaling axis in that physical interaction between Hes or Hey proteins and Runx2 reduced Runx2 transcriptional activity (Hilton et al., 2008). In agreement with Hilton group, Tu et al., also showed that deletion of Notch2 or Rbpjk in mesenchymal progenitors and not the more mature osteoblast-lineage cells led to the high-bone mass phenotype similar to PPS and PNN mice. The progenitor specific Rbpjk deficiency exhibited an enhancement of osteoblast numbers along with enhancement in mRNA levels of Runx2, Osx, BSP, OC and OPN, a reduction in progenitor pool, but rapid age-related bone loss which suggests canonical Notch signaling is the regulator of osteoblast formation. They showed that not only Notch2 and Rbpjk deletion but also Hey1 and HeyL deficiency exhibited high bone mass in mice suggesting an inhibitory role of Notch on osteogenic differentiation (Tu et al., 2012). Further, inactivation of Notch signaling not only by deletion of Notch receptors but also by deletion of Notch target genes including Hes1 in limb bud in Hes3 and Hes5 null background and Hey2 in osteoblasts precursors caused an initial high bone volume which were declined later by age (Zanotti & Canalis, 2013, 2014; Zanotti et al., 2011).

Notch signaling functions also during bone fracture repair. For instance, overexpression of dnMAML during tibial fracture healing showed no effect of canonical Notch inhibition in early bone formation, instead it altered bone remodeling during the later stages of repair resulted in abrogation of osteoblast maturation and function in mice (Dishowitz et al., 2013; Dishowitz, Terkhorn, Bostic, & Hankenson, 2012). Dishowits et al, reported that systemic Notch inhibition during fracture healing caused prolonged inflammatory cell infiltration and expression of cytokines such as Tumor Necrosis Factor-α (TNFα), and IL-1β. Moreover, a recent research by Wang et al., reported that transiently inactivation of Notch by using gamma-secretase inhibitor, DAPT, two days following fracture for a single day exhibits an early enhancement in local MSC

differentiation and more rapid bone remodeling, resulting in accelerated fracture repair (Wang et al., 2015).

The *in vitro* studies of Notch signaling role on osteogenic cell differentiation also revealed conflicting results supporting both stimulatory and inhibitory effects. These studies have investigated Notch signaling during osteogenic differentiation by cell manipulation leading to activation or inactivation of Notch signaling components including Notch receptor intracellular domain (NICD), Notch ligands (DLL1 or Jagged1) or some of Notch nuclear genes such as Hey1. The *in vitro* gain of Notch function studies by NICD1 or Jagged1 transfection induced early and strong osteogenic differentiation while suppressed osteocalcin expression (Tezuka et al., 2002; Ugarte et al., 2009). Other studies confirmed the stimulatory role of Notch in osteogenic differentiation as such immobilized jagged1 favored osteogenic differentiation of hMSC (Dishowitz et al., 2014; Zhu, Sweetwyne, & Hankenson, 2013). Furthermore, Zhu et al, showed that NICD2 overexpression alone or with Jagged-1 immobilization in hMSCs enhanced osteogenic differentiation of hMSCs, evidenced by increase in the levels of ALP, BSP expression and mineralized matrix. Zhu et al., suggested that Jagged1 induced hMSC differentiation directly via the canonical CSL-NICD-MAML complex, as a dominant negative form of MAML overexpression abolished Jagged1 induced osteoblast formation (Zhu et al., 2013). Differently, some studies showed suppressive effects of Notch gain of function on osteogenic differentiation. For instance NICD1 overexpression via transfection in mouse mesenchymal progenitor cells (Kusa A and Kusa O) (Shindo et al., 2003) and in hMSCs (Fei et al., 2015) or through jagged1 immobilization in mouse MSCs suppressed osteoblast differentiation (Zhu, Sweetwyne, & Hankenson, 2013). Moreover, Notch activation by NICD1 transfection impaired BMP2-induced osteogenic differentiation of mouse stromal cells (ST-2 and MC3T3) (Deregowski, Gazzerro, Priest, Rydziel, & Canalis, 2006; Sciaudone, Gazzerro, Priest, Delany, & Canalis, 2003).

Also the results of in vitro studies which evaluated the effects of inactivation of Notch signaling showed different effects on osteogenic differentiation. For instance, inactivation of Notch signaling by using dominant negative form of MAML1 although had no effect on the early phase of differentiation, inhibited mineralization but increased osteocalcin expression levels (Ugarte et al., 2009). Moreover, overexpression of dominant negative form of extracellular domain of Notch1 or incubation with γ-secretase inhibitor L685,458 as well as transfection of three distinct small

interference RNA of Notch1 (siRNA-Notch1), resulted in a strong inhibition of BMP2-induced osteogenic differentiation in MC3T3-E1 cells (Nobta et al., 2005).

On the other hand, some studies showed that inactivation of Notch signaling by using γ-secretase inhibitor; DAPT, or Notch1 siRNAs in C3H10T1/2 cells (Jung et al., 2013) or by deletion of RBPjk via transfection of shRNA-RBPjK in mesenchymal stem cells promoted osteogenic activity through up-regulation of BMP signaling shown by increased responsive reporter activity and phosphor-smad1/5/8 expression level (Shang et al., 2015).

Collectively, both *in vivo* and *in vitro* studies suggest that the Notch pathway plays important role in osteogenic differentiation, although its effects are dependent on some factors such as the cell line studied and the method or time and duration of Notch loss or gain of function. Moreover, previous in vitro studies have investigated Notch pathway by using activation or inactivation of some of Notch components during a short period of differentiation period, generally for 2 weeks. There are limited number of studies in which the role of Notch have been investigated during osteogenic differentiation without manipulating the cells (Schnabel, Fichtel, Gotzen, & Schlegel, 2002; Zamurovic, Cappellen, Rohner, & Susa, 2004), however, those studies have only shown changing in some of Notch components, indeed limited to the Notch receptors. Therefore, to better understand of exact changes in whole differentiation period, investigation of all Notch components including receptors, ligands and Notch nuclear genes will be clinically important for finding new treatment strategies in bone healing process.

1.6. Pulsed electromagnetic field and osteogenic differentiation

Pulsed electromagnetic fields (PEMF) are biophysical stimuli largely used in clinics to promote bone repair processes. In particular, clinical applications of PEMFs is considered an useful method especially for the treatment of delayed union or non-union fractures and this therapy method has been approved by FDA (Aaron, Ciombor, & Simon, 2004).

The use of such biophysical stimuli in clinical setting is supported by the results of several studies performed in vivo in animal models and in vitro, in different cell models, which have identified

the effects of PEMFs on bone tissue and on the different cell types involved in bone repair processes including osteoblasts, osteoclasts and mesenchymal stem cells (MSCs). The use of in vitro models, as that used in this study, has been relevant allowing the identification of the biological responses of specific cell types, as well as the use of methodological approaches leading to the identification of possible molecular mechanisms by which the biophysical stimulus is able to modulate cellular responses.

In vitro studies have been mainly focused on the effects of PEMFs on osteoblasts and mesenchymal stem cells, related to the essential events occurring during bone repair including cell proliferation, cell differentiation and synthesis of bone matrix components. Most of these studies have shown that electromagnetic stimuli, characterized by specific physical characteristics of the signal, can induce several biological effects on bone cells such as increased proliferation, stimulation of bone matrix production, changes in gene expression and release of molecules, such as growth factors and small molecular mediators with important regulatory activities both on osteoblasts and on other major cell types involved in bone reparative processes.

Although most of the results concerning the effects of PEMFs on bone cells have been obtained in osteoblasts or osteoblast cell lines, some studies investigated the effects of PEMFs on the differentiation of mesenchymal stem cells toward the osteoblastic lineage. These studies show that PEMFs are able to support the different stages of the osteogenic differentiation in both rat and human mesenchymal stem cells by increasing their proliferation, and stimulating the production of bone matrix and mineralization. In one of the first studies performed in human stem cells cultured on calcium phosphate substrates, it was observed that the exposure to PEMFs induces an increase in the activity of ALP and OC production, but only in the presence of the growth factor BMP-2, suggesting that PEMFs play a synergistic action with this important bone regulator (Schwartz et al., 2008). Subsequently, the role of PEMFs in favoring osteogenesis has been also confirmed in the absence of BMP-2, both in animal and human bone marrow mesenchymal stem cells. In fact, several authors have shown that PEMF exposure, in the presence of an osteoinductive medium, in the absence of added growth factors, can induce the increase in several biological markers of osteogenic differentiation, such as the activity of ALP, OC and other matrix protein including OPN and collagen production and gene expression, as well as the deposition of mineral salts (Esposito et al., 2012; Jansen et al., 2010; C. Liu et al., 2013; Lu, Huang, Zhang, Chai, &

Zhang, 2015; Ongaro et al., 2014; Tsai, Li, Tuan, & Chang, 2009; Yan, Dong, Zhang, & Qi, 2010; Yang et al., 2010). Further in some studies, these effects were associated with a significant increase in the expression of osteogenic TFs that play essential roles during differentiation such Runx-2 (Song et al., 2014; Tsai et al., 2009)

In spite of the several studies confirming that PEMF exposure can stimulate osteogenic differentiation, to date still few data are present in literature concerning the PEMF action mechanisms. Similarly to what reported in osteoblast-like cells also in mesenchymal stem cells it has been shown that PEMFs can stimulate the release of regulatory molecules. In particular, it has been reported that PEMFs increase TGF-beta, BMP-2 and osteoprogeterin (OPG) production (Jansen et al., 2010; Yu et al., 2014). These data suggest that PEMFs can influence the differentiation process of stem cells in an autocrine way, as well as the activity of other bone cells through the release of growth factors which have important roles in bone. The most generally accepted theory concerning the PEMF action mechanism is that PEMFs can influence molecules at the plasma membrane which then can favor signal transduction through ligand and receptor binding at cells surfaces (Luben, 1991; Panagopoulos, Karabarbounis, & Margaritis, 2002; Sun et al., 2009), however to date the membrane target molecules which may sense PEMFs have not been identified. Some data have been reported concerning the potential involvement of cellular signaling transduction in the differentiation induced by PEMFs. In particular, it has been shown that the exposure can increase the activity of certain kinases belonging to known intracellular signaling pathways such as the protein kinase A (PKA) and the MAPK ERK1/2 (Song et al., 2014). Moreover, recently Zhou YJ, et al. have suggested the involvement of the Wnt/β-catenin Signaling Pathway by identifying changes in the expression of molecules belonging to this pathway (Wnt1, Wnt3a, LRP5, β-catenin) during differentiation (Zhou et al., 2015). However, to date these studies are too limited and do not permit to derive definitive conclusions. On the other hand, the osteogenic effects induced by PEMFs in mesenchymal stem cells appear of particular interest, as these cells are involved in normal bone repair processes, and for their possible use in tissue engineering approaches. Therefore, further studies aimed to clarify the action mechanism by which PEMFs can influence osteogenic cell differentiation are required and may be important to help the optimization in the use of this biophysical stimulus.

1.7. Aim of the study

The aim of this study was to investigate the potential involvement of Notch signaling pathway in osteogenic differentiation of human mesenchymal stem cells (hMSCs). Moreover, we aimed to investigate the possible connection between the Notch pathway and the biophysical stimulation by pulsed electromagnetic fields (PEMFs) during differentiation of hMSCs, as it is known that PEMFs can favor osteogenic differentiation as well as bone healing (Aaron et al., 2004; Ongaro et al., 2014).

MSCs are used as they are the cellular key players both in bone formation and in bone regeneration, through their differentiation towards the osteoblast lineage. Moreover, osteogenic differentiation process of MSC cells is largely studied as they are essential components in bone tissue engineering approaches.

The osteogenic differentiation is a complex process which involves several signaling pathways such as BMP, Wnt/β-catenin and Notch signaling. However, the exact role of these pathways as well as the interplay among them remain to be clarified (Deng et al., 2008; Javed et al., 2010).

In this study, we focused on Notch pathway, an evolutionarily conserved signaling pathway which seems to be essential for proper skeletal development and bone renewal. In spite of the several previous in vivo and in vitro studies on Notch signaling pathway during bone formation and osteogenic differentiation, still several conflicting results are present in literature, as both inhibitory and stimulatory effects have been reported (Dishowitz et al., 2014; Hilton et al., 2008).

Moreover, most previous in vitro studies have investigated the role of Notch pathway by using cell manipulation protocols leading to the overexpression or inactivation of specific Notch components or only during a short period of the whole differentiation period.

From this background, the research objective of this study was to clarify the role of Notch signaling during the spontaneous in vitro differentiation of human MSCs that is without any genetic cell manipulation.

To this aim we'll specifically:

1. Induce the osteogenic differentiation of MSCs and verify the differentiative process by the analysis of typical early and late osteogenic markers (ALP. Osteocalcin, mineralization), as well as the gene expression of the main osteogenic transcription factors (Runx2, Osterix and Dlx5).
2. Investigate potential changes in the expression of Notch signaling pathway components including receptors, ligands and Notch nuclear genes, during the whole period of hMSCs differentiation in culture (28 days).
3. Analyze the possible correlations among progression of differentiation and changes in Notch signaling pathway component expression.
4. Evaluate the effects of the inhibition of the Notch signaling on the differentiation process in order to confirm the potential involvement of the pathway.

In addition, the same investigations will be also performed in cell exposed to PEMFs during the differentiation period, in order to investigate if the known PEMF-induced osteogenic effects may be related to changes in the Notch signaling pathway.

We retain that the results of this study may help to add new information concerning the involvement of Notch pathway in osteogenic differentiation as well as to clarify the mechanism by which PEMFs favor osteogenic differentiation. New knowledge about the molecular mechanisms that govern osteogenic differentiation may help to manipulate osteogenesis for clinical applications in pathological conditions such as osteoporosis, osteogenesis imperfecta, osteolytic lesions in metastatic cancers, primary bone tumors and bone regeneration

2. Materials and methods

2.1. Reagents and Materials

Human osteosarcoma cell line (MG63) was purchased from the American Type Culture Collection (ATCC). Human bone marrow mesenchymal derived stem cells (hMSCs) were purchased from Lonza (Walkersville, MD, USA). Cell culture basal medium components for MG63 cells including Dulbecco's modified Eagle's medium (DMEM/F12), Fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from GIBCO by Life Technologies, (Carlsbad, CA, USA). Mesenchymal stem cell basal medium components including mesenchymal cell growth supplement (MCGS), Gentamicin sulphate amphotericin-B and L-Glutamine were from Lonza. Osteogenic medium components including mesenchymal cell growth supplement (MCGS), Ascorbate, Dexamethasone, L-Glutamine, β-Glycerophosphate and Penicillin-Streptomycin were from Lonza. The γ-Secretase inhibitor; GSI-IX, LY-374973, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was from Sigma Aldrich (St. Louis, MS, USA) (. Reagents for western blot analysis including Notch4 (H-225) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-DLL4 (ab7280) was from Abcam (Cambridge, UK). β-actin antibody was from Sigma Aldrich, Running and Transfer Buffers, ECL Plus Western Blotting Detection Reagents were from Life Technologies (Carlsbad, CA, USA). Reagents for RT-PCR including SuperScript III reverse transcriptase, Random Hexamers, dNTPs, RNase Out and PureLink RNA microkit were from Invitrogen by Life Technologies. PerfeCta SYBR Green SuperMix with ROX kit was from Quanta Biosciences (Gaithersburg, MD, US). RT-PCR primers for Notch1, Notch2, Notch3, Notch4, Hes1, Hes5, Hey1, Hey2, DLL1, DLL4, Jagged1, and GUSB were purchased from IDT (primers from IDT, Tema Ricerca, Bologna, Italy). RT-PCR primers for, RUNX2, Col1a1, DLX5 and SP7 (Osx) were purchased from Sigma Aldrich. Prestoblue Cell Viability Reagent was from Invitrogen by Life Technologies. Other materials were purchased from Sigma Aldrich. All the other chemicals and solvents were of the highest analytical grade. T75 tissue culture polystyrene flasks was from Falcon BD (Franklin Lakes, NJ, USA), 96-well plates and 35 mm cell culture dishes were from Nest Biotech Co.(Rhaway, NJ, USA), and 4-well plates were from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Cell culture and osteogenic differentiation

Human osteosarcoma cell line (MG63) was purchased from the American Type Culture Collection (ATCC). Cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 10% FBS, penicillin (100Uml^{-1}) and streptomycin (100 μgml^{-1}). Human bone marrow mesenchymal derived stem cells (hMSCs) were purchased from Lonza and grown in complete Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza) according to the product specifications. All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C. In all experiments of cell differentiation, MG63 cells or hMSCs (after first passage) were seeded in 4-well plates for Osteocalcin, ALP activity and Alizarin Red staining assays, in 96-well plate for cell proliferation assay or in 35 mm cell culture dishes for RT-PCR and Western blot assays. MG63 and hMSCs were cultured at 2×10^3 cells/cm^2 or 4×10^3 cells/cm^2 respectively, and treated with Osteogenic Differentiation Medium (OM) (Lonza) for 28 days and set as OM group. For control samples, MG63 cells (at 2×10^3 cells/cm2) treated with growth medium (DMEM/F12 supplemented with 4 mM L-glutamine, 10% FBS, penicillin (100Uml^{-1}) and streptomycin (100 μgml^{-1}) and hMSCs (at 4×10^3 cells/cm2) treated with growth medium (Mesenchymal Stem Cell Growth Medium supplemented with Gentamicin sulphate amphotericin-B and L-Glutamine (MSCGM)) for 28 days and set as Ctrl group. For all experiments, culture medium was changed every 3 days. Samples were harvested at days 0, 1, 3, 7, 14, 21 and 28 for assays.

2.3. Characteristics of PEMFs and exposure conditions

To evaluate a possible involvement of Notch pathway in PEMF-induced osteogenic differentiation, we performed all the analysis described before also in PEMF exposed cells. To our aim, among the treatment groups (undifferentiated controls, OM group and OM group with DAPT) a subset of cell cultures were assigned to PEMF exposure. The PEMF generator system was the same as used in previous studies (De Mattei et al., 2007; De Mattei et al., 2004; De Mattei et al., 2009; Ongaro et al., 2014; Ongaro et al., 2011; Ongaro, Pellati, et al., 2012; Ongaro, Varani, et al., 2012).

The PEMF generator consisted of a pair of circular Helmoltz coils of copper wire placed opposite to each other and in a signal generator (IGEA S.p.A., Carpi, Italy). The 4-well plates, 96-well plates and 35 mm culture dishes were placed between the pair of Helmoltz 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 (LE, Gaussmeter DG500, USA) with a reading sensitivity of 0.2%. The shape of the induced electric field and its impulse length were kept constant. The intensity value of the magnetic field measured between two coils was uniform in the whole area in which the cell cultures were placed. 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. Figure 2-2 shows the generator system and its position inside the cell incubator.

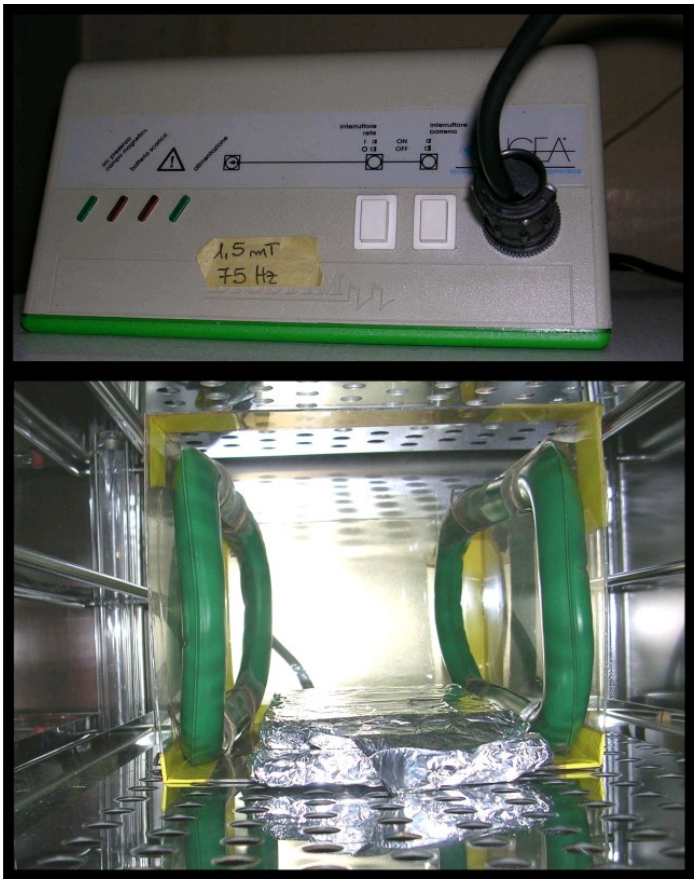


Figure 2-1: PEMF generator system furnished by IGEA (Carpi, Italy).

2.4. Inhibition of Notch pathway by DAPT treatment

DAPT, blocks Notch signaling by inhibition of γ-Secretase complex (Kanungo, Zheng, Amin, & Pant, 2008). For DAPT treatment (LY-374973, Sigma Aldrich), after constituting a 5mM stock solution in DMSO (Sigma, Aldrich), DAPT added to the MG63 or hMSCs cultures at 1:500 dilution, producing final concentration of 10 μM DAPT (Jung et al., 2013). In all cases when DAPT treatment was used, DAPT was applied throughout the whole growth and differentiation period.

2.5. Osteogenic Markers

Cell proliferation assay

Cell proliferation was assessed by Prestoblue Cell Viability Reagent (Invitrogen by Life Technologies). PrestoBlue™ reagent is a resazurin-based solution that functions as a cell viability indicator by using the reducing power of living cells to quantitatively measure cell proliferation. MG63 or hMSCs cells were seeded in 96-well plate dishes at a density of 2×10^3 cells/cm^2 or 4×10^3 cells/cm^2 respectively in growth medium or OM in the presence or absence of DAPT. At the different time points during differentiation (0, 1, 3, 7, 14, 21, 28 days), PrestoBlue Cell Viability Reagent solution was added to each well of all experimental groups including Ctrl group, OM group and OM + DAPT group, under PEMF exposure or without exposure to PEMF 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.

Alkaline phosphatase (ALP) activity

MG63 or hMSCs cells were seeded in 4-well plate at density of 2×10^3 or 4×10^3 cells/cm^2 respectively, in five groups of treatment; Ctrl, OM, OM + DAPT, OM + PEMF and OM + PEMF + DAPT. At each time point (days 3, 7, 11, 14, 21 and 28), the cells 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 micro-plate reader (TECAN). ALP activity was normalized to total protein quantity measured using the bicinchoninic acid assay (BCA), a protein assay reagent kit (Quantum Protein Euroclone Milano, Italy) according to the manufacturer's instructions. ALP activity was expressed as μMol/(min×g protein) (U/g).

Alizarin Red staining

Alizarin red, an anthraquinone derivative, is used to evaluate calcium salts in cell cultures. For the assay, MG63 or hMSCs cells cultured in 4-well plates at density of 2×10^3 or 4×10^3 cells/cm^2 respectively, and were harvested at days 14, 21 and 28 of differentiation, washed in phosphate buffer saline (PBS) and fixed in 4% formalin for 5 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. Each well was rinsed three times with distilled water. 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.

Detection of Osteocalcin levels by enzyme-linked immunosorbent assay (ELISA)

At day 7, 14, 21 and 28 of differentiation, MG63 or hMSCs cells which were seeded in 4-well plates at a density of 2×10^3 or 4×10^3 cells/cm^2 respectively, 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 is a solid phase enzyme-amplified sensitivity immunoassay performed on a microtiter plate. The assay uses monoclonal antibodies, directed against distinct epitopes of human Osteocalcin. A schematic representation of the assay

has been shown in figure 2-1. Samples and standards were assayed in duplicate. OC levels were normalized to the total protein content and expressed as ng OC/mg protein.

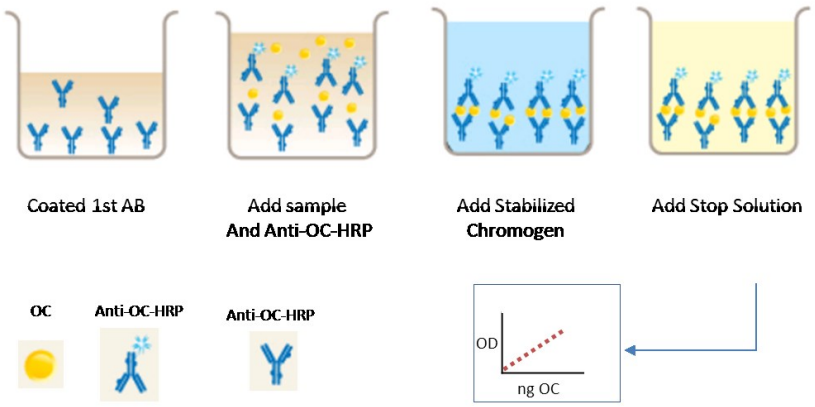


Figure 2-2: Schematic representation of quantitative Osteocalcin ELISA test.

(Adapted from Invitrogen manual of OC ELISA kit).

2.6. Real-Time PCR

MG63 or hMSCs cells were seeded in 35 mm culture dishes at density of 2×10^3 or 4×10^3 cells/cm^2 respectively, and harvested at days 0, 1, 3, 7, 14, 21 and 28. Cells were washed three times with Earle`s Balanced Salt Solution (containing Calcium Chloride (1.8 mM), Potassium Chloride (5.3 mM), Magnesium Sulfate (0.8 mM), Sodium Chloride (117mM), Sodium Bicarbonate (26 mM), Sodium Phosphate Monobasic (1.0 mM), Glucose (5.6 mM) and Phenol Red (0.1 mM)) and total RNA was extracted using commercially available kit (PureLink RNA microkit Invitrogen by Life Technologies). RNA concentration and purity were determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 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 with ROX kit (Quanta Biosciences) according to the manufacturer's protocol in a final volume of 20 µl. Real-time PCR

was carried out for GUSB, Notch1, Notch2, Notch3, Notch4, Hes1, Hes5, Hey1, Hey2, Jagged1, DLL1, DLL4, Osx, DLX5, Runx2 and Col1A1 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). Concentrations of 500 nM of primers listed in Table 1 (all from IDT Tema Ricerca, Bologna, Italy) were used. 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 at day 1 or 3 for MG63 or hMSCs respectively.

2.7. Western Blot analysis

MG63 or hMSCs cells were seeded in 35 mm culture dishes at density of 2×10^3 or 4×10^3 cells/cm^2 respectively, and harvested at days 0, 3, 7, 14, 21 and 28. Western blot analysis were carried out to detect Notch4, DLL4, and β-actin proteins by using the corresponding antibodies. Whole cell lysates from MG63 or hMSCs cells were prepared using radioimmunoprecipitation assay buffer (RIPA buffer containing 0,1% SDS, 1% NP40, while 0.05% sodium-deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM sodium Orthovanadate were freshly added) on ice for 30 minutes. Protein samples were denatured by incubation at 70°C for 10 minutes in loading buffer and reducing agent (Life Technologies) and electrophoresed on 7% Tris-Acetate Protein gels (NuPAGE by Life Technologies). For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane (Westran Clear Signal, PVDF, WHATMAN INC. Florham Park, N.J USA) at 30 V for 150 min. Non-specific binding was blocked by incubating membranes with Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and5% nonfat dry milk for 1 hour at room temperature. PVDF membranes were then incubated overnight at 4°C with primary antibodies, washed 4 times in TBS/T and incubated for 60 minutes at room temperature with secondary antibodies peroxidase-conjugated in TBS/T containing 5% nonfat dry milk. Membranes were washed and developed with a chemiluminescent substrate, ECL Western Lightning Ultra Blotting Detection Reagents (Perkin Elmer,Waltham, MA, USA). Images were obtained by ChemiDoc MP imaging system (Bio-Rad Laboratory Inc.Hercules,CA, USA) and protein bands were analyzed and quantitated by using the Image Lab analysis software. Values were expressed as the ratio of each band density to β-actin band density normalized to control band.

Table 2-1: RT-PCR primers		
Name	Forward	Reversed
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′
Jagged-1	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′
Hes1	5′-CGGACATTCTGGAAATGACA-3′	5′-CATTGATCTGGGTCATGCAG-3′
Hes5	5′-AAGCACAGCAAAGCCTTCGT-3′	5′-TGGAGCGTCAGGAACTGCAC-3′
Col I	5′-GCTATGATGAGAAATCAACCG-3′	5′-TCATCTCCATTCTTTCCAGG-3′
Runx2	5′-AAGCTTGATGACTCTAAACC-3′	5′-TCTGTAATCTGACTCTGTCC-3′
DLX5	5′-GCATTACAGAGAAGGTTTCAG-3′	5′-TTTTCACCTGTGTTTGTGTC-3′
Osterix	5′-TGAGGAGGAAGTTCACTATG-3′	5′-CATTAGTGCTTGTAAAGGGG-3′
GUSB	5′-CCCGCGGTCGTCATGTGGTC-3′	5′-GCCGGGAGGGGTCCAAGGAT-3′

2.8. 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, OM versus OM + DAPT, OM versus OM + PEMF and OM+PEMF+DAPT versus OM+PEMF at the different time points investigated (1, 3, 7, 14, 21, 28 days) as reported in figure legends.

3. Results

3.1. Effects of osteogenic medium on MG63 cells differentiation

In our study, we used MG63 cell line as a cell model to investigate the potential correlation between osteogenic differentiation and Notch pathway. We first analyzed early and late osteogenic markers of MG63 cells in response to standard osteogenic medium. Results showed that MG63 cells can be differentiated to osteoblasts evidenced by the increasing levels of both early and late osteogenic markers.

3.1.1. Osteogenic markers

ALP Activity: ALP activity is an early marker of osteogenic differentiation and is used to monitor the differentiation of osteoprogenitor cells toward mature osteoblasts (Orimo, 2010). MG63 cells as immature osteoblasts show alkaline phosphatase activity even in the absence of osteogenic medium (figure 3-1 A). ALP activity exhibited no significant changes during the period of 28 days in growth medium (Ctrl group). Whilst, it increased significantly at days 7 (1.77 fold) and 14 (2.09 fold) of differentiation in cells cultured in osteogenic medium (OM group). No significant differences between control and OM groups were observed at the late phase of differentiation (21 and 28 days).

Osteocalcin level: Results show that unlike the ALP activity, OC, a late marker of osteogenic differentiation (Ryoo et al., 1997), was not detectable in cells cultured in growth medium in the absence of osteogenic supplement. Figure 3-1B shows OC levels in MG63 cells during the 28 days of differentiation. OC was detectable in our cultures in OM from day 14 and it increased significantly until day 28. OC levels were significantly higher in OM compared to control group at days 14 (5.81 fold), 21 (60.74 fold) and 28 (85.97 fold).

Alizarin Red Staining: For evaluation of calcium nodules and mineralization process, another late osteogenic marker (Anderson, 2003), alizarin red staining has been done during the 28 days of differentiation. Figure 3-1C shows that there is no mineral deposition in control group in the whole period; however MG63 cells in osteogenic medium show mineral nodules starting from day 14 that increased until day 28. These data were confirmed by quantification of deposited minerals

obtained by solubilization of calcium nodules which increased 0.398, 1.35 and 5.12 folds at days 14, 21 and 28 respectively.

Proliferation: To verify if the changes observed in OM group respect to the control group were due to differences in the 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 3-1D).

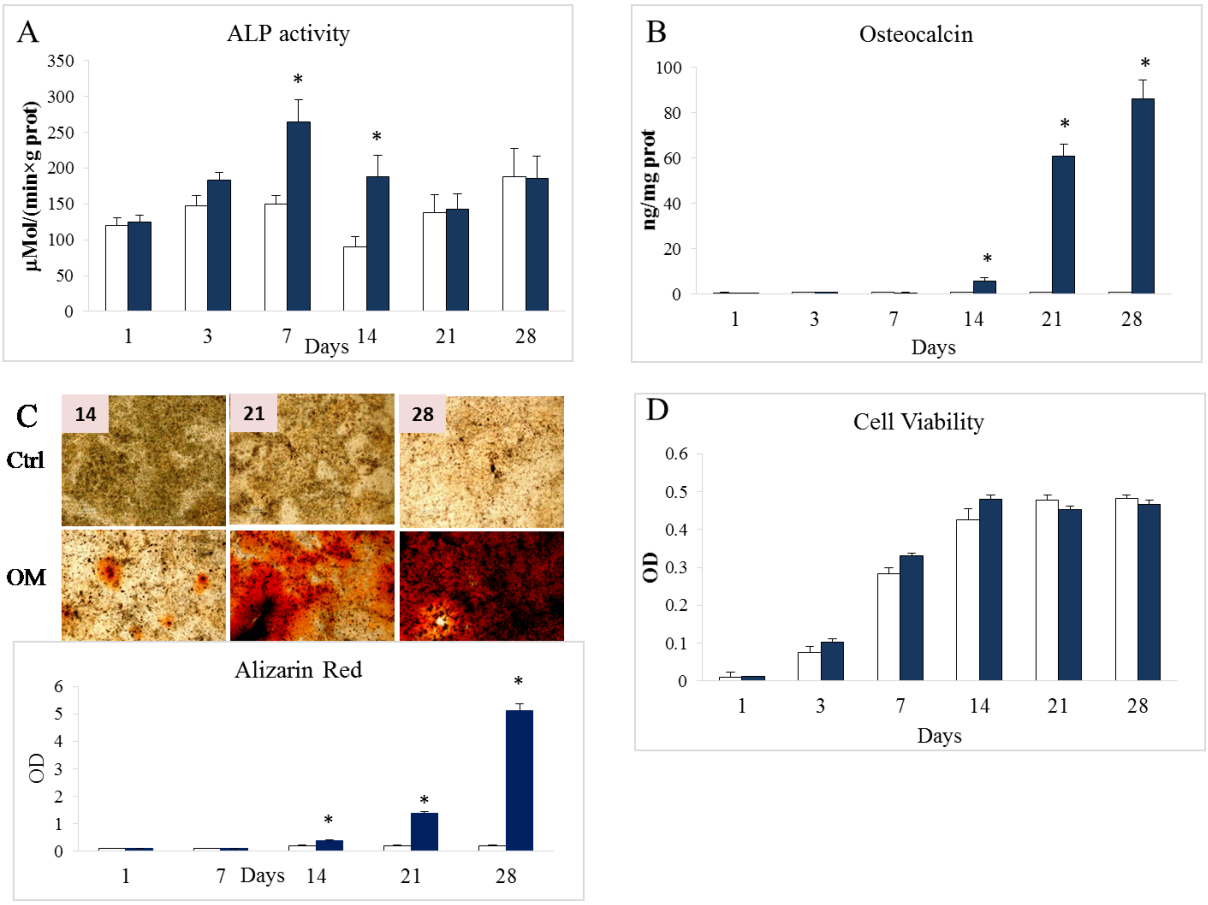


Figure 3-1: Biomarkers during MG63 cells differentiation

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, 28). Cells were tested for ALP activity (A), osteocalcin levels (B), matrix mineralization by Alizarin red (C) and cell viability level (D). The quantification of Alizarin red staining was done spectrophotometrically and reported in the graph under the panel. *Statistical significance OM versus control, at the corresponding time point.

3.1.2. Osteogenic Transcription Factors

Gene expression levels of the osteogenic marker Collagen type I and the master osteogenic TFs including Runx2, DLX5, Osx have been investigated by Real Time PCR at days 1, 3, 7, 14, 21 and 28. Results show that osteogenic differentiation of MG63 cells involves increasing in the mRNA levels of Runx2, DLX5, Osx and Collagen type I, according to previous studies on differentiation (Javed et al., 2010).

Runx2: RUNX2 is believed as the main osteogenic transcription factor which is expressed in osteoprogenitor cells (Ducy et al., 1997). Comparing to day 1, expression levels of Runx2 did not significantly change in control group in the whole experimental period. However, Runx2 expression in osteogenic medium significantly increased from day 7 to a maximum at day 28 compared to the control at the same time (Figure 3-2A).

DLX5: DLX5 is another transcription factor that is expressed in osteoblasts and involved in differentiation (Ryoo et al., 1997). RT-PCR results show that expression levels of DLX5 in osteogenic medium are higher than in growth medium at all-time points, showing upregulation of this gene from day 7. Figure 3-2B shows induction of DLX5 that are statistically significant from days 7 to 28 comparing to the control group at the same days.

Osterix: When monitoring Osx expression during the 28 days of growth, we observed that expression of Osx in OM group was significantly increased only at day 7 comparing to control (2.25 fold, $p=0.008$) (Figure 3-2C).

Collagen type I: Results in figure 3-2D shows that while no change in expression levels of Col I were observed in control group, a significant up-regulation from day 3 to day 28 in osteogenic

medium according to the progression of osteogenic differentiation occurred (Viguet-Carrin et al., 2006).

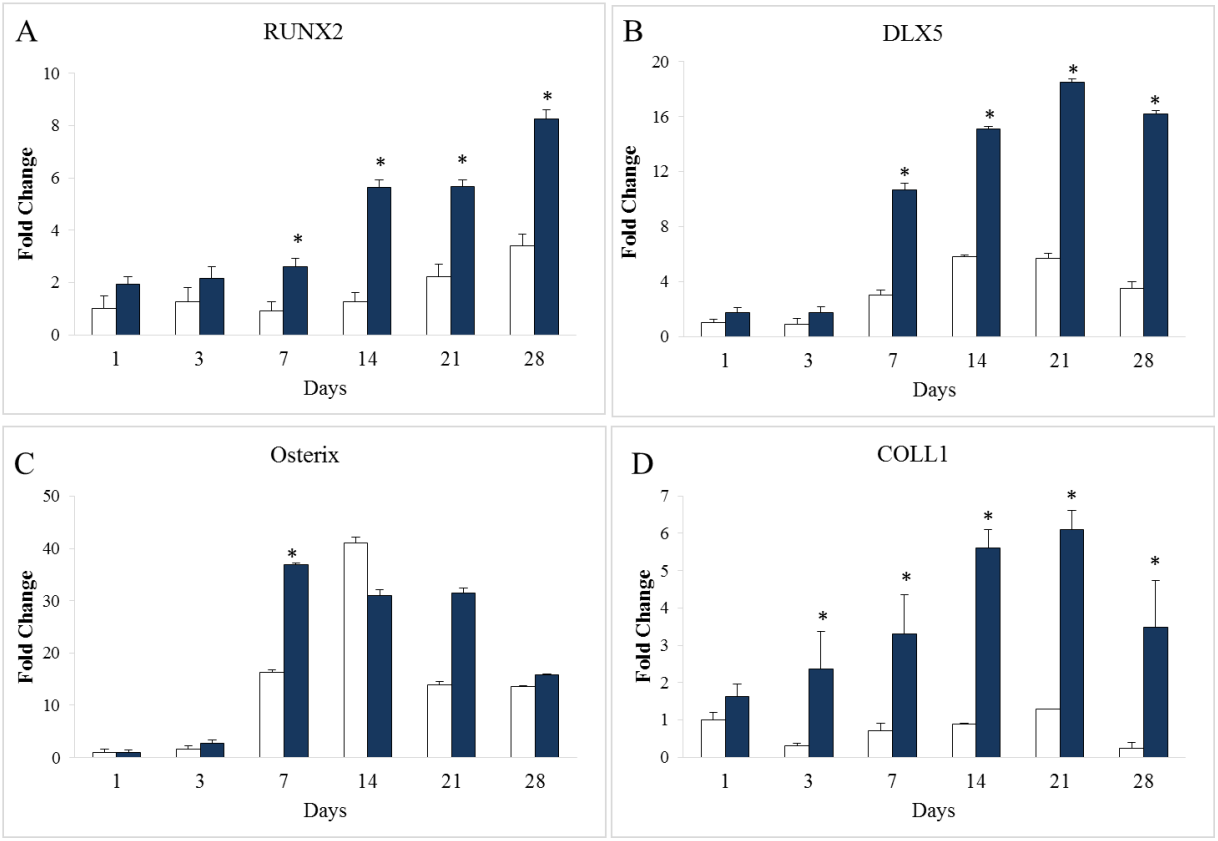


Figure 3-2: Collagen type I and osteogenic Transcription Factors gene expression by RT-PCR at different times during MG63 cells differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 28) cells were tested for Col type I and transcription factors gene expression. *Statistical significance OM versus control, at the corresponding time point.

3.2. Gene expression of Notch signaling components during MG63 differentiation

Notch nuclear genes: Among the Notch nuclear target genes investigated, during osteogenic differentiation significant variations were obtained for Hey1 and Hes5 comparing OM versus control (Figure 3-3A and D). Specifically, a significant overexpression of Hey1 respect to undifferentiated cells was observed from day 7 to day 28 with a maximum increase at day 21 (7.60 fold, $p<0.0001$). Differently, 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, $p=0.011$). Hey2 and Hes1 expression levels showed no change compared with the control at all the time points investigated (Figure 3-3B and C).

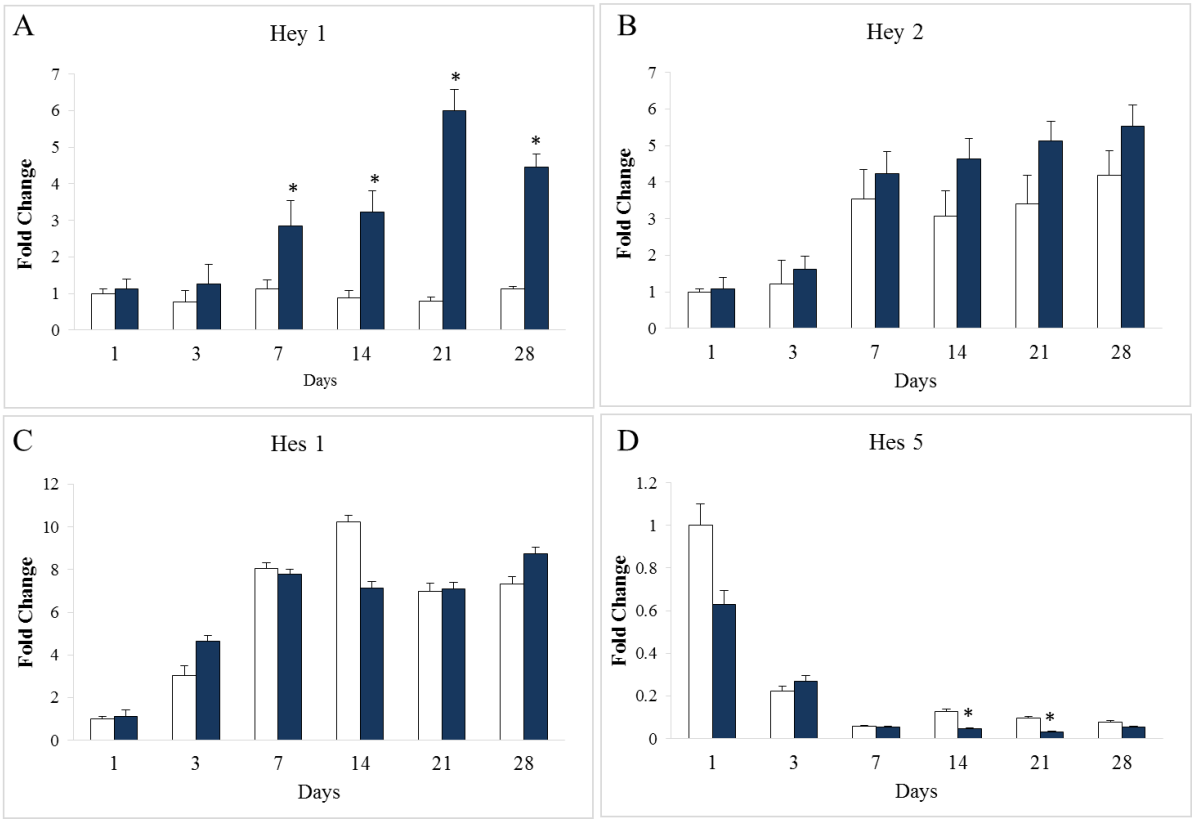


Figure 3-3: Notch Nuclear genes expression by RT-PCR at different times during MG63 cells differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 28) cells were tested for Notch nuclear genes expression. *Statistical significance OM versus control, at the corresponding time point.

Notch receptors: Notch receptors mRNA levels were evaluated in MG63 cells by RT-PCR during a period of 28 days in growth medium and in the presence of osteogenic supplements at days 1, 3, 7, 14, 21 and 28. Cells grown in osteogenic medium compared to control cells, showed changes in the expression of all the Notch receptors at specific times (Figures 3-4A-D). Specifically, Notch1 and Notch3 were down-regulated in osteogenic medium compared control group during the first two weeks from day 7 to14 (Figure 3-4A and C). On the other hand, Notch2 and Notch4 were up-regulated during the third and fourth weeks of the differentiation period comparing to the control group (Figure 3-4B and D).

Notch ligands: We also monitored the Notch ligands, Jagged1 and DLL4 in MG63 during 28 days of proliferation and differentiation. Results showed no significant changes in the expression levels of DLL4 and Jagged1 ligands in cells cultured in growth medium and in osteogenic differentiation medium (Figure 3-4E and F).

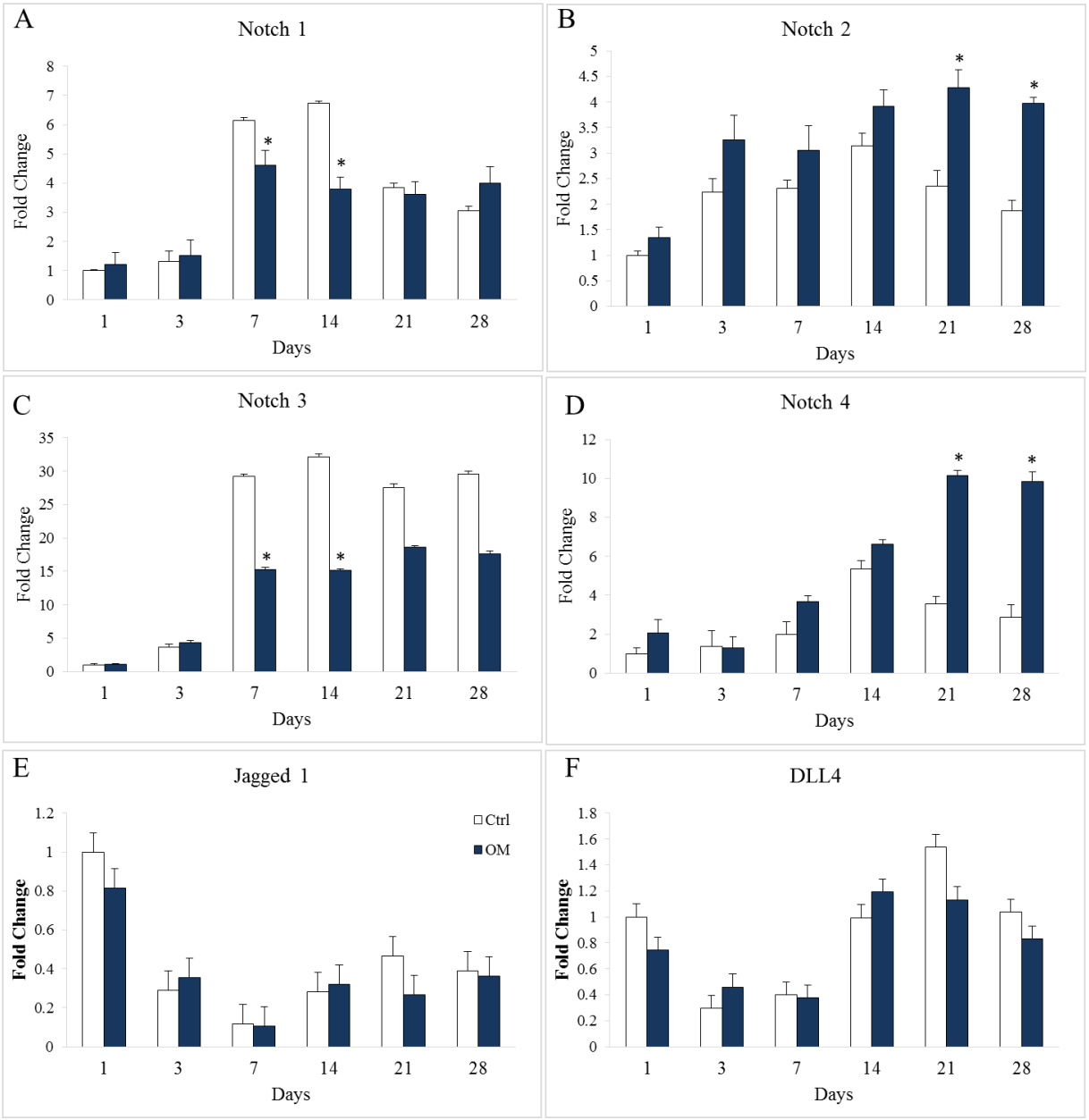


Figure 3-4: Notch receptors and ligands gene expression by RT-PCR at different times during MG63 cells differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 28) cells were tested for Notch receptors and ligands genes expression. *Statistical significance OM versus control, at the corresponding time point.

3.3. Effect of the Notch pathway inhibitor on MG63 cells

As reported in introduction, Notch signaling activation is dependent on two proteolytic cleavages catalyzed by ADAM and γ-secretase. Therefore, one way to inhibit Notch pathway is using γ-secretase inhibitors (Kanungo et al., 2008). To investigate the effects of loss of Notch signaling on MG63 cell osteogenic differentiation, we blocked Notch pathway by treating cells with a known γ-secretase inhibitor; DAPT and monitored the effects of early and late osteogenic markers. Results showed significant reduction in ALP activity, OC and mineralization in DAPT treated samples from day 14 to 28 of differentiation. DAPT decreased ALP activity at all time-points with significant reductions from day 14 to day 28 (Figure 3-5A). At the same time points, DAPT caused also a significant decrease in OC levels with a complete inhibition of OC production at day 14 and 21 during differentiation (Figure 3-5B). Similar results were obtained also by Alizarin red staining, as calcium deposits were reduced in the presence of DAPT. The reduction was more appreciable in the middle stage of differentiation (day 14-21) as shown in Figure 3-5C, reporting the spectrophotometric measurment of the dissolved Alizarin red staining. The reduction in the osteogenic biomarkers observed in the presence of DAPT were independent from the cell number because DAPT did not influence cell proliferation at any time point investigated (Figure 3-5D).

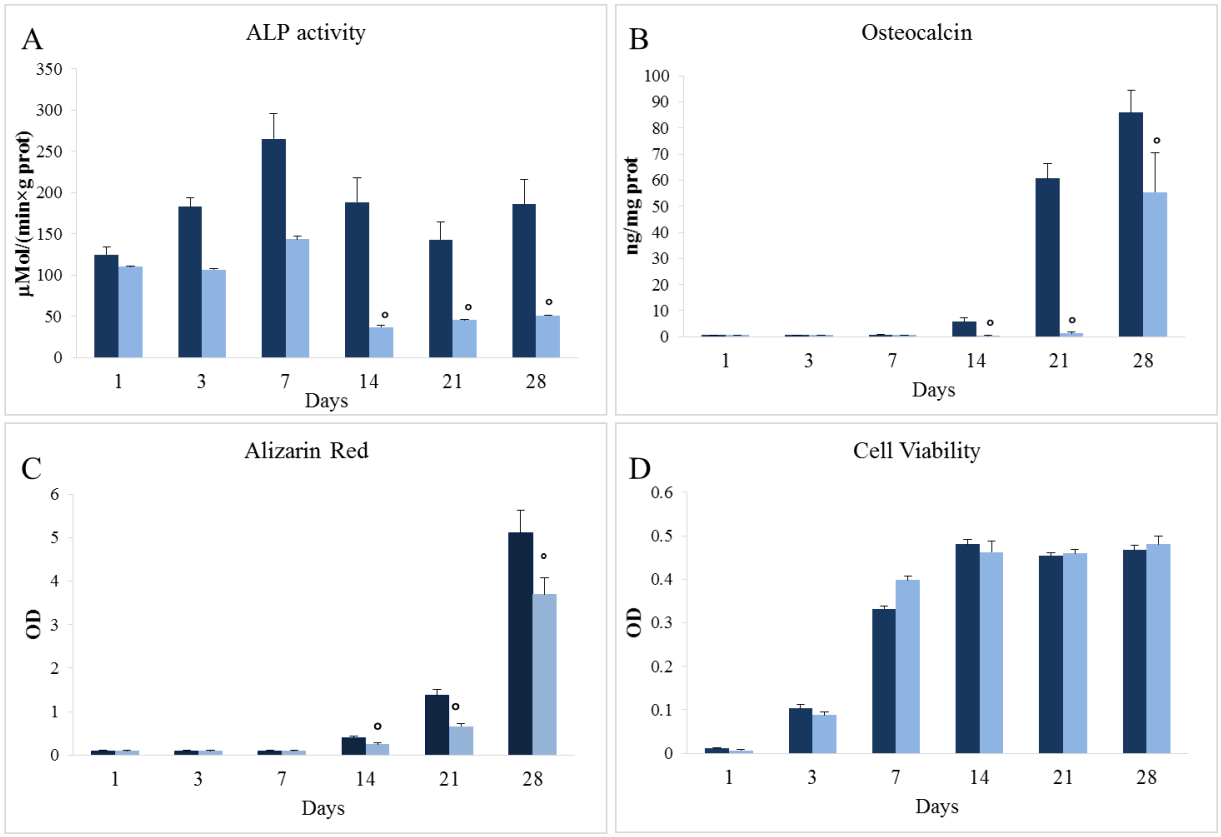


Figure 3-5: Biomarkers in the presence of DAPT during MG63 cells differentiation.

Cells were cultured in OM (black bar) or in OM+DAPT for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for ALP activity (A), osteocalcin levels (B), matrix mineralization by Alizarin red (C) and cell viability levels (D). The quantification of Alizarin red staining was done spectrophotometrically. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point.

3.4. Effects of osteogenic medium on hMSCs differentiation

Data obtained by using MG63 cell line as a model to investigate Notch pathway during osteogenic differentiation suggested that Notch signaling might be involved in this process and that inhibition of Notch pathway impairs osteogenic differentiation. To verify Notch signaling pathway involvement in differentiation of hMSCs, we next studied the response of hMSCs from bone marrow to osteogenic medium and evaluated changes in the levels of early and late osteogenic markers, as well as mRNA levels of the main osteogenic transcription factors (Runx2, DLX5, Osx) and Notch components including Notch nuclear genes, receptors and ligands.

3.4.1. Osteogenic markers

ALP activity: Alkaline phosphatase activity is an early marker usually used to monitor cell differentiation toward osteoblast lineage in vitro (Orimo, 2010). As Figure 3-6A shows, ALP enzyme activity was very low in hMSCs cultured in growth medium at all-time points (3, 7, 14, 21, and 28) of cell growth. However, ALP activity increased in the presence of the osteogenic medium from day 3 to 28 with differences which were statistically significant from day 7 to 28.

Osteocalcin: In Figure 3-6B we show osteocalcin levels, a late osteogenic marker (Ryoo et al., 1997), measured from day 7 to day 28, as OC levels were not detectable at day 3 (data not shown). As expected, OC was not detected in hMSCs cultured in growth medium at any time point. However OC levels were elevated significantly from day 21 (137 fold) to 28 (229 fold) in osteogenic medium respect to control.

Alizarin Red Staining: Deposited mineral is another marker of osteogenic differentiation that is detected during the late phase of differentiation (Marom et al., 2005). Here we examined deposited mineral by alizarin red staining from day 7 to 28. As expected, in the absence of osteogenic medium no nodule formation observed, however, cells cultured in osteogenic medium exhibited nodules from day 14 that increased until day 28. The quantification of Alizarin red staining was done spectrophotometrically and showed significant increases at 21 (0.72 fold) and 28 days (1.6 fold) (Figure 3-6C).

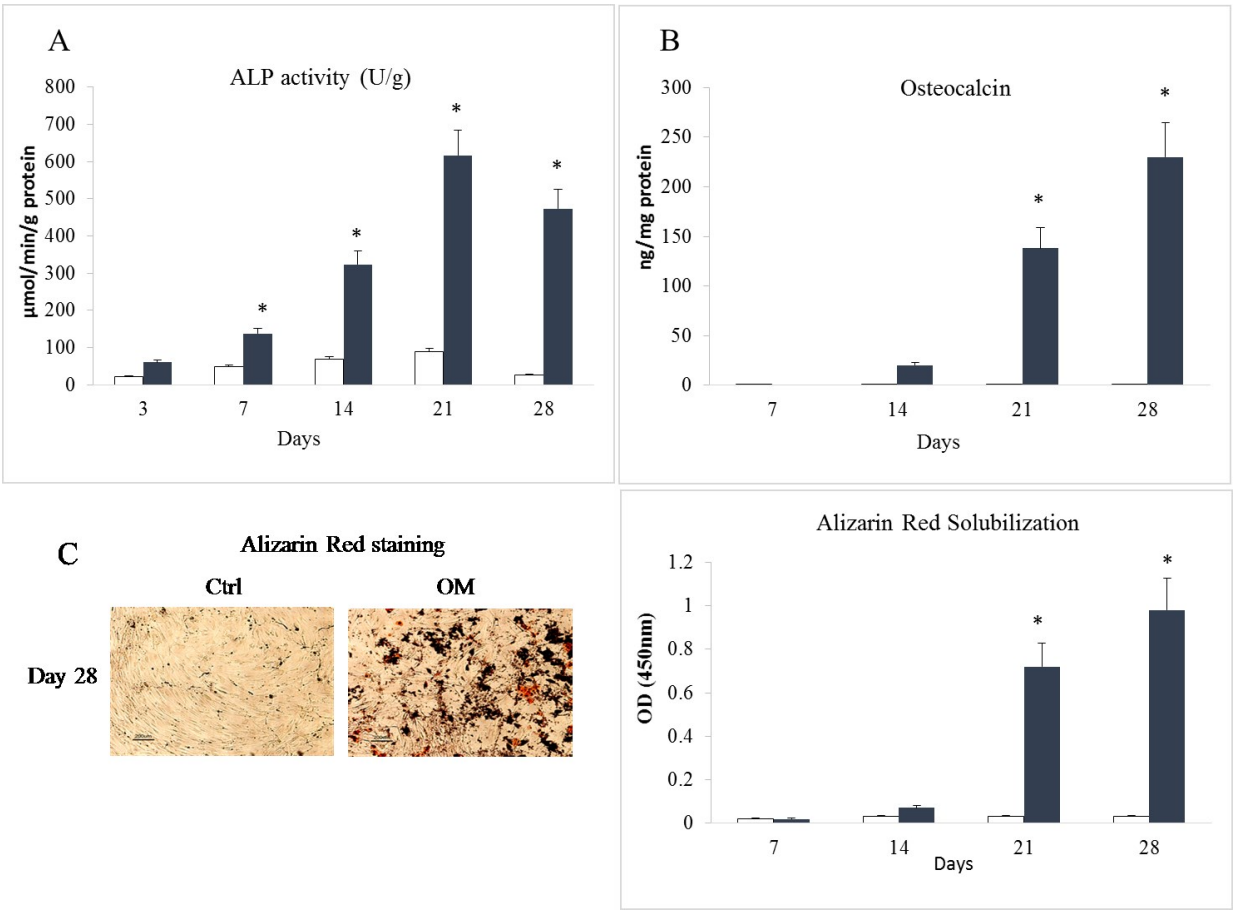


Figure 3-6: Biomarkers during hMSC differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 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. *Statistical significance OM versus control, at the corresponding time point.

3.4.2. Osteogenic transcription factors

Data obtained by analyzing mRNA levels of the specific TFs including Runx2, Osx and DLX5 that are expressed during osteogenesis (Marom et al., 2005) are shown in Figure 3-7A-C. During differentiation of hMSCs, a significant increase in the expression of Runx2 (4.31-fold), DLX5

(2.11-fold) and Osx (4.66-fold) was observed at day 3 of differentiation, comparing hMSCs cultured in osteogenic medium to control. Osx upregulation showed a pattern different from Runx2 and DLX5, as it remained upregulated in OM group until day 14 (2.32 fold at day 7; 3.05 fold at day 14) compared to control group (Figure 3-7C).

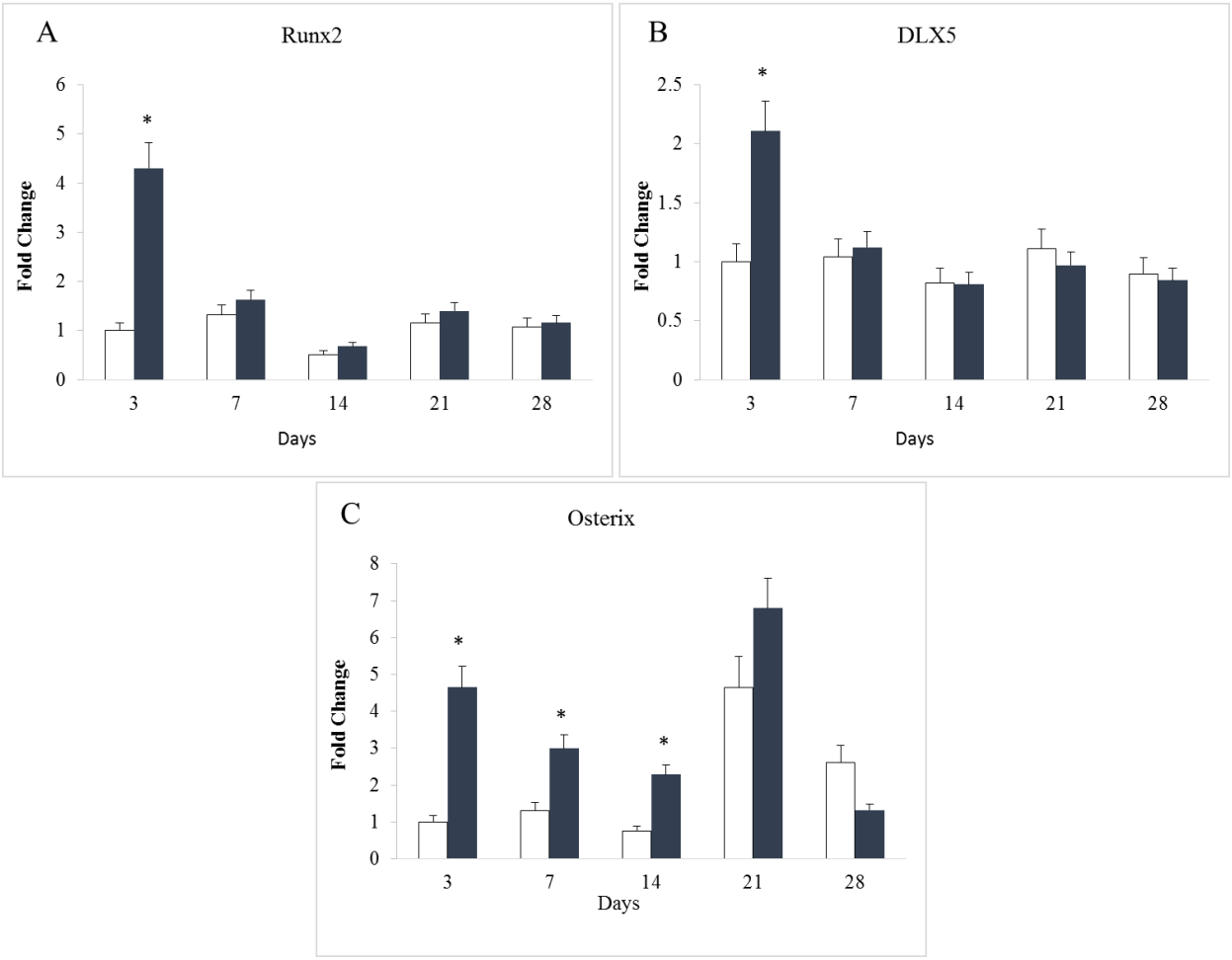


Figure 3-7: Osteogenic Transcription Factors gene expression by RT-PCR at different times during hMSCs differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 28) cells were tested for transcription factors genes expression. *Statistical significance OM versus control, at the corresponding time point.

3.5. Gene expression of Notch components during hMSCs differentiation

3.5.1. Notch nuclear genes

In order to elucidate the potential role of Notch pathway in osteogenic differentiation of hMSC, similarly to what performed on MG63, we also investigated the expression levels of Notch nuclear genes including Hey1, Hey2, Hes1 and Hes5 during the whole period of differentiation. Figure 3-8A – D shows the expression of the Notch nuclear genes evaluated at different times in cell treated with both osteogenic and control medium. Notch nuclear genes expression changed differently during differentiation. Specifically Hes5 exhibited a significant up-regulation in osteogenic medium compared to control from day 7 to 28 (Figure 3-8A). Differently, Hey1 (2.34-fold at day 21; 3.39-fold at day 28) and Hes1 (3.41-fold at day 21; 5.20-fold at day 28) were significantly up-regulated in the late phase of differentiation in osteogenic medium respect to control (Figure 3-8B-C). Hey2 showed no significant change in gene expression during the whole differentiation period (Figure 3-8 D).

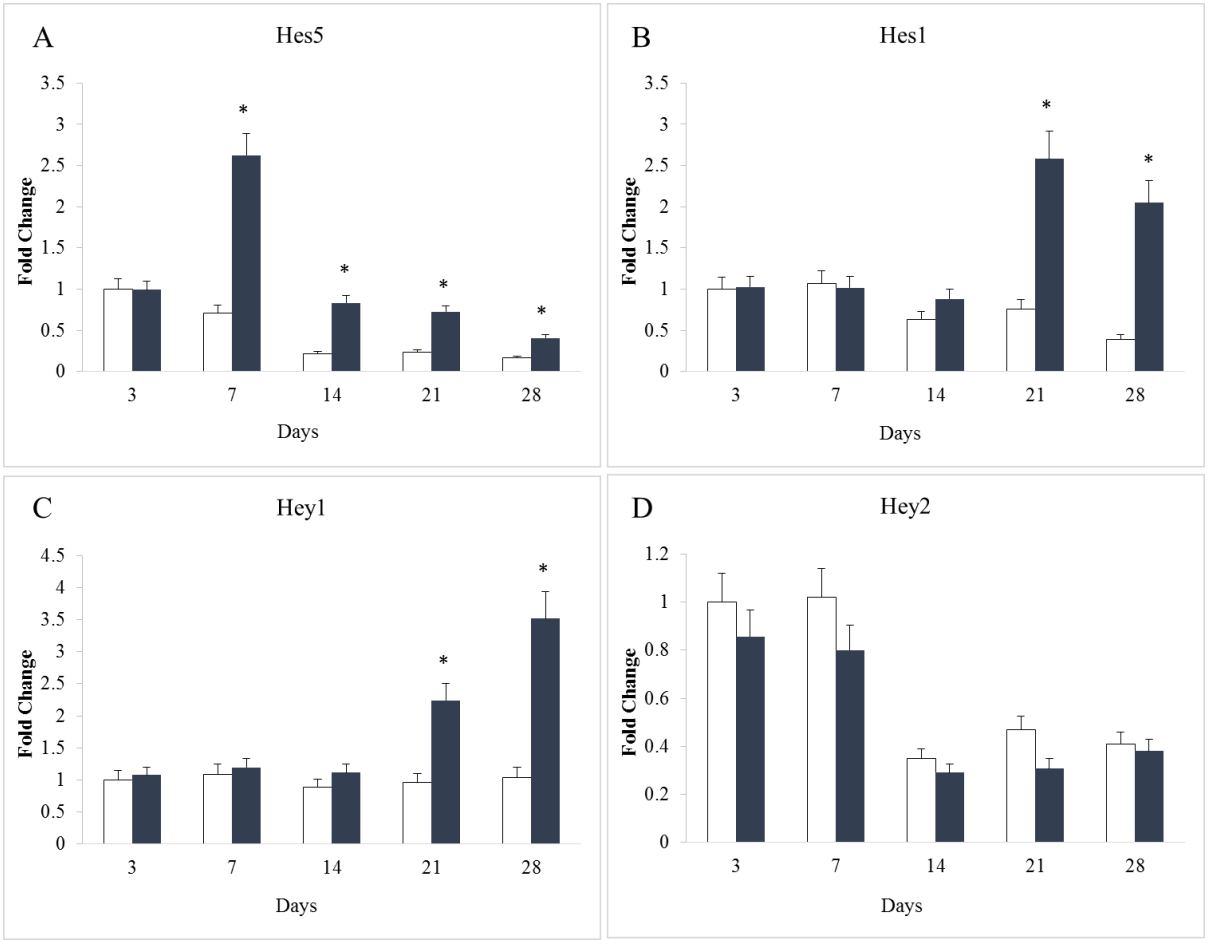


Figure 3-8: Notch Nuclear Genes expression by RT-PCR at different times during hMSC differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 28) cells were tested for Notch nuclear genes expression. *Statistical significance OM versus control, at the corresponding time point.

3.5.2. Notch Receptors and Ligands

Gene expression levels of the four Notch receptors, Notch1-4, and the Notch ligands including Jagged1, DLL1 and DLL4 were investigated during 28 days of human mesenchymal stem cell differentiation. When we compared the gene expression levels in cells cultured in osteogenic medium and in control cells, we did not observe any significant change in Notch 1, Notch 2, Notch3 receptors and in the ligands Jagged and DLLl at all the time points investigated (data not shown). In comparison to control cells, cells treated with ostegenic medium showed significant changes only in the expression of the Notch4 receptor and the ligand DLL4 at specific times. Specifically, while Notch4 was significantly increased (4.53-fold) at day 3 in the early phase of differentiation comparing to the control, DLL4 expression levels were significantly elevated in OM compared to control, in the late phase of differentiation from day 21 (5.18 fold) to 28 (4.40 fold). (Figure 3-9 A-B).

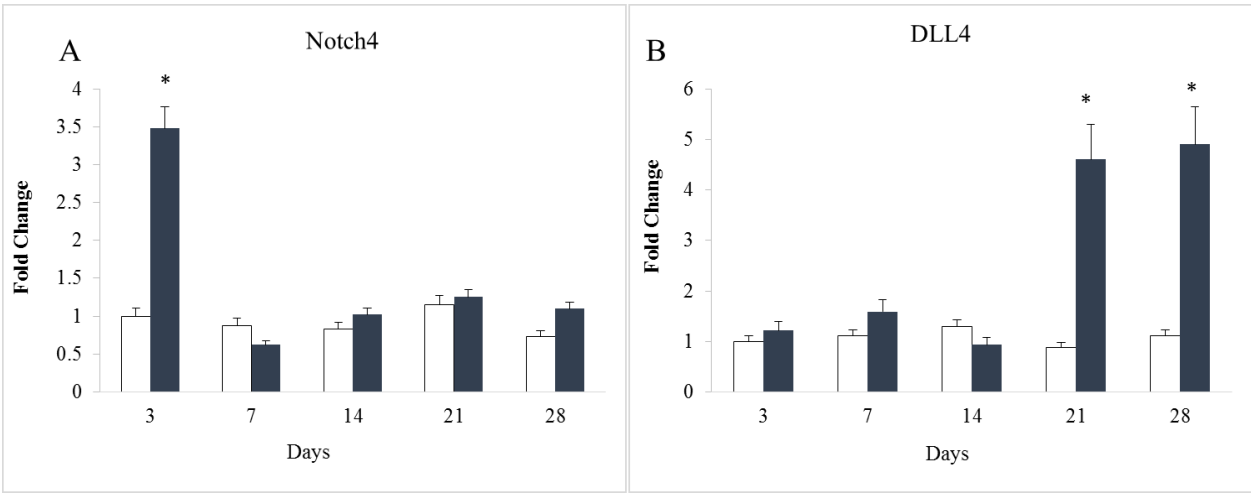


Figure 3-9: Notch receptor and ligand gene expression by RT-PCR at different times during hMSC differentiation.

Cells were cultured in control (white bar) or in OM (black bar) for 28 days. At different time points (1, 3, 7, 14, 21, and 28) cells were tested for Notch receptor and ligand genes expression. *Statistical significance OM versus control, at the corresponding time point.

3.6. Effects of the Notch inhibitor DAPT on hMSCs osteogenic differentiation and Notch components gene expression

Changes in gene expression of Notch molecules during hMSCs osteogenic differentiation suggested involvement of Notch pathway in the process. To investigate the role of Notch signaling more in depth, similarly to what performed on MG63, we blocked Notch pathway also in hMSCs by using a γ-secretase inhibitor; DAPT (Kanungo et al., 2008).

3.6.1. Osteogenic markers

ALP activity, OC and mineral contents of hMSCs were measured during the whole period of 28 days in osteogenic supplement with and without DAPT. Results are shown in Figure 3-10A – C. The presence of DAPT caused a significant reduction in ALP activity at days 14 (-61%) and 21 (-66%) of differentiation in comparison to cells cultured in osteogenic medium alone. Further, DAPT treatment also induced significant decrease in OC levels at days 21 (-77%) and 28 (-82%) of differentiation comparing to OM group as well as a in mineral contents at the same time points.

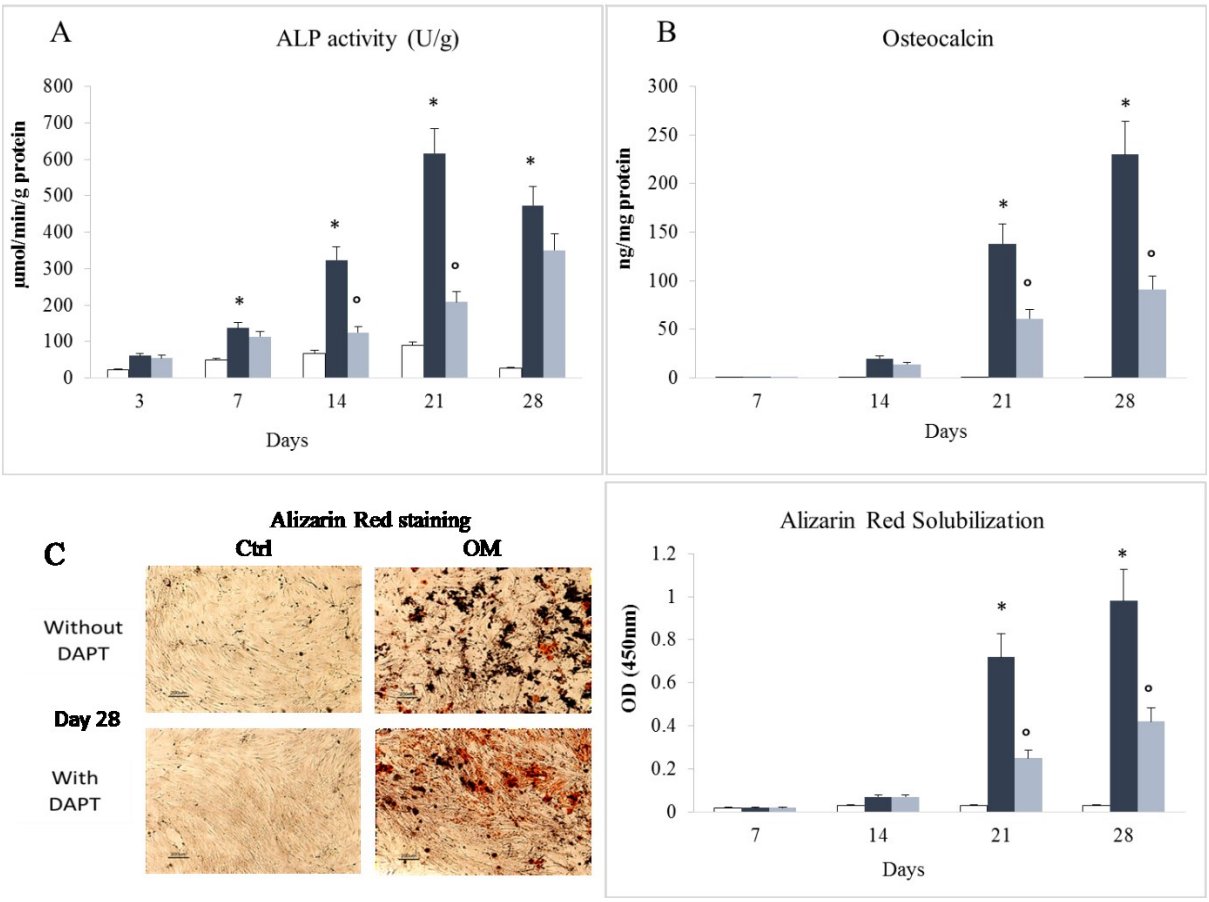


Figure 3-10: Biomarkers in the presence of DAPT during hMSC differentiation.

Cells were cultured in control (white bar) or in OM (dark blue bar) or in OM+DAPT (light blue bar) for 28 days. At different time points (3, 7, 14, 21, and 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. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point.

3.6.2. Osteogenic Transcription factors

We also investigated DAPT effects on osteogenic TFs; RUNX2, DLX5 and Osx. DAPT treatment significantly reduced the expression levels of Runx2, DLX5 and Osx from day 7 to 21 (Figure 3-11A – C).

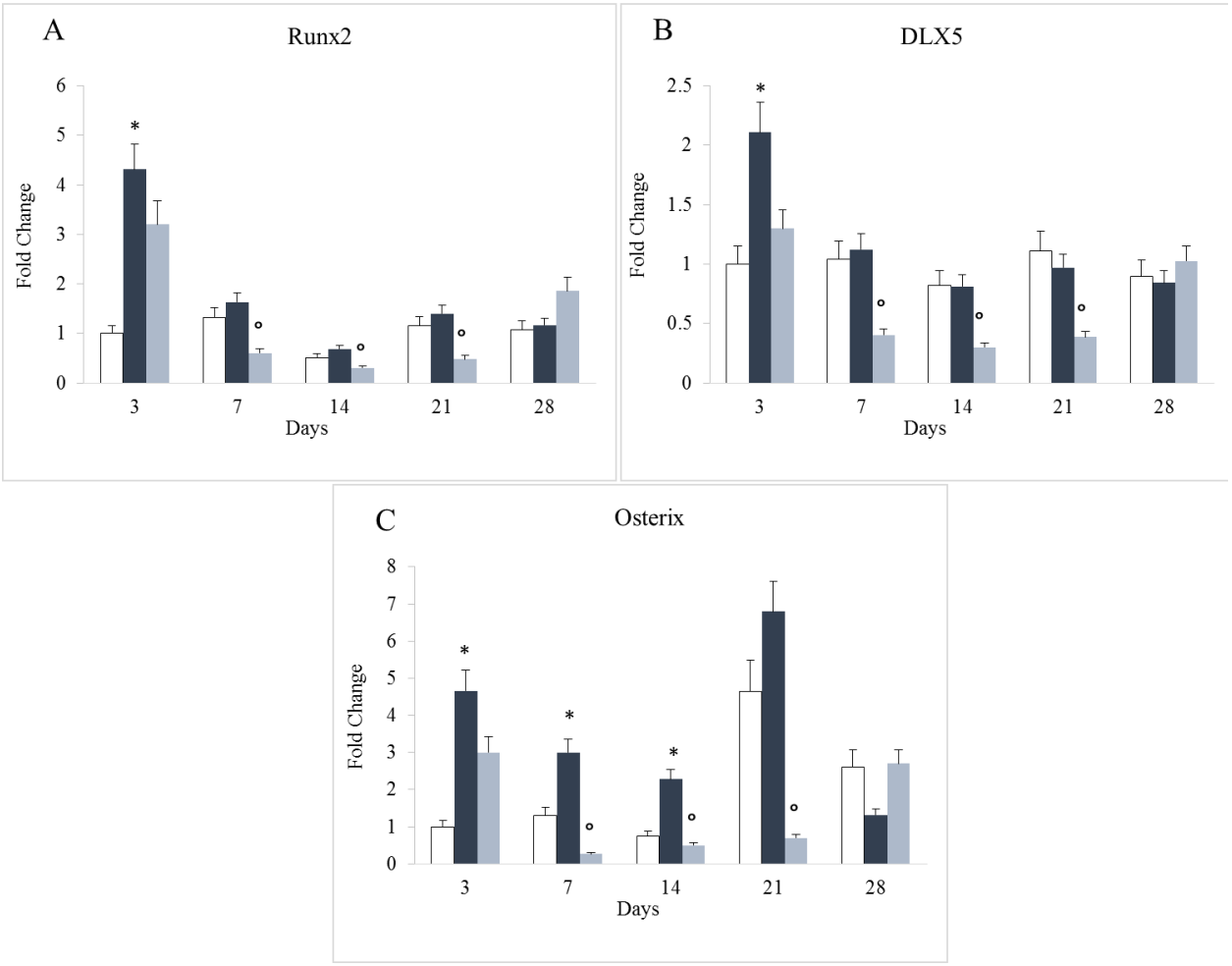


Figure 3-11: Transcription Factors in the presence of DAPT during hMSC differentiation.

Cells were cultured in control (white bar) or in OM (dark blue bar) or in OM+DAPT (light blue bar) for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for transcription factors genes expression. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point.

3.6.3. Notch signaling components

The inhibition of osteogenic differentiation by DAPT treatment suggests that Notch signaling is involved in the process. Therefore, we also studied the expression of Notch target nuclear genes by analyzing of their expression levels. In the presence of DAPT, we observed a significant reduction in the expression of Hes5 and Hes1 levels which were increased in osteogenic medium compared to control medium (Figure 3-12A-B). On the other hand, Hey1 which was upregulated

at the late phase of differentiation showed no reduction by DAPT treatment, suggesting the potential involvement of a Notch-independent pathway in the regulation of Hey1 expression during differentiation (Figure 3-12C).

Differently from the effects observed in Notch target genes, in the presence of DAPT we did not observe any significant change in the expression of Notch receptors and ligands (data not shown).

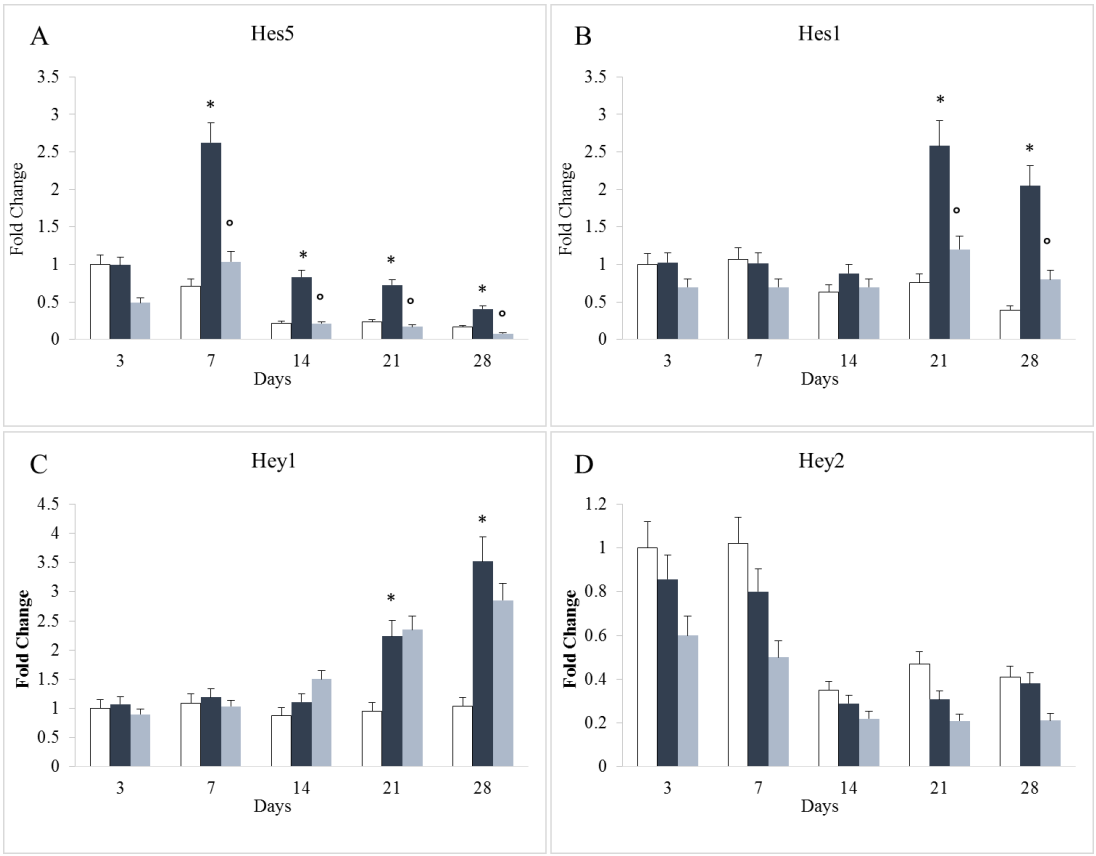


Figure 3-12: Notch Nuclear genes in the presence of DAPT during hMSC differentiation.

Cells were cultured in control (white bar) or in OM (dark blue bar) or in OM+DAPT (light blue bar) for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for Notch nuclear genes expression. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point.

3.7. PEMF effects on hMSCs osteogenic differentiation and Notch signaling

3.7.1. Osteogenic markers

The effects of PEMFs on differentiation of hMSCs toward osteoblast lineage were evaluated by comparing the levels of osteogenic markers; ALP activity, OC and nodule formation in PEMF exposed cells compared to cells cultured in OM. As previously reported (Ongaro et al., 2014), an enhancement of osteogenic differentiation with time-dependent variations of osteogenic markers was observed when hMSCs grown in OM were exposed to PEMFs.

Results in figure 3-13A-C show that PEMFs stimulate ALP activity at days 7 (+41%), 14 (+51%) and 21 (+66%) comparing to OM group. In addition, PEMFs determined a significant enhancement of OC production at day 21 (3.16-fold) and 28 (4.71-fold) respect to unexposed cells cultured in OM. Similarly, alizarin red staining increased in PEMFs-exposed hMSCs, confirming a greater matrix mineralization in the late stage of differentiation under PEMF exposure compared to OM group.

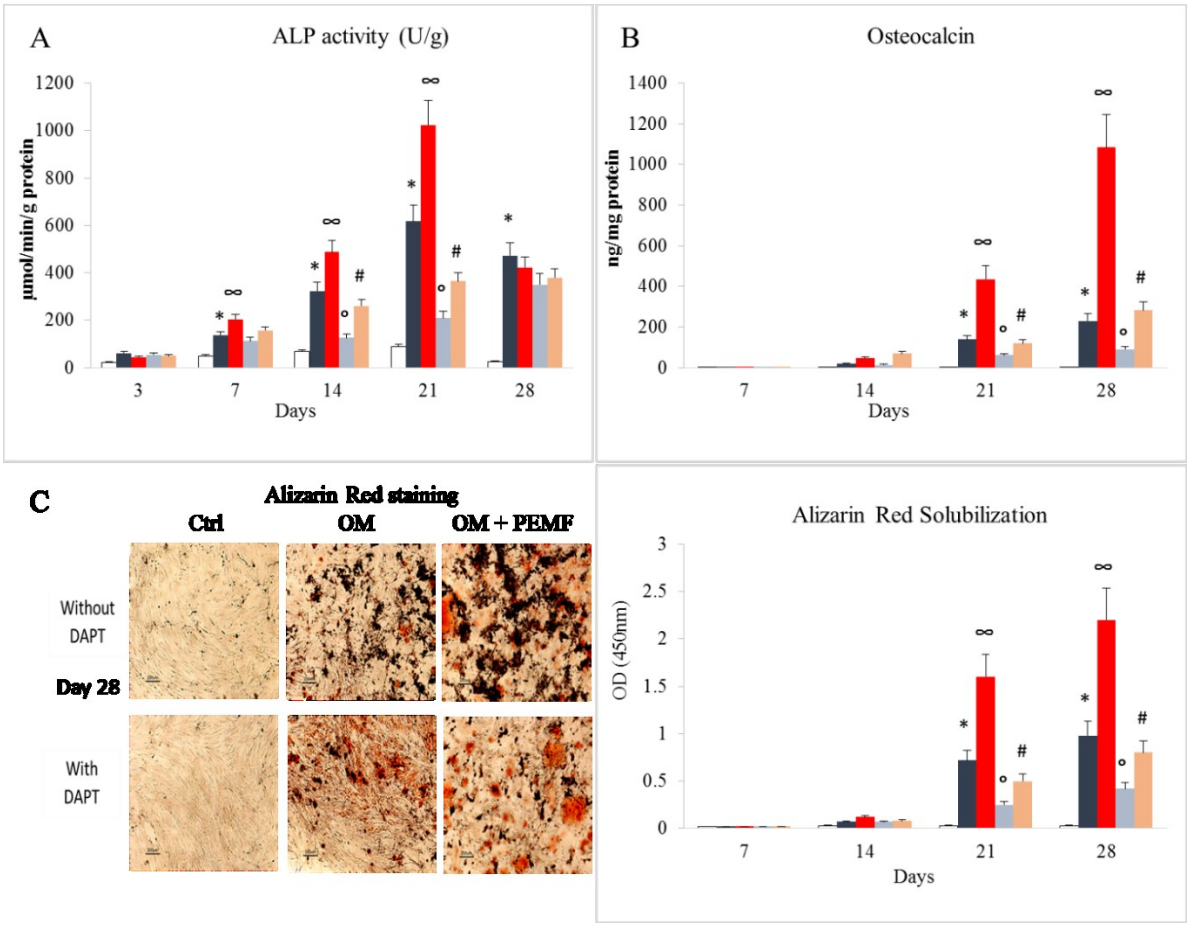


Figure 3-13: Biomarkers under the PEMF exposure during hMSC differentiation in the absence and in the presence of DAPT.

Cells were cultured in control (white bar) or in OM (dark blue bar), OM+DAPT (light blue bar), OM+PEMF (red bar), or in OM+PEMF+DAPT (pink bar) for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for ALP activity (A), osteocalcin levels (B) and matrix mineralization by Alizarin red (C). *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point. ∞ Statistical significance OM+PEMF versus OM, at the corresponding time point. # Statistical significance OM+PEMF+DAPT versus OM+PEMF, at the corresponding time point.

3.7.2. Osteogenic transcription factors

Expression levels of TFs including Runx2, DLX5 and Osx in the samples under PEMFs exposure were investigated. Figure 3-14A-C shows significant increase in the early activation of all the TFs by PEMFs at day 3 (Runx2: +2.80-fold; Dlx5: +2.30-fold; Osx: +3.09-fold). In addition, PEMFs prolonged their overexpression also at day 7 for Runx2 (+2.02-fold), Osx (+3.35-fold) and DLX5 (+1.84-fold) and at days 14 (+2.22-fold), 21(+2.47-fold) and 28(+4.74-fold) only for DLX5 (Figure 3-14A-C).

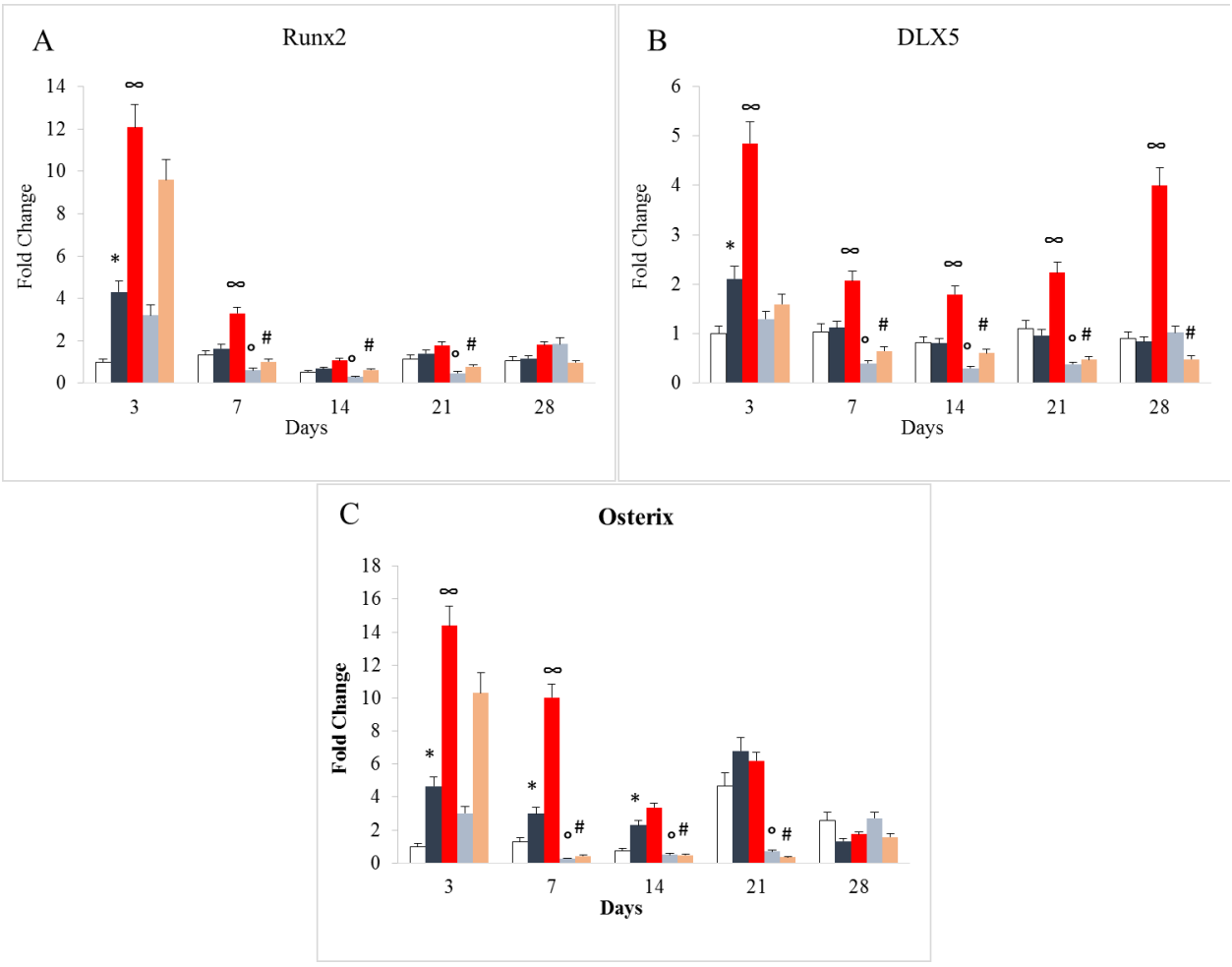


Figure 3-14: Osteogenic Transcription Factors under the PEMF exposure during hMSC differentiation in the absence and in the presence of DAPT.

Cells were cultured in control (white bar) or in OM (dark blue bar), OM+DAPT (light blue bar), OM+PEMF (red bar), or in OM+PEMF+DAPT (pink bar) for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for transcription factors genes expression. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point. ∞ Statistical significance OM+PEMF versus OM, at the corresponding time point. # Statistical significance OM+PEMF+DAPT versus OM+PEMF, at the corresponding time point.

3.7.3. Notch signaling components

In order to investigate how pulsed electromagnetic fields elevated and accelerated osteogenic differentiation of hMSCs evidenced by increasing ALP activity, OC and mineralization, we questioned whether changes in osteogenic markers by PEMFs may be related to changes in Notch signaling.

The expression patterns of Notch nuclear genes in PEMFs-exposed cells compared to cells cultured in OM are shown in figure 3-15A-C. Interestingly, in a specific timing (day 14 and 21), PEMF exposure induced a significant overexpression in the same Notch nuclear genes which appeared upregulated during cell differentiation in OM in the absence of PEMFs. Comparing to OM group, PEMFs stimulated Hes5 mRNA expression from day 14 (+2.18-fold) to 21(+2.19-fold) (Figure 3-15A). We also found that up-regulation in the expression levels of Hey1 and Hes1 were anticipated one week in the samples under the PEMF exposure. Indeed, PEMF exposure induced the significant overexpression of both Hey1 (+2.49-fold at day 14 and +2.44-fold at day 21) and Hes1 (+3.64-fold at day 14 and +2.01-fold at day 21) (Figure 3-15B-C).

These results suggested a role of PEMFs in promoting changes in Notch nuclear genes expression at specific times that appeared to be temporally associated with the middle-late stage of differentiation process.

Figure 3-16A-B shows the results for Notch4 receptor and the ligand DLL4 gene expression changes in the presence of PEMFs. PEMFs upregulated DLL4 expression at day 14 (11.13 fold) and 21 (4.68 fold) comparing to the OM group. Notably, PEMFs exposure maintained a higher Notch4 expression also at day 7 (+2.59-fold) in exposed cells respect to cells maintained in OM

alone, prolonging the increased expression of this receptor observed in OM, in the early phase of differentiation (at day3). No significant effect of PEMFs was observed on the other ligands (Jagged1 or DLL1) and Notch receptors (Notch1, Notch2 and Notch3) mRNA expression.

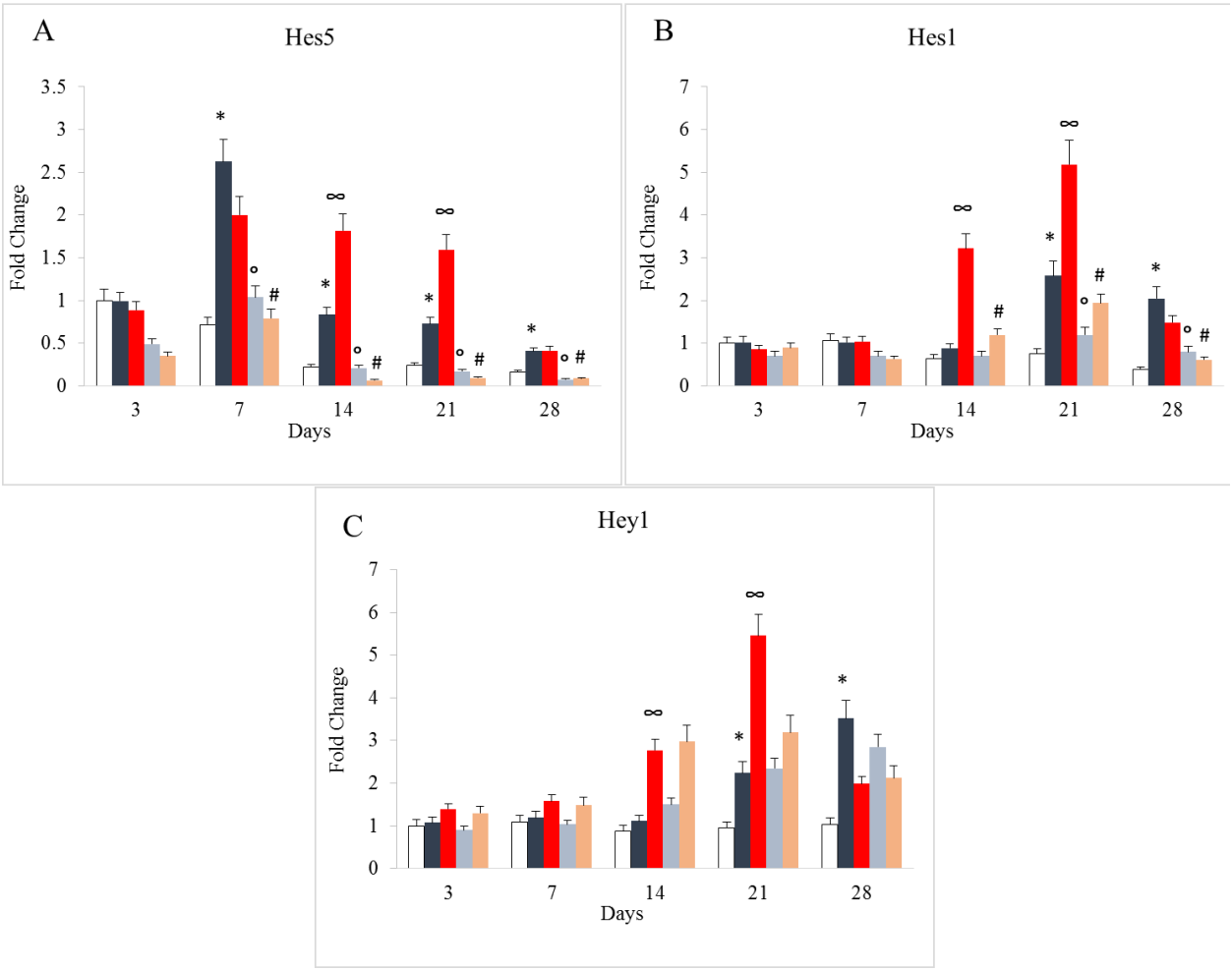


Figure 3-15: Notch Nuclear genes under the PEMFs exposure during hMSC differentiation in the absence and in the presence of DAPT.

Cells were cultured in control (white bar) or in OM (dark blue bar), OM+DAPT (light blue bar), OM+PEMF (red bar), or in OM+PEMF+DAPT (pink bar) for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for Notch nuclear genes expression. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance

OM+DAPT versus OM, at the corresponding time point. ∞ Statistical significance OM+PEMF versus OM, at the corresponding time point. # Statistical significance OM+PEMF+DAPT versus OM+PEMF, at the corresponding time point.

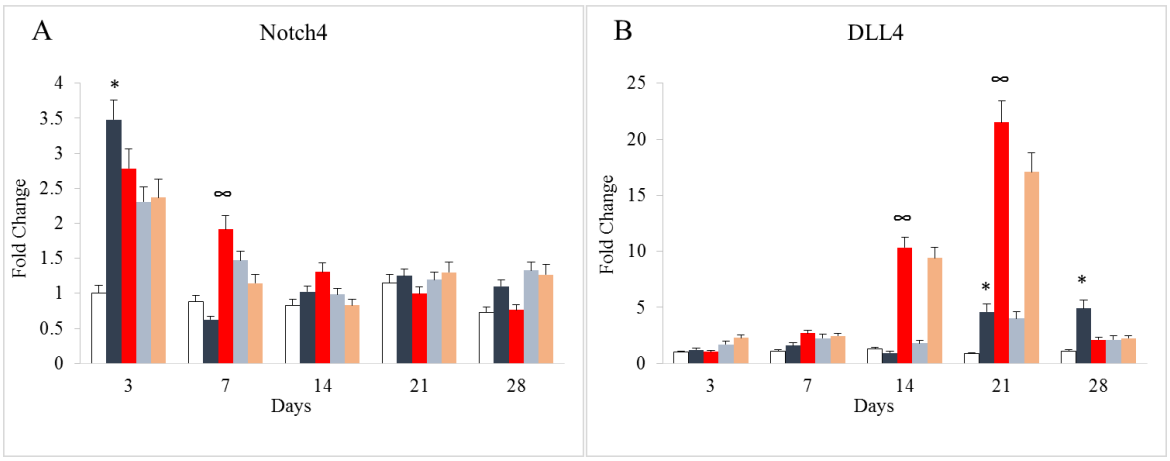


Figure 3-16: Notch ligand and receptor under the PEMFs exposure during hMSC differentiation in the absence and in the presence of DAPT.

Cells were cultured in control (white bar) or in OM (dark blue bar), OM+DAPT (light blue bar), OM+PEMF (red bar), or in OM+PEMF+DAPT (pink bar) for 28 days. At different time points (3, 7, 14, 21, and 28) cells were tested for Notch receptor and ligand genes expression. *Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point. ∞ Statistical significance OM+PEMF versus OM, at the corresponding time point. # Statistical significance OM+PEMF+DAPT versus OM+PEMF, at the corresponding time point.

3.8. Effects of the Notch inhibitor DAPT on PEMF-induced osteogenic differentiation of hMSCs

Also in PEMFs exposed cells, treatment with DAPT induced effects similar to those observed in the absence of PEMFs, by inhibiting osteogenic markers. In the presence of DAPT and PEMFs, ALP was reduced at day 14(-47%) and at day 21 (-64%) compared with OM+PEMFs. Similarly, DAPT significantly inhibited also OC levels in OM+DAPT+PEMFs group at days 21 (-72%) and also 28 (-74%) respect to OM+PEMFs group, as well as mineral deposition (Figure 3-13A-C).

Further, DAPT treatment reduced significantly the expression of the osteogenic transcription factors from day 7 to 21, except for DLX5 in which the reduction was observed also at day 28, comparing OM+DAPT+PEMFs and OM+PEMFs groups, suggesting a partial control of Notch pathway on these transcription factors also in the presence of PEMFs (Figure 3-14A-C).

Finally, under DAPT treatment, we observed a decrease in Hes5 and Hes1 expression comparing OM+DAPT+PEMF with OM+PEMF suggesting that PEMFs could stimulate osteogenesis at least partially through Notch signaling. Differently, no modulation by DAPT was found on Hey1, the Notch ligands and receptors (Figure 3-15A-C).

3.9. Immunoblotting analysis

As the analysis of gene expression on Notch pathway related molecules during differentiation of hMSCs in the absence and in the presence absence showed differences in the expression of the Notch4 receptor and the ligand DLL4 at specific times, we also evaluated the effects of osteogenic medium and PEMF exposure on the Notch4 receptor and the ligand DLL4 by Western blotting analysis. The immunoblot images, reported in Figure 3-20 A, show the pattern bands of Notch4 and DLL4 in lysates from hMSCs cultured in different conditions (Ctrl, OM, OM+DAPT) in the absence of PEMF exposure. By using an antibody against the C-terminus of the Notch4 protein that detected a fragment of 64 KDa, corresponding to the active form of Notch4 (Caliceti et al., 2013), we observed a significant difference among specific treatments. In fact, at three days in OM an increase of the active Notch4 was observed respect to control, which corresponded to the increase in gene expression induced by OM, previously described. Further, in DAPT we observed

a decrease in the active form of the receptor. The analysis at day 21 of resulted bands corresponding to the full-length precursor (74 KDa) of the ligand DLL4 showed an increase in the intensity of the band in OM versus control, which also corresponded to the increase observed in gene expression. No effect of DAPT was identified in the intensity of the band.

The immunoblotting of samples obtained from PEMF-exposed cells showed also that PEMFs could increase the active form of the Notch4 receptor at day 7 as well as DLL4 at day 21 respect to cells cultured in OM, in line with gene expression results (Figure 3-20 B). Also in the presence of PEMFs, DAPT inhibited the amount of the active form of the Notch4 receptor, whilst did not modify DLL4.

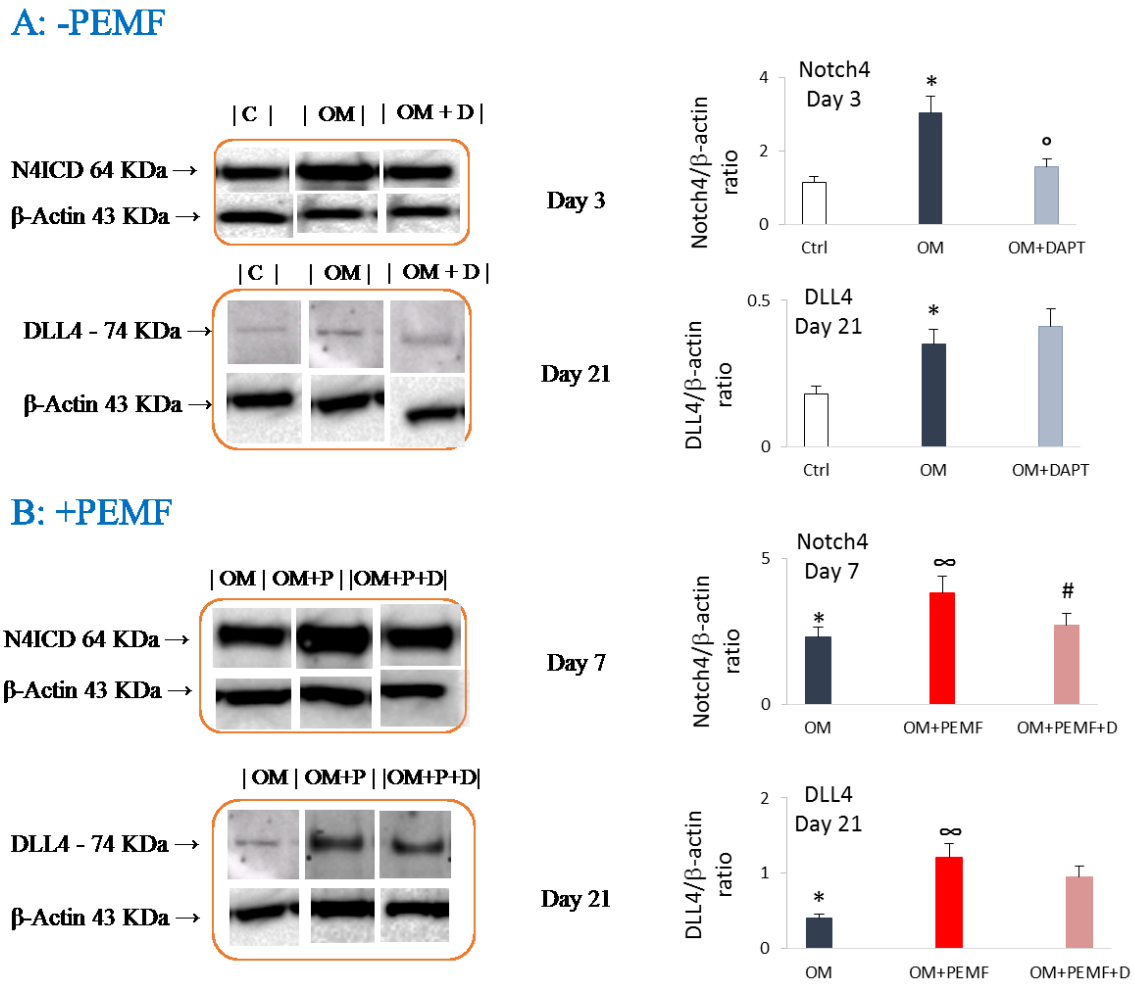


Figure 3-17: Immunoblotting for the active form of the Notch4 receptor and for the ligand DLL4 in hMSCs in the absence and presence of PEMFs.

A) Immunoblotting for the active form of the Notch4 receptor (at day 3) and for the ligand DLL4 (at day 21) in hMSCs in the absence of PEMFs. Cells were cultured in control medium (Ctrl), in OM without (OM) and with DAPT (OM+D). **B**) Immunoblotting for the active form of the Notch4 receptor (at day 7) and for the ligand DLL4 (at day 21) in hMSCs in the presence of PEMFs. Cells were cultured in OM (OM), in OM and PEMF exposure without (OM+PEMF) and with DAPT (OM+PEMF+DAPT). Cell lysates were electrophoresed and immunoblotted with the antibodies for Notch4, and DLL4. β-actin antibody was used to ensure equal loading. Densitometric analysis of Western blot assay quantified protein levels. Results are expressed as mean ±SEM of three independent experiments. * Statistical significance OM versus control, at the corresponding time point. ° Statistical significance OM+DAPT versus OM, at the corresponding time point. $^{\infty}$ Statistical significance OM+PEMF versus OM, at the corresponding time point. $^{\#}$ Statistical significance OM+PEMF+DAPT versus OM+PEMF, at the corresponding time point.

4. Discussion

Osteogenic differentiation of MSCs is a complex process which naturally occurs in embryos during bone development as well as in adults during bone healing and repair. Further, large bone defects due to bone diseases or non-union fractures necessitate the use of bone tissue engineering approaches in which MSCs and their ability to differentiate towards osteoblasts play essential role. (Fayaz et al., 2011; Marolt, Knezevic, & Novakovic, 2010; Rosenbaum, Grande, & Dines, 2008).

The osteogenic differentiation process is modulated by several growth factors including bone morphogenetic proteins (BMPs), transforming growth factor (TGF) beta, fibroblast growth factor (FGF), insulin-like growth factor (IGF), plateletderived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (A. H. Simpson, Mills, & Noble, 2006) and several signal transduction pathways have been identified acting during osteogenic differentiation. They include BMP signaling, Wnt/b-catenin signaling, Hedgehog (HH) signaling, and Notch signaling pathways (Ai-Aql, Alagl, Graves, Gerstenfeld, & Einhorn, 2008; Javed et al., 2010). However the exact involvement of each of these pathways, as well as the integration and cooperation among them remains to be clarified. In particular, in this study we aimed to clarify the potential involvement of the Notch signaling pathway in osteogenic differentiation and the results collected reveal several novel findings concerning the significant role of this pathway during MSCs osteogenic differentiation.

Notch pathway is a conservative pathway involved in several essential cell events including cell fate and differentiation (Artavanis-Tsakonas et al., 1999). In particular, Notch signaling has been implicated in bone development during embryogenesis as well as in bone fracture healing in adults (Dishowitz et al., 2013).

However, in spite of the previous in vitro and in vivo studies investigating the role of Notch pathway in osteogenic differentiation, to date no definitive and clear conclusion can be derived (Chen et al., 2014). Some of previous 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, as both inhibitory (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 gain or loss of function in specific and single Notch receptors or Notch related genes. Further, the effects mostly were evaluated only in early phase of the differentiation process. Therefore, in this study, we aimed to characterize the Notch signaling pathway in spontaneous osteogenic differentiation that is without any genetic manipulation of cells and during the whole period of cell differentiation in vitro. To this aim we analyzed the expression of all four Notch receptors, some of the Notch ligands and the main nuclear target genes and evaluated the possible correlation with the expression of the typical osteogenic markers.

Due to the limited number of hMSCs, at first we investigated potential changes in the gene expression of Notch signaling molecules during the differentiation of the osteosarcoma MG63 cell line, used as an in vitro cellular model of differentiation (Kraus et al., 2012). To our aims we used a typical protocol reported in several differentiative studies to induce osteogenic differentiation (Cheng et al., 2011; Kyllonen et al., 2013). As previously reported (Kumarasuriyar, Murali, Nurcombe, & Cool, 2009; Luo, Liao, & Zhou, 2001; Xing et al., 2007), during the osteoinductive treatment, MG63 showed the ability to differentiate, as indicated by the increase in the production of both early (ALP activity, Coll I) and late osteogenic markers (OC, matrix mineralization). Further, as expected, differentiation was associated to the increased expression of known specific osteogenic TFs, specifically Runx2, DLX5 and Osterix (Javed et al., 2010). 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, suggesting the involvement of this pathway in the process. In particular, in the osteoinductive condition a significant increase of Notch2 and Notch4 receptor expression was observed compared to untreated control cells at the later stage (days 21-28) of differentiation, whilst an under-expression 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. during 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 the day 3 (Zamurovic et al., 2004). Further, although only few studies have investigated Notch2 and Notch4 receptors during osteogenic differentiation and none in our cellular model, 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 data on MG63 also showed that the observed differences in Notch receptors expression were related to specific changes in Notch nuclear target genes expression; as we found the upregulation of Hey1 (from day 7 to day 28) and the downregulation of Hes5 (at days 14-21) during differentiation. A way to clarify the involvement of a signaling pathway is based on the use of pathway inhibitors. As the γ-secretase-mediated cleavage is the rate-limiting step of initiating Notch signaling, we investigated the effects of the γ-secretase inhibitor DAPT (Kanungo et al., 2008) on cell differentiation. In DAPT treated cells, differentiation appeared significantly inhibited as indicated by the decrease in the evaluated osteogenic markers.

Therefore, collectively, data obtained in MG63 cell line, supported the involvement of the Notch pathway in osteogenic differentiation and represented the basis to further investigate the involvement of Notch pathway in the differentiation of hMSCs, which are the cells naturally involved in bone development and repair. An experimental approach similar to that used in MG63 cell line was used for the *in vitro* induction of hMSCs differentiation in the presence of the osteoinductive medium, following the analysis of differentiation markers and Notch related gene expression at different time points during the whole period of differentiation (28 days).

During the osteoinductive treatment, hMSC differentiated towards osteoblastic phenotype, shown by the increase in the production of early (ALP activity) and late osteogenic markers (OC, matrix mineralization) and these changes, as previously reported (Hamidouche, Fromigue, Ringe, Haupl, & Marie, 2010; G. Liu et al., 2009) were associated to the early increase (3 days) in the expression of the known specific osteogenic transcription factors (Runx2, DLX5, Osx), with prolonged increased expression of Osx. Then, in order to clarify the potential involvement of Notch signaling in the events associated to the cell differentiation we investigated changes in the expression of Notch related genes. Our results showed an increased expression of several Notch nuclear target genes in differentiating cells compared to control cells, with an early (7 days) increase for Hes5, maintained until the end of the differentiation period, and a later induction (21 and 28 days) for Hes1 and Hey1 genes. These data suggested the activation of the canonical Notch pathway, in which specific nuclear target genes are expressed following the activation of Notch receptors, during the osteogenic differentiation process (Ayaz & Osborne, 2014).

Similar to MG63 cells, we also analyzed possible changes in the expression of the four Notch receptors and several Notch ligands. In agreement with the results on Notch nuclear target genes expression, this analysis showed a significant increase in Notch4 receptor (at day 3) and the ligand DLL4 (at days 21 and 28) expression in differentiating cells respect to control cells. Differently no significant changes were identified in the expression of the other Notch receptors and ligands investigated.

The association among cell differentiation and changes in the expression of Notch related genes at specific times during differentiation suggested a role for the Notch pathway also in hMSCs differentiation. Therefore to gain more information concerning the involvement of the pathway, we also evaluated the effects of DAPT (Kanungo et al., 2008) on hMSCs differentiation and gene expression. Treatment with DAPT inhibited cell differentiation, as shown by the significant decrease in all osteogenic markers as well as in osteogenic TFs expression. Interestingly, DAPT inhibited also the expression of Notch target genes Hes5 and Hes1. Altogether these biochemical and gene expression data, collected in the presence of DAPT, confirmed that Notch pathway activation was involved in MSCs osteogenic differentiation, in agreement with previous studies showing a stimulatory role of this pathway on osteoblast differentiation and function (Dishowitz et al., 2014; Nobta et al., 2005; Tezuka et al., 2002; Ugarte et al., 2009; Zhu et al., 2013). Further, they suggest a role of Hes1 and Hes5 Notch target genes in osteogenic differentiation. Differently from Hes1 and Hes5, the absence of DAPT effect on Hey1 expression indicated that the increase in Hey1 expression observed during differentiation was not directly related to the Notch pathway suggesting that in MSCs, Hey1 modulation may be due to alternative signaling pathways. Indeed our data showing an increase in Hey1 expression during differentiation are in line with previous studies on mesenchymal stem cells and C2C12 cell line showing an increase for this gene during osteogenic differentiation under BMP2 or BMP9 stimulation (de Jong, Steegenga, et al., 2004; Lavery et al., 2009; Sharff et al., 2009). It is worth to note that in those studies as well as in endothelial cells, Hey1 modulation has been associated to the BMP pathway (Woltje, Jabs, & Fischer, 2015). Therefore, it is possible that the changes in Hey1 expression identified in our study are due to the activation of BMP pathway. In agreement with knowledge about osteogenic differentiation, other results from our work suggest the involvement of alternative signaling pathways (Lamplot et al., 2013; Lavery et al., 2008; G. Liu et al., 2009; Marcellini et al., 2012; Plaisant et al., 2009). In fact, when we analyzed the temporal relationship among the changes in

Notch target genes and the increased expression of the osteogenic TFs, we found that TFs expression increased earlier than the Notch nuclear target genes. Therefore, although our data do not give us definitive conclusions about the relationship among osteogenic TFs and Notch target genes, they seem to indicate that osteogenic TFs are initially induced by alternative pathways such as BMP pathway, as previously reported. For instance, BMP7, BMP2, 6 and 9 can induce Runx2 (Celil & Campbell, 2005; Ducy et al., 1997; Luu et al., 2007; Minamizato et al., 2007; Peng et al., 2003). Osterix has been reported to be induced by BMP2 (Minamizato et al., 2007; Nakashima et al., 2002; Yagi et al., 2003) or BMP9 (Celil & Campbell, 2005). BMP2 also can induce DLX5 (S. E. Harris, Guo, Harris, Krishnaswamy, & Lichtler, 2003; Nishimura et al., 2008). On the other hand, the decrease in the expression of the osteogenic TFs in cells treated with the inhibitor of Notch pathway; DAPT, suggests that Notch pathway may be involved in their regulation (de Jong, Vaes, et al., 2004; Hilton et al., 2008; Salie et al., 2010; Sharff et al., 2009; Zamurovic et al., 2004). In this view, it will be of interest to better elucidate the correlation among the Notch and BMP signaling pathways.

Independently from the signaling pathway involved, our results confirm a role for specific transcriptional regulators including the members Hes1 and Hes5 belonging to the human Hes family which are expressed in many tissues (Kobayashi & Kageyama, 2014), as well as Hey 1, one of the three mammalian Hey proteins (Weber, Wiese, & Gessler, 2014), in the osteogenic differentiation of hMSCs.

Although it is known that all of these factors are transcriptional regulators mainly involved in processes such as cell fate decisions and differentiation, however, to date the exact role for each of these proteins in osteogenic differentiation remains to be elucidated. It has been reported that, Hes1 accelerated osteogenesis and stimulated the expression of osteogenic marker genes by increasing the transcriptional activity of Runx2 (J. S. Lee et al., 2006; Suh, Lee, Lee, & Kim, 2008; Tokuzawa et al., 2010). While in other studies Hes1 overexpression inhibits OC expression or osteopontin expression (Matsue, Kageyama, Denhardt, & Noda, 1997; Zhang, Lian, Stein, van Wijnen, & Stein, 2009). Moreover, Hey1 has been involved in osteogenic differentiation although with conflict results (de Jong, Steegenga, et al., 2004; Salie et al., 2010; Sharff et al., 2009; Zamurovic et al., 2004), the molecular mechanism behind the Hey1 functional role in osteogenesis remains to be defined. In fact, it has been proposed that Hey1 and Runx2 can act synergistically in

BMP9-induced osteogenic differentiation (Sharff et al., 2009) and Hey1 knockdown completely blocked the BMP7 induced-mineralizing capacity of the hMSCs (Lavery et al., 2009). In contrary, other authors found that Hey1 inhibited osteoblast maturation via interaction with Runx2 (Hilton et al., 2008; Zamurovic et al., 2004). In addition, Hes1 or Hey1 transient transfection decreases Runx2 trans-activity by direct bonding to Runx2 (Hilton et al., 2008).

Finally, to further clarify the involvement of Notch pathway in hMSCs differentiation, we also investigated the Notch4 receptor in all our experimental conditions by western blotting. As Notch4 expression, evaluated by real-time PCR, increased early during differentiation (at day 3) it appeared as a good candidate in mediating the effects of Notch pathway. Western blotting results showed that the receptor was activated at early differentiation time and that its activation was maintained to the late phase of differentiation (data not shown). Further, the activated form of the receptor was reduced in the presence of DAPT. Therefore our results suggest that changes in the Notch nuclear genes during differentiation may be linked to the increased expression and activation of Notch4 receptor, as well as to the increase in the DLL4 ligand which also increased in line with gene expression data. To our knowledge, only few studies have investigated the Notch4 receptor and DLL4 ligand 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 Notch4 expression found in our results. Moreover, both DLL4 and Notch4 have been reported to be upregulated during both endochondral and intramembranous bone regeneration (Dishowitz et al., 2012).

Overall, data obtained in hMSCs, investigating in vitro the relationship between differentiation and Notch related genes during the whole period of osteogenic differentiation, appears to add new information concerning the complex involvement of Notch pathway in osteogenic differentiation showing the temporally expression of specific genes. Our data confirm a role for this pathway in favoring osteogenesis and suggest that Hes5 and Hes1 might be the target genes involved, following the activation of Notch4 receptor and DLL4 ligand.

Finally in this project, we also investigated the potential involvement of Notch pathway in PEMF-induced osteogenic differentiation. PEMFs are commonly used in clinics to promote bone healing alone or in combination with BMPs and it is has been proved that they can modulate cell behavior and in particular they can favor osteogenic differentiation (Schwartz et al., 2008;

Selvamurugan, Kwok, Vasilov, Jefcoat, & Partridge, 2007). However, to date how differentiation is increased by PEMFs remains largely unknown. As during this project, we identified a role for Notch pathway in favoring osteogenesis, we hypothesized that the same signaling pathway might be modulated by PEMFs. Therefore we performed our investigations on Notch pathway also in hMSCs exposed to PEMFs during the whole period of differentiation, as previously reported (Ongaro et al., 2014).

The data collected appear to indicate that PEMFs can act, at least partially, by the Notch pathway. In agreement with previous studies (Jansen et al., 2010; Ongaro et al., 2014; Schwartz, Fisher, Lohmann, Simon, & Boyan, 2009; Schwartz et al., 2008; Tsai et al., 2009) stimulation with PEMFs induced a significant increase in all the osteogenic markers investigated, as well as the enhancement in the expression of the osteogenic TFs which increased in osteogenic medium alone. These data confirmed and extended our previous findings obtained in human bone marrow MSCs (Ongaro et al., 2014) showing that the PEMFs effects on osteogenic differentiation are related to a PEMF-induced increase in osteogenic TFs (Jansen et al., 2010; Sun, Hsieh, Lin, Chiu, & Chiou, 2010; Tsai et al., 2009). When we investigated changes in the expression of Notch related genes, we observed that PEMFs induced the overexpression of the Notch target genes Hes5, Hey1 and Hes1 in the middle-late period of differentiation (14 and 21 days), in comparison to cells differentiated in the absence of PEMFs. Further, PEMFs increased the expression of the Notch4 receptor to day 7 and also DLL4 at days 14 and 21.

These results showed that PEMFs could induce changes in the expression of the same Notch related genes which appeared modulated during MSCs differentiation in the absence of the physical stimulus, suggesting that the modulation of Notch pathway may be involved in the PEMF-induced osteogenesis. Moreover, in the presence of PEMFs, treatment with DAPT inhibited cell differentiation, shown by the decrease in osteogenic markers and TFs expression as well as the expression of the Notch nuclear target genes Hes5 and Hes1. In addition, similar to what observed during differentiation in the absence of PEMFs, the western blotting analysis showed that the activation of the Notch4 receptor could be increased by PEMFs comparing to cells differentiated in the absence of PEMFs and inhibited by DAPT.

Altogether data obtained in cells exposed to PEMFs suggest that the osteogenic effects of PEMFs are at least partially linked to the activation of Notch pathway, with a prominent role of Notch

target genes Hes5 and Hes1 probably through the activation of the Notch4 receptor and the ligand DLL4. Differently, also in the presence of PEMFs, the absence of an effect of DAPT on Hey1 expression indicated that the increase in Hey1 expression during differentiation in OM and its further increase in the presence of PEMFs, was not directly related to the Notch pathway. For what concerns the PEMF effect, the novelty of this study is to reveal Notch pathway is involved in mediating its effects on osteogenesis. Further, the observation that PEMFs can induce Hey1 expression suggest that this gene may be involved in PEMFs-induced osteogenesis. As reported before, increases in Hey1 expression related to osteogenesis have been already described and associated to BMP signaling. Further, although mechanisms have not been elucidated, the PEMF effects have been previously associated to BMP pathway, as it has been observed that PEMFs act synergistically with BMPs when both stimuli are used to induce differentiation (Ongaro et al., 2014; Schwartz et al., 2008). Also some studies reported changes in the expression of specific BMPs as well as BMPs inhibitors during osteogenic differentiation in vitro (Choi, Noh, Park, Lee, & Suh, 2011; Qi et al., 2003). Therefore, results of this study concerning the effects of PEMFs suggest future investigations to elucidate the correlation among PEMFs effects, BMP pathway and Hey1 expression/activity during osteogenic differentiation.

5. Conclusions

In conclusion, results of this study add new important information concerning the osteogenic differentiation of human mesenchymal stem cells as well as the molecular mechanisms by which PEMFs can stimulate their differentiation. Data collected have shown that Notch pathway is involved in favouring osteogenic differentiation and identified Hes5 and Hes1 as the Notch target genes involved, following the activation by the Notch4 receptor. On the other hand, in the late phase of differentiation, we have also identified a significant increase in the expression of Hey1, which appears as another important gene involved in osteogenic differentiation, although its regulation, as mediated, did not appear by the canonical Notch pathway. In agreement with the complexity of the signal transduction pathways involved in osteogenic differentiation, our results confirm that other signaling pathways are involved, as suggested by the temporal shift in the increase of osteogenic TFs expression and Notch nuclear target genes, as well as by the Notch-independent increase in Hey1, demonstrated by the use of Notch signaling inhibitors. Based on the results of various studies, we suggest that BMP pathway might be involved.

Further, for the first time in this study we show that the PEMF-induced effects on osteogenesis can be associated to the modulation of Notch pathway which plays essential roles in cell fate and differentiation, as well as to the increased expression of the nuclear gene Hey1 by adding important knowledge concerning the molecular mechanisms by which PEMFs can modulate osteogenesis.

Although still not clarified, previous studies suggests a crosslink among the Notch and BMP signaling pathways .Further studies may better elucidate the relationship between these pathways in the osteogenic differentiation process as well as in mediating the effects of PEMFs. Finally, since PEMFs are efficacious in facilitating non-united fracture repair in animals and humans, clarification concerning the mechanisms by which PEMFs affect osteogenesis may be of interest from a clinical point of view for the evaluation of new strategies in bone repair and tissue engineering optimization and application.

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