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ROLE OF miR-204 AND BIOACTIVE COMPOUNDS IN MODULATING CHONDROGENIC AND OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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“In the middle of every difficulty lies opportunity.”

Albert Einstein

ABSTRACT

In aging-related diseases, such as osteoporosis and osteoarthritis (OA), cartilage and bone are the most commonly damaged tissues. Chondrocytes and osteocytes originate from pluripotent mesenchymal stem cells (MSCs) through chondrogenic and osteogenic differentiation; two interrelated processes. RUNX2 and SOX9 are the major transcription factors for osteogenesis and chondrogenesis, respectively. They directly interact and can inhibit each other's transactivation. Epigenetic regulation by miRNA greatly influences the differentiation fate of MSCs. It has been found that miR-204 binds to the 3'-UTR of RUNX2 mRNA and that its expression is promoted by SOX9. Inflammation has been shown to impair MSCs chondrogenic and osteogenic differentiation. Methylsulfonylmethane (MSM) and fisetin, two nutraceutical compounds with anti-inflammatory and antioxidant properties, have been proved to be effective in the management of cartilage and bone degenerative disorders. In this study, we elucidated the miR-204 role during MSCs' commitment and differentiation steps and investigated the overall effects of MSM and fisetin on chondrogenesis and osteogenesis. The analyses were conducted both in vitro and in vivo.

The silencing of miR-204 activity in MSCs enhanced the expression of chondrogenic (SOX9, COMP, COL2A1) and osteogenic (COL1A1) maturation-related genes during the early MSCs differentiation phase. On the contrary, the absence of miR-204 activity considerably affected the chondroblasts' and osteoblasts' maturation during the late MSCs differentiation phase. RUNX2 expression was slightly altered in miR-204 silenced cells compared to control, although RUNX2 protein levels were increased. *In vivo*, miR-204 expression increased during zebrafish larval development and up to six months of growth, but then it dropped as zebrafish grew older. Interestingly, β-catenin and p-ERK protein levels decreased during *in vitro* MSCs chondrogenic differentiation. Conversely, in 2 years old zebrafish, high levels of p-ERK and low levels of aggrecan, a chondrogenic maturation marker, suggested a reduced chondrogenic commitment. Upon IL-1β treatment, miR-204 and SOX9 expression decreased in MSCs and mature chondrocytes. Interestingly, the addition of MSM reversed IL-1β effects in both cell types by increasing miR-204, SOX9, SESN1 and COMP expression. MSM promoted chondrogenesis in both adult zebrafish and larvae. Moreover, MSM stimulated osteoblasts maturation in vitro and osteogenesis in vivo, as shown by the more intense calcein and Alizarin Red (AR) staining in MSM-treated adult zebrafish and larvae compared to untreated. Interestingly, p-ERK levels were lower in the fins and scales of MSM-treated zebrafish compared to untreated. Fisetin supplementation promoted MSCs osteogenic differentiation in vitro. Moreover, fisetin stimulated bone formation and mineralization in vivo, as shown by the higher AR and calcein staining in fisetin-treated adult zebrafish and larvae compared to untreated. Remarkably, fisetin significantly enhanced osteogenic maturation in primary fibroblasts of patients with Cleidocranial Dysplasia, a genetic skeletal disease caused by RUNX2 mutations. A delivery system with PLGA (poly lactic-co-glycolic acid) nanoparticles was generated to encapsulate fisetin [PLGA-(Fis)]. Surprisingly, PLGA nanoparticles improved fisetin stability and its biological effects on gene expression. Moreover, in a microfluidic organ-on-a-chip experiment, PLGA-(Fis) nanoparticles showed the ability to cross the human intestinal epithelial tissue, preventing fisetin degradation.

In conclusion, this study shows that miR-204 negatively regulates the MSCs' commitment to the osteochondrogenic lineage, while it positively influences the maturation of chondroblasts and osteoblasts. Moreover, our data show that MSM and fisetin act by influencing MSC commitment and differentiation and have beneficial effects on osteogenesis, suggesting that patients affected by skeletal degenerative diseases of both genetic and metabolic origins may benefit from their administration.

RIASSUNTO

Nelle malattie associate all'invecchiamento, come l'osteoporosi e l'artrosi, la cartilagine e l'osso sono i tessuti più comunemente danneggiati. I condrociti e gli osteociti originano da cellule staminali mesenchimali pluripotenti (MSCs) attraverso il differenziamento condrogenico e osteogenico; due processi correlati. RUNX2 e SOX9 sono i principali fattori di trascrizione per l'osteogenesi e la condrogenesi, rispettivamente. Essi interagiscono direttamente e possono inibire reciprocamente la loro attivazione. La regolazione epigenetica mediata dai miRNA influenza notevolmente la direzione del differenziamento delle MSCs. È stato scoperto che il miR-204 ha come bersaglio molecolare l'mRNA di RUNX2 e che la sua espressione è promossa da SOX9. È risaputo che lo stato di infiammazione altera il differenziamento condrogenico e osteogenico delle MSCs. Il metilsulfonilmetano (MSM) e la fisetina, due composti nutraceutici con proprietà anti-infiammatorie e antiossidanti, sono efficaci nella gestione terapeutica delle malattie degenerative della cartilagine e dell'osso. In questo studio, abbiamo delucidato il ruolo del miR-204 durante il commissionamento e il differenziamento delle MSCs e analizzato gli effetti complessivi dell'MSM e della fisetina sulla condrogenesi e osteogenesi. Le analisi sperimentali sono state condotte sia in vitro che in vivo.

Il silenziamento dell'attività del miR-204 nelle MSCs aumentava l'espressione di geni associati alla maturazione condrogenica (SOX9, COMP, COL2A1) e osteogenica (COL1A1) durante la fase iniziale del differenziamento delle MSCs. Al contrario, l'assenza di attività del miR-204 influenzava notevolmente la maturazione dei condroblasti e degli osteoblasti durante la fase tardiva del differenziamento. L'espressione di RUNX2 risultava leggermente alterata rispetto al controllo, mentre il livello di espressione della proteina aumentava nelle cellule in cui il miR-204 era silenziato. In vivo, l'espressione del miR-204 aumentava durante lo sviluppo larvale degli zebrafish e fino a 6 mesi di età, per poi diminuire quando gli zebrafish anziani. È interessante notare come i livelli di espressione della proteina □-catenina e di p-ERK diminuivano durante il differenziamento condrogenico delle MSCs, mentre invece negli zebrafish aventi 2 anni di età alti livelli di p-ERK e bassi livelli di aggrecano, un marcatore di maturazione condrogenica, suggerivano un ridotto processo di condrogenesi. In seguito a trattamento con IL-1□, l'espressione del miR-204 e di SOX9 diminuiva sia nelle MSCs che nei condrociti maturi. È interessante notare come l'aggiunta dell'MSM abbia annullato gli effetti negativi dell'IL-1□ in entrambi i tipi cellulari aumentando l'espressione di miR-204, SOX9, SESN1 e COMP. L'MSM promuoveva la condrogenesi sia nelle larve che negli zebrafish adulti. Inoltre, l'MSM stimolava la maturazione degli osteoblasti in vitro e l'osteogenesi in vivo, come dimostrato dalla maggiore intensità della colorazione con calceina e Alizarin Red (AR) negli zebrafish adulti e nelle larve trattate con MSM rispetto ai non trattati. Inoltre i livelli di p-ERK erano più bassi nelle pinne e nelle squame degli zebrafish trattati con MSM rispetto ai non trattati. Il trattamento con fisetina promuoveva il differenziamento osteogenico delle MSCs. Inoltre, la fisetina favoriva la formazione e la mineralizzazione dell'osso in vivo, come evidenziato dalla maggiore intensità della colorazione con calceina e AR negli zebrafish adulti e nelle larve trattate con fisetina rispetto ai non trattati. Sorprendentemente, la fisetina aumentava sensibilmente la maturazione osteogenica di fibroblasti primari isolati da pazienti affetti da Displasia Cleidocranica, una malattia genetica dello sviluppo osseo causata da mutazioni nel gene RUNX2. Abbiamo generato delle nanoparticelle costituite da acido lattico e glicolico (PLGA) con incapsulata la fisetina PLGA-(Fis). Sorprendentemente, le

nanoparticelle PLGA hanno migliorato la stabilità della fisetina, amplificando i suoi effetti sull'espressione genica. Inoltre, mediante un esperimento di microfluidica organ-on-a-chip, abbiamo dimostrato come PLGA-(Fis) erano in grado di attraversare l'epitelio intestinale umano preservando la fisetina dalla degradazione.

In conclusione, questo studio evidenzia come il miR-204 regola negativamente il commissionamento osteo-condrogenico delle MSCs, mentre regola positivamente la maturazione dei condroblasti e degli osteoblasti. Inoltre, i dati sperimentali dimostrano come l'MSM e la fisetina agiscano influenzando il commissionamento e il differenziamento delle MSCs e hanno effetti positivi sull'osteogenesi, suggerendo che pazienti affetti da malattie scheletriche sia di origine genetica che metabolica possono trarre beneficio dalla loro somministrazione.

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1. INTRODUCTION

1.1 Cartilage and bone

1.1.1 Structure and composition of articular cartilage

Cartilage is a specialized connective tissue present in several areas of the body. It is histologically classified into hyaline, elastic, and fibrocartilaginous cartilage depending on the molecular composition. The most prevalent type of cartilage is hyaline cartilage, which lines and covers the ends of articulating bones in diarthrodial joints. [1]
Its smooth and viscoelastic features allow articular cartilage to act as a lubricant to support the flexible joint movement, absorb shock and facilitate the transmission of loads with a low frictional coefficient. [2]

Human articular cartilage is stratified into four zones, known as the superficial zone, middle zone, deep zone and calcified zone, according to differences in chondrocyte morphology and distribution, and collagen fibers and proteoglycan orientation. Within each zone, three regions can be identified: the pericellular region, the territorial region and the interterritorial region. (Figure 1)
Chondrocytes are the sole resident cells within articular cartilage, accounting for about 2% of the total tissue volume. They are sparsely distributed in cavities, known as lacunae, within the cartilage extracellular matrix (ECM). Each chondrocyte establishes a unique microenvironment and is responsible for the turnover of the ECM in its immediate vicinity, by synthetizing ECM components. This microenvironment basically traps the chondrocyte within its own matrix, avoiding any migration to adjacent areas of cartilage and cell-to-cell contacts between neighboring chondrocytes. [2], [3]

Cartilage ECM is highly dense and supports the entire cartilage structure. It is mainly composed of water (80% of the total cartilage wet weight), collagen (60-90% of cartilage dry weight) and proteoglycans (10-15% of cartilage wet weight), with other lipids, phospholipids, non-collagenous proteins and glycoproteins present in lesser amounts. Together, these components facilitate in the retention of water within the ECM, which is essential to maintain the distinctive mechanical properties of cartilage ECM. [4]

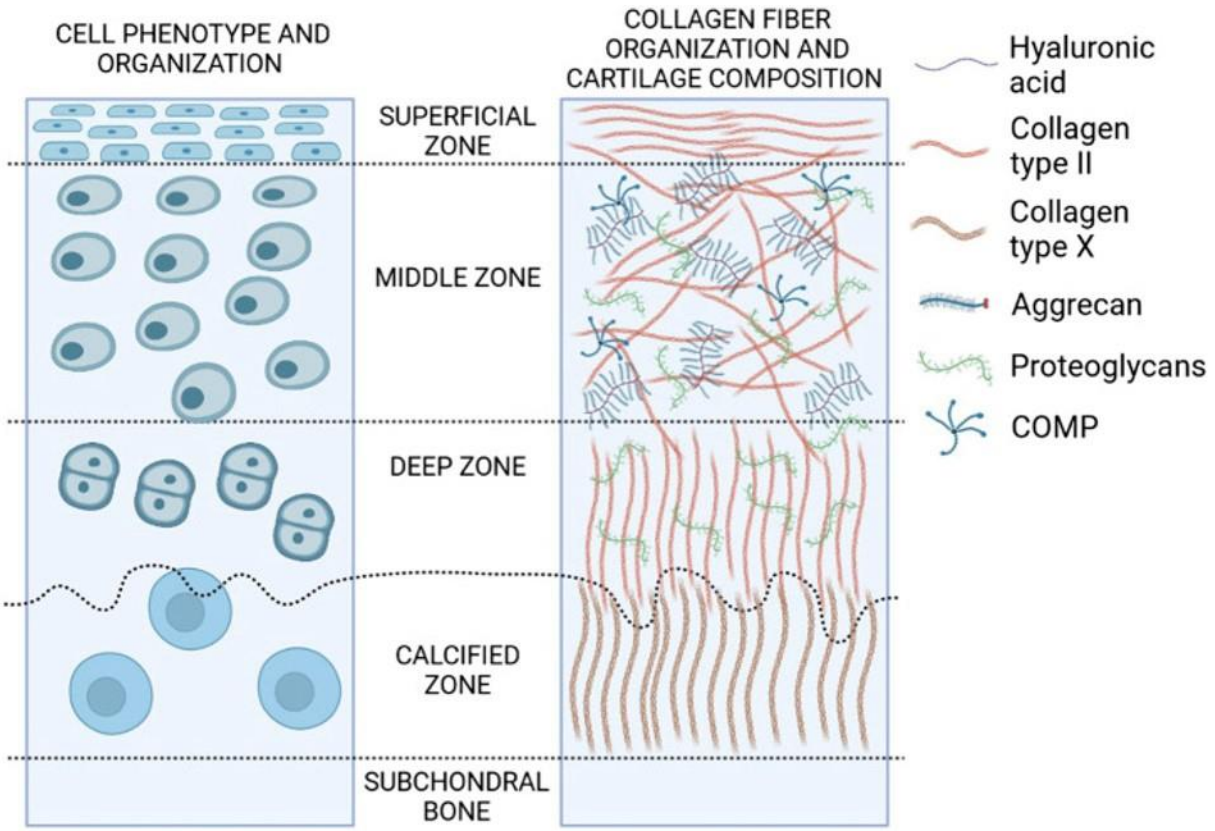

Figure 1. Structure and composition of hyaline cartilage. Figure shows the zones of hyaline cartilage with varying cellular distribution, shape, collagen organization. On the right, the ECM proteins are depicted. Adapted from M. Szustak et al, 2021 [5]

Collagen is the most abundant structural macromolecule present in cartilage ECM. Type II collagen is the major isoform, representing about 90% of the collagen in the tissue. This isoform confers resistance to compressive forces in cartilage. Thus, its role in maintaining ECM homeostasis is crucial, and loss causes perturbations in cartilage physical and mechanical properties, resulting in arthritis. The remaining isoforms of collagen (collagen types I, IV, V, VI, IX and XI) assist the collagen type II fibril network formation. [4]

Proteoglycans are the second largest group of macromolecules in cartilage ECM. They are formed of glycosaminoglycans (GAGs), which interact with hyaluronic acid (HA) via link proteins to form highly negatively charged cartilage aggregating proteoglycans. Aggrecan, the most abundant cartilage-specific proteoglycan, is a macromolecular aggregate, consisting of a polypeptide backbone covalently attached to several linear sulfonated glycosaminoglycan (GAG) chains.

Sulfonated GAG chains, such as chondroitin sulfate, keratan, and dermatan sulfate, are responsible for attracting a significant amount of water molecules with fixed charge density and high osmotic pressure, which fills the interfibrillar spaces of the cartilage ECM, ensuring

flexibility and viscoelasticity and enabling the articular cartilage to resist high compressive loads.

Decorin, biglycan and fibromodulin are non-aggregating proteoglycans, characterized by their ability to interact with collagen. Decorin and fibromodulin interact with collagen type II fibrils in the matrix and play a role in fibrillogenesis and interfibril interactions. Biglycans are mainly found in the immediate surrounding of the chondrocytes, where they may interact with collagen VI. [6]

Within cartilage ECM there are also present several non-collagenous proteins and glycoproteins, but their specific function has not been fully characterized. Some of these molecules (such as fibronectin and CII, a chondrocyte surface protein) likely play a role in the organization and maintenance of the macromolecular structure of the ECM. [4]

1.1.2 Structure and composition of bone tissue

Bone is a mineralized connective tissue that exerts important functions in the body such as locomotion, organ protection and support, calcium and phosphate storage, and the generation of hematopoietic cells.

Bone tissue is composed of an organic and an inorganic phase. The inorganic phase is constituted by calcium phosphate, whereas the organic phase is composed predominantly by type I collagen, a variety of non-collagenous proteins and for a small percentage by cells. [7]

Bone, despite its inert appearance, is a highly dynamic organ. To constantly maintain the structure-function relationship, bone tissue undergoes to two processes, bone modeling and bone remodeling. Bone modeling controls the growth and the adaptation of bone, instead bone remodeling controls the removal and the repair of old or damaged bone and it is a highly complex process that requires the coordinated actions of all bone cells. The preservation of tissue homeostasis depends on both processes. [8]

Normal bone modeling and remodeling is essential for fracture healing and skeleton adaptation to mechanical use, as well as for calcium homeostasis. On the contrary, an imbalance of bone formation and resorption results in several bone diseases. Thus, the equilibrium between these two processes is crucial and depends on the action of several local and systemic factors including hormones, chemokines, cytokines, and biomechanical stimulation. [7]

There are four main types of cells in bone tissue:

- **Osteoblasts:** they are cuboidal cells derived from mesenchymal stem cells (MSCs). Osteoblasts participate in the bone formation process and are located in the growing portions of the bone comprising 4-6% of the total resident bone cells. Osteoblasts produce the bone matrix composed by collagen proteins, mainly type I collagen, noncollagen proteins (OCN, osteonectin, BSP II, and osteopontin), proteoglycans including decorin and biglycan, and calcium salts. The osteoid, the protein matrix formed by osteoblast, creates a template for the production and the mineralization of the mature bone. [7], [8]

- **Osteocytes:** they are the most abundant and long-lived cells in the bone, with a lifespan of up to 25 years, accounting for 90-95% of the total bone cells. Osteocytes are derived from MSCs lineage through osteoblast differentiation. When the secreted matrix surrounding the osteoblast starts to calcify, osteoblasts change their morphology, reduce the production of matrix and become osteocytes incorporated into mineralized matrix. Osteocytes reside within small cavities (lacunae) surrounded by mineralized bone matrix, wherein they show long cytoplasmic processes that extend throughout tiny tunnels (canaliculi) that originate from the lacuna space. Osteocytes's dendritic-like processes allow intercellular communications and the formation of an interconnected network between neighboring osteocytes and surface-lining osteoblasts. [9]

- **Osteoclasts:** they are terminally differentiated multinucleated cells, which originate from hematopoietic cell lineage (monocyte-macrophage cells). Osteoclasts take part in the bone resorption process, removing mineralized bone matrix through the release of alkaline phosphatase. RANK ligand (RANKL) is a key factor for osteoclastogenesis and is expressed by osteoblasts, osteocytes, and stromal cells. The binding of RANKL to its RANK receptor in osteoclast precursors induces osteoclast maturation, whereas osteoprotegerin (OPG), which is produced by osteoblasts and stromal cells, binds to RANK to prevent interaction with its receptor and consequently inhibits osteoclastogenesis. [8] [9]

- **Bone lining cells:** they are quiescent flat-shaped osteoblasts that surround the bone surfaces, where neither bone formation nor bone resorption occurs. They have a reduced secretory activity. Their cytoplasm can extend into bone canaliculi and is in communication with adjacent bone lining cells and osteocytes. [10] Depending on the bone physiological status, these cells can reacquire their secretory activity, becoming bigger and with a cuboidal appearance. Although the functions of bone lining cells are still not fully understood, it has been shown that these cells regulate bone resorption by avoiding the direct interaction between osteoclasts and bone matrix. They also take part in osteoclast differentiation by producing osteoprotegerin (OPG) and RANKL. [11] [12]

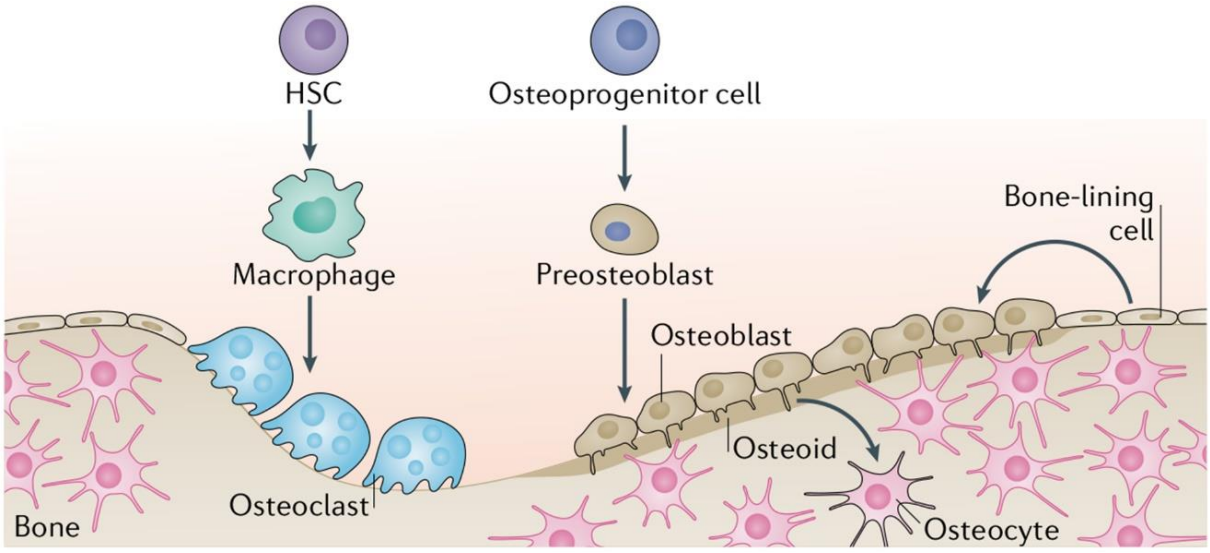

Figure 2. Bone homeostasis and cellular components of bone tissue. Bone homeostasis is achieved through the combined activity of osteoblast and osteoclast lineage cells. HSC (haematopoietic stem cell); Osteoclast (resorbs bone); Osteoprogenitor cell (derived from msesenchymal stem cell); Osteoblast (forms bone matrix); Osteocyte (maintains bone tissue); Bone-lining cell (resting cell). Adapted from A. Salhotra et al., 2020 [13]

1.2 Mesenchymal stem cells (MSCs)

1.2.1 Characteristics of mesenchymal stem cells

First described by Friedenstein in 1967, mesenchymal stem cells (MSCs) are adult multipotent stem cells, capable of self-renewal and differentiation in specialized cells of the mesenchymal lineage like osteoblasts, chondroblasts, and adipocytes, as well as cells of other embryonic lineages (ectoderm and endoderm). MSCs can be isolated from a variety of tissues, such as bone marrow, umbilical cord, adipose tissue, peripheral blood, etc. [14]

MSCs lack a single distinct marker that can reliably discriminate them from other cells. Indeed, they are distinguished immunophenotypically by the expression of surface antigens such as CD44, CD73, CD29, CD90 and CD105, and by the absence of hematopoietic and endothelial markers such as CD11, CD14, CD31, CD34 and CD45. [15]

The direction of MSC differentiation toward a specific cell type is precisely regulated by a multitude of stimuli and inhibitors, which play important roles in the initial commitment and in the subsequent stages of differentiation. The factors involved are various biological and chemical factors, including cytokines, growth factors, extracellular matrix molecules and transcription factors. Changes in any of these components will result in different outcomes for MSCs differentiation. [16]

MSCs represent a promising cell source for regenerative medicine and cell therapy for degenerative diseases. Bone Marrow-derived MSCs (BM-MSCs) are the most commonly used. They are simple to isolate, possess a relatively high expansion rates and migratory capacity, and can avoid allogeneic responses after transplantation. Indeed, in response to injury signals, BM-MSCs can migrate from their niche into the peripheral circulation and then to the injured site (homing ability). There, BM-MSCs can differentiate into local components of injured site helping the tissue to regenerate. The efficacy of BM-MSCs in cell therapy depends on their homing ability and engraftment into the injured site for a long time. [17]

1.2.2 Chondrogenic differentiation of MSCs

The chondrocyte is the only type of resident cell in articular cartilage. Chondrocytes are highly specialized, metabolically active cells that play a unique role in cartilage ECM development, maintenance and repair. Unfortunately, chondrocytes have a restricted proliferation potential, which contributes to cartilage's limited intrinsic healing capability in response to injury. [18]

Chondrocytes originate from MSCs. During the early phases of embryogenesis, cartilage development starts with MSCs condensation and proliferation to form chondrification centers. Then, MSCs differentiate into chondroprogenitor cells, which later become chondroblasts. Chondroblasts develop into chondrocytes, which start to synthesize and secrete cartilage-specific ECM components such as collagen type II (COL2A1), IX (COL9A1), XI (COL11A1) and aggrecan (ACAN). Finally, chondrocyte maturation involves the transition of proliferating chondrocytes to non-proliferating and hypertrophic chondrocytes, which have an ECM rich in collagen type X (COL10A1). [5]

Chondrogenic differentiation of MSCs is a complex developmental process influenced by a variety of chemical factors, including soluble growth factors, chemokines and cytokines. Transforming growth factor β (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and bone morphogenetic protein (BMP) have all been shown to induce chondrogenesis. [19]
Chondrogenesis is distinguished by cell-specific markers that play critical roles during the chondrogenic differentiation steps of MSCs. For instance, COL2A1 and ACAN genes are mostly expressed in proliferating chondrocytes, whereas parathyroid hormone 1 receptor (PTH1R) and Indian hedgehog (Ihh) are mainly expressed in pre-hypertrophic chondrocytes. Instead, the expression pattern of hypertrophic chondrocytes is characterized by COL10A1 and matrix metalloproteinase 13 (MMP13). [20]

Several transcription factors are involved in the chondrogenic stages and they must all be activated in a specific spatiotemporal sequence for proper chondrocyte formation. Among them, SOX9 and RUNX2 are two key regulators that are essential for the early stage of chondrogenesis and for the hypertrophic maturation of chondrocytes, respectively.
The SRY-box containing gene (Sox) family, including SOX5, SOX6, and SOX9, control gene expression during chondroprogenitor cells differentiation and chondroblast maturation. In particular SOX9 binds and activates chondrocyte-specific promoters of extracellular matrix genes, including COL2A1, COL11A2 and ACAN. [21]

RUNX2, which belong to the Runt related transcription factor (RUNX) family, guides the expression of COL10A1 and MMP13, but once hypertrophic maturation is complete, SOX9 suppresses RUNX2 activity. RUNX2 also plays an essential role in regulating the Hedgehog (Hh) signaling pathway by binding to Hh promoter region. Among Hh signaling pathway, Wnt/β-catenin is another important pathway involved in cartilage microenvironment formation. [5]

In addition to genes and proteins regulation, numerous studies have revealed that also non-coding RNAs (ncRNAs), especially miRNAs and long ncRNAs, play important roles in chondrogenesis. [22]

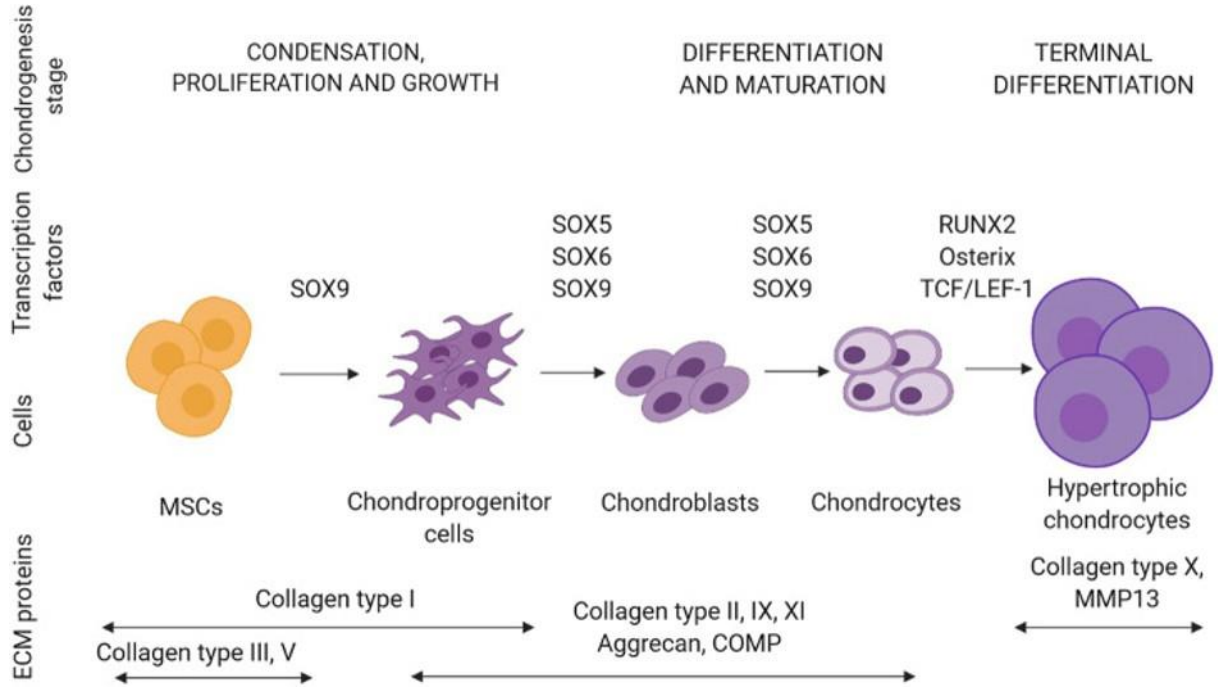

Figure 3. Schematic representation of the events that lead to the differentiation of MSCs toward chondrocytes. The figure illustrates the temporal expression profiles of ECM proteins and transcription factors at each stage of chondrogenesis. Adapted from M. Szustak et al, 2021 [5]

1.2.3 Osteogenic differentiation of MSCs

Bone ossification, or osteogenesis, is the process of bone formation. This process is controlled by a wide range of stimulators and inhibitors, which occur both at the transcriptional level and through extracellular signaling pathways.

Osteogenesis can be divided into intramembranous and endochondral ossification processes. The first process (direct ossification), which occurs in the flat bones of the skull and clavicles, is characterized by the condensation of MSCs which, after the commitment to osteo-progenitors, become osteoblasts. Mature osteoblasts can become bone lining cells or osteocytes or may go through apoptosis. Conversely, during endochondral ossification (indirect ossification), which occurs in the long, short, and irregular bones of the axial and appendicular skeleton, the MSCs differentiate into chondrocytes to form the cartilage growth plate, which is later gradually replaced by new bone tissue. [23]

Osteoblasts and osteocytes, the bone cells responsible for bone development and homeostasis, originate from MSCs. Bone marrow and periosteum are the main sources of MSCs. The osteogenesis process begins with the involvement of MSCs, which are stimulated by systemic and local factors, to migrate and proliferate to form cell condensation centers. Cell condensation centers are characterized by closely packed cells expressing specific condensation markers and their organization will determine the position, shape and size of the skeletal elements. After condensation, MSCs decreased their proliferation rate and differentiate into osteoprogenitor cells, then into pre-osteoblasts and mature osteoblasts, which start to secrete bone matrix proteins. At the end of the osteogenic phase, osteoblasts become osteocytes, which remain trapped in the bone ECM, or become bone lining cells. Osteocytes are the most abundant cells in adult bone, with a small cell body and numerous long, dendritic-like cytoplasmic prolongations. They are characterized by the expression of bone-alkaline phosphatase (bALP) and PTH receptors, and have mechanosensory functions. [24]

Osteogenic differentiation of MSCs is a complicated process, normally regulated either by systemic hormones, such as parathyroid hormone (PTH), glucocorticoids, cytokines, steroids or by local growth factors, such as fibroblast growth factor (FGF), transforming growth factor-β (TGF-β1/2), bone morphogenetic proteins (BMPs) and insulin-like growth factor (IGF), the same growth factors that induce MSCs chondrogenic differentiation. All these factors activate intracellular signaling pathways that regulate osteoblastic differentiation by directing the expression and activation of specific transcription factors. [25]

BMPs represent a group of signaling molecules belonging to the TGF-β superfamily. BMPs have been proved to increase the expression of osteogenic markers in MSCs, including the early-stage markers like bone alkaline phosphatase (bALP), RUNX2, osterix (Osx), and COL1A1 and the late-stage markers like osteopontin (Opn) and osteocalcin (Oc). Opn and Oc are major noncollagenous proteins (NCPs) involved in bone matrix organization, mineralization and deposition.

FGF proteins, don't directly affect osteogenic differentiation, rather, they modulate it, acting as osteogenesis accelerators. They foster MSCs differentiation into the osteogenic cell lineage and stimulate osteoblast proliferation. Some studies demonstrated that FGF-2 and BMP-2 act synergistically in bone regeneration, enhancing the effectiveness of bone formation. [25]

TGF-β proteins family, are the most abundant cytokines in bone and also cartilage. TGF-β1 plays a role in the initial phase of osteogenesis, enhancing the expansion of MSCs, the proliferation of osteoprogenitor cells and their early osteogenic differentiation stages. Interestingly, interplay was found between the signaling of TGF-βs and FGFs or BMPs in the bone. For example, TGF-β1 and FGF-2 stimulate osteoblasts proliferation, but inhibit bALP activity and mineralization. Besides, TGF-β1 strongly promotes BMP-2-induced osteogenic functions in bone formation in vitro. Consequently, it has been proposed that these cytokines can be potentially applied in tissue engineering for the induction of bone growth in vitro. [25], [26]

The insulin-like growth factor (IGF) axis comprises two polypeptide growth factors (IGF1 and IGF2) and two cellular surface receptors (IGF1R and IGF2R). The IGF axis regulates both osteoblast and osteoclast differentiation and IGFs are the most abundant growth factors in bone tissue. IGF-1 is upregulated in osteocytes in response to mechanical loading. [25]

The commitment and differentiation of MSCs towards the osteogenic lineage are regulated by several transcription factors, among which the most important is RUNX2. When RUNX2 is activated in human MSCs, it promotes the expression of specific osteoblastic markers such as COL1A1, ALP, Oc during osteoblast maturation. Since RUNX2 plays a critical role in MSCs osteogenic differentiation, RUNX2 deficient mice ($RUNX2^{-/-}$) lack of mature osteoblasts and a barely mineralized bone matrix.

Osx, another important zinc finger transcription factor in osteogenesis, acts downstream RUNX2. Osx-deficient mice ($Osx^{-/-}$) lack of mature osteoblasts in the same way that

RUNX2$^{-/-}$ mice do. Instead, overexpression of Osx in C2C12 cells boosted the expression of bALP and Oc, resulting in bone calcification. [27]

Other transcription factors that are involved in osteogenic differentiation are FRZB and HOXB7. FRZB is a Wnt modulator that increases the expression of osteogenic-related markers and calcium deposition. HOXB7 enhance osteogenesis through the upregulation of RUNX2. HOXB7 overexpression also had an effect on other osteogenic transcription factors and proteins such as osteonectin (ON), COL1A1, BSP, and Oc leading to the promotion of osteogenesis. [23]

Multiple signaling pathways regulate osteogenesis, including Wnt, Notch, PTH, and Hedgehog (Hh). The Wnt signaling pathway is recognize to play a pivotal role in skeletal development and homeostasis, through the promotion of osteoblast proliferation, differentiation, and maturation. In the canonical pathway, in the presence of Wnt ligand binding, β-catenin translocate into the nucleus and induces the upregulation of RUNX2 and Sp7/Osterix gene expression. In turn, RUNX2 and Sp7/Osterix positively regulate the gene expression of other osteogenic transcription factors, such as bALP, Opn, and Oc.

Notch signaling pathway directly affects osteoblast maturation. It was displayed that the inhibition of the Notch signaling in osteoprogenitor cells led to the reduction of bone marrow-derived MSCs and bone loss. Additionally, the treatment of MSCs with Jag-1, one of the Notch signaling ligands, increased the expression of osteoblast-related genes such as bALP and BSPs. Interestingly, a study conducted by Cao J. et al. showed that the Notch signaling pathway enhanced the osteogenic differentiation of MSCs in vitro and in vivo through the induction of BMP-9/SMAD signaling. [25]

1.2.4 SOX9 and RUNX2 control the fate of osteo-chondroprogenitor cells

SOX9 (SRY-Box Transcription Factor 9) and RUNX2 (Runt related transcription factor 2) are master transcription factors essential for cartilage and bone development, respectively, However, RUNX2 also has a role in chondrogenesis, and SOX9 and RUNX2 expression levels determine the fate of the osteo-chondroprogenitor cells. [5]

- **SOX9**

SOX9 is a member of the sex-determining region Y-type (SRY) box transcription factors family of DNA binding proteins. This family is composed also by SOX5 and SOX6, which play various roles during chondrogenic differentiation. However, among SOX family members, SOX9 is the primary determinant during chondrogenesis and it cooperatively bind with SOX5 and SOX6 on enhancers to promote the transcription of chondrogenic associated genes. Indeed, SOX9 promotes MSCs differentiation into chondrocytes by upregulating early chondrogenic-specific genes, including collagen type II (COL2A1), collagen type IX (COL9A1), collagen type XI (COL11A2), and aggrecan (ACAN). [28]

Genetic deletion of SOX9 in mice blocks chondrocyte differentiation at the point of mesenchymal stem cell condensation, indicating that SOX9 is necessary for the early stage of chondrogenic differentiation. [24]

- **RUNX2**

RUNX2 is one of the three members of RUNX family, along with RUNX1 and RUNX3. All RUNX family members have an evolutionary conserved domain, the Runt domain, that is the homologous of the Drosophila pair-rule gene runt. [29]

RUNX2 plays a multitude of roles in different tissues, however its main role is to be the regulator of the early stages of osteoblast differentiation. It has a central role for the commitment of MSCs to osteogenic lineage by controlling the expression of osteoblast-specific genes through the binding to OSE2, the RUNX2 binding site, which is found in the promoter region of all major osteoblast marker genes.
However, during osteogenesis, RUNX2 can act as a positive or negative regulator of osteoblast differentiation. It has a positive role at the early stages of osteoblast differentiation and a negative role at the later stages. [30]

Osteo-chondroprogenitor cells are MSCs that can differentiate into chondrocytes or osteoblasts. The expression of RUNX2, together with OSX, is essential in the commitment of MSCs to differentiate into osteoblast, passing through the osteo-chondroblast progenitor cell, while the expression of SOX9, together with SOX5 and SOX6, is necessary for the commitment of MSCs to differentiate into chondroblast. [31]

In addition to its main role in MSCs osteogenic differentiation, RUNX2 plays a role also in MSCs chondrogenic differentiation. Indeed, it supervises the differentiation of immature chondrocytes towards hypertrophic and mature chondrocytes. [30], [32]
Chondrocyte maturation is an important aspect of the chondrogenesis process that occurs in the late stages. This involves the transition of proliferating chondrocytes, to non-proliferating, hypertrophic, and eventually apoptotic chondrocytes. The expression of SOX9, along with the activation of Camp response element binding protein (CREB) and c-Fos, maintains the chondrocytes in a proliferative state. In order for the chondrocytes to exit the cell cycle and become hypertrophic, RUNX2 must be upregulated and SOX9 suppressed.
RUNX2 promotes chondrocytes maturation by regulating the expression of collagen type X (COL10A1) and matrix metalloprotease 13 (MMP13). The exit from the cell cycle and apoptosis is necessary for the eventual invasion of cartilage by osteoblasts during the endochondral ossification process. [24]

It has been demonstrated that RUNX2 and SOX9 physically interact and SOX9 can inhibit the transactivation of RUNX2 as well as RUNX2 can inhibit the transactivation of SOX9. [33] [34] Moreover, the expression of RUNX2 and SOX9 can be influenced by some proteins. For instance, several bone morphogenetic proteins (BMPs) play important roles in cartilage homeostasis. BMP2, BMP4, and BMP7 can induce chondrogenic differentiation by regulating SOX9 expression and can stimulate endochondral ossification by regulating RUNX2 transcription. Moreover, BMP3 promotes the maturation of terminal hypertrophic chondrocytes. [35]

It has been discovered that when β-catenin is activated, it upregulates RUNX2 expression and MSCs differentiate into osteoblasts, rather than chondroblasts. These results implicate β-catenin in the determination of osteogenesis versus chondrogenesis. [23]

Although many previous studies have investigated MSCs differentiation, the precise molecular mechanisms that control chondrogenic and osteogenic differentiation are still largely undefined. [36]

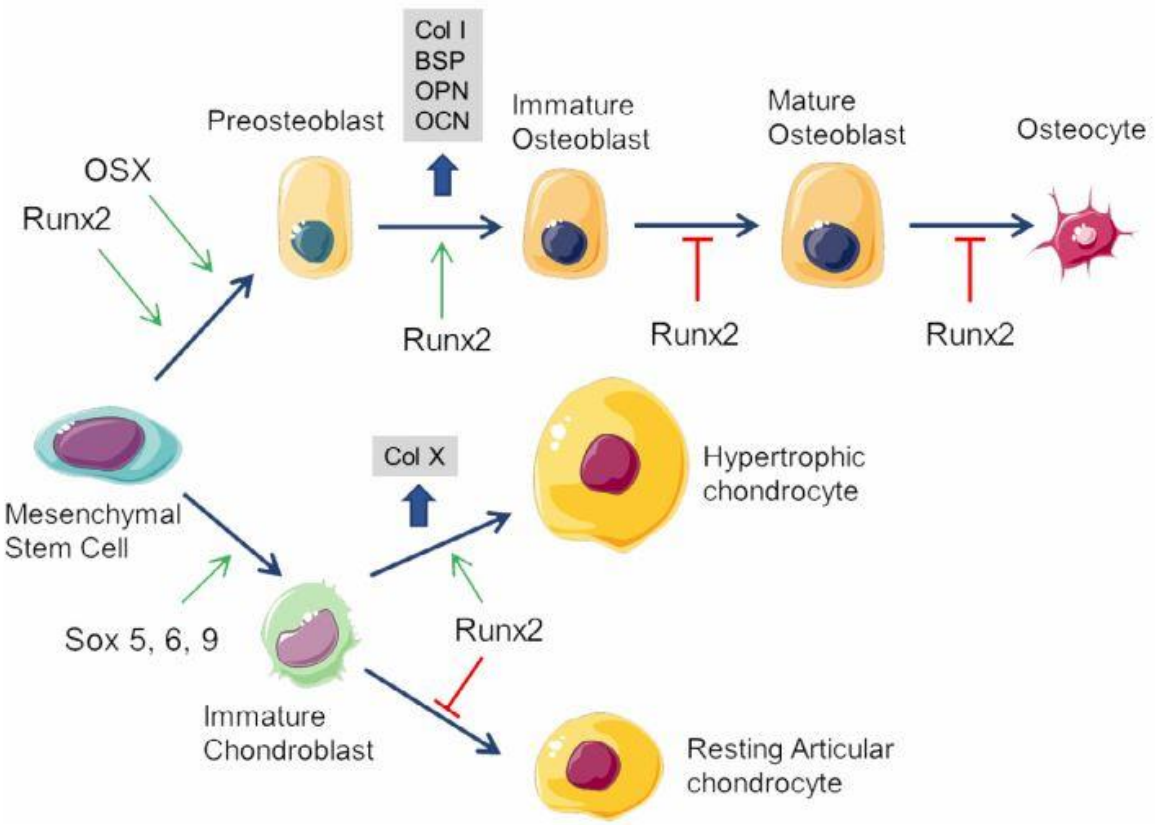

Figure 4. SOX9 and RUNX2 regulation during chondroblast and osteoblast differentiation. RUNX2 is required for MSCs commitment to the osteogenic lineage and positively influences early stages of osteoblast differentiation. Osterix (OSX) starts to play an important role in osteoblast differentiation following RUNX2-mediated mesenchymal condensation. During osteoblast differentiation, RUNX2 promotes the expression of collagen type 1 (COL1), osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN). In the late phases of osteoblast differentiation, RUNX2 expression must be downregulated to promote osteoblasts maturation. During the process of chondroblasts differentiation initiated by SOX9, which mediates mesenchymal condensation, RUNX2 is essential for chondroblasts maturation from immature to terminal hypertrophic chondrocytes, and prevents immature chondrocytes from adopting the phenotype of permanent cartilage. Adapted from M. Bruderer et al., 2014 [30]

1.3 Cartilage and bone diseases

1.3.1 Osteoarthritis (OA)

Osteoarthritis (OA) is the most prevalent chronic joint disease, characterized by pain and a degenerative pathological evolution. It affects over 250 million people globally; the incidence is higher in women than in men, especially beyond age fifty. Worldwide estimates indicate that 10% of men and 18% of women aged ≥ 60 years have symptomatic OA. [37]

OA etiology is largely unknown, but OA it is most likely considered as a multifaceted and complex disease that affects all synovial joint components. Pathological features of OA typically include manifestations such as the progressive articular cartilage destruction, synovial membrane inflammation, narrowed joint space, osteophyte formation, and subchondral bone sclerosis. OA can affect any joint, however most commonly it affects the knee, hand, hip, spine and foot. [38]

OA can be regarded as a mesenchymal disease as it affects not only the articular cartilage but also other joint tissues, like meniscus and synovium. All these tissues, have a mesenchymal origin or contain significant portions of mesenchymal stem cells (MSCs) or mesenchymal progenitor cells (MPCs), which play important roles in joint homeostasis and maintenance. For example, the formation of osteophytes, a prominent radiographic feature of OA and a considerable source of pain and joint dysfunction, requires MPCs derived from peripheral areas including periosteum and synovium to recapitulate endochondral ossification process. [39]

There are many risk factors identified for OA development and progression, such as age, gender, acute joint trauma and chronic joint overload, obesity, inflammation and genetic predisposition. Age is considered the most influential risk factor for OA development, whereas traumatic knee injuries are known to increase the risk of OA by more than four times. [38]
Recently, also oxidative stress has been included among the factors promoting OA pathogenesis.
Aging and aberrant mechanical stress can promote the accumulation of oxidative stress in chondrocytes. This involves the successive activation of multiple downstream pathways leading to cellular senescence, dedifferentiation, and apoptosis in chondrocytes. Oxidative stress typically results from the production of reactive oxygen species (ROS) that exceeds the

cellular antioxidant capacity. Both preclinical and clinical studies suggest that an increase in the quantity of ROS in articular cartilage is closely linked to OA development. [40], [41]

Although several risk factors are known, the exact etiology of OA remains unknown. However, currently available studies suggest that OA pathogenesis is associate to dramatic changes in cartilage homeostasis, identified by an imbalance between degradation and synthesis of articular cartilage by chondrocytes.

In healthy adult cartilage, chondrocytes are in a quiescent phase distinguished by a very little turnover of the cartilage matrix, with a fine balance between anabolic and catabolic activities, that allows chondrocytes to synthesize and degrade the articular extracellular matrix (ECM) rich in collagen (type II, IX, and XI), proteoglycans, and other related macromolecules. Instead, in ageing and degenerative joint diseases, among them OA, there is an up-regulation of catabolic pathways, that leads to the progressive articular cartilage degradation. This dysregulation induces cellular senescence, dedifferentiation and apoptosis, in joint cells. [42]

Early events during OA development are represented by the activation of quiescent chondrocytes to form clusters and increase the production of matrix proteins such as non-aggregated proteoglycan, and COL1A1, as well as matrix-degrading enzymes. When there is a joint tissue injury, it triggers the production of many inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. As a consequence, articular cartilage degenerates due to the acceleration of catabolic activities such as proteolysis of aggrecan by aggrecanases (ADAMTS 4 and ADAMTS 5) and degradation of collagen type II by matrix metalloproteinases (MM1, MMP3 and MMP13) (Figure 4). [43]

A further hallmark of deteriorated cartilage is the modification of chondrocyte differentiation stage. Indeed, chondrocyte phenotype switches toward a hypertrophic phenotype. Hypertrophic chondrocytes showed the expression of terminal differentiation markers, including RUNX2, COL10A1, MMP-13 and Indian hedgehog (Ihh), and the acquirement of an “autolytic” phenotype, displayed by their ability to induce the degradation of pericellular cartilage matrix. Conversely, in the physiological condition, the expression of hypertrophy-related genes is downregulated by cartilage protective mechanism such as Dickkopf (Dkk-1), which acts by inhibiting Wnt signaling.

The fate of chondrocytes to remain in the quiescent phase or undergo hypertrophic maturation prior to ossification is subject to complex regulation by the interplay of the FGF, TGF-β, BMP and Wnt signaling pathways. Particularly, whereas FGFs signaling have been shown to accelerate the rate of terminal hypertrophic differentiation, BMPs have been shown to slow

this process. Therefore, FGF signal acts as antagonist to BMP signal and negatively regulate Ihh expression. [19]

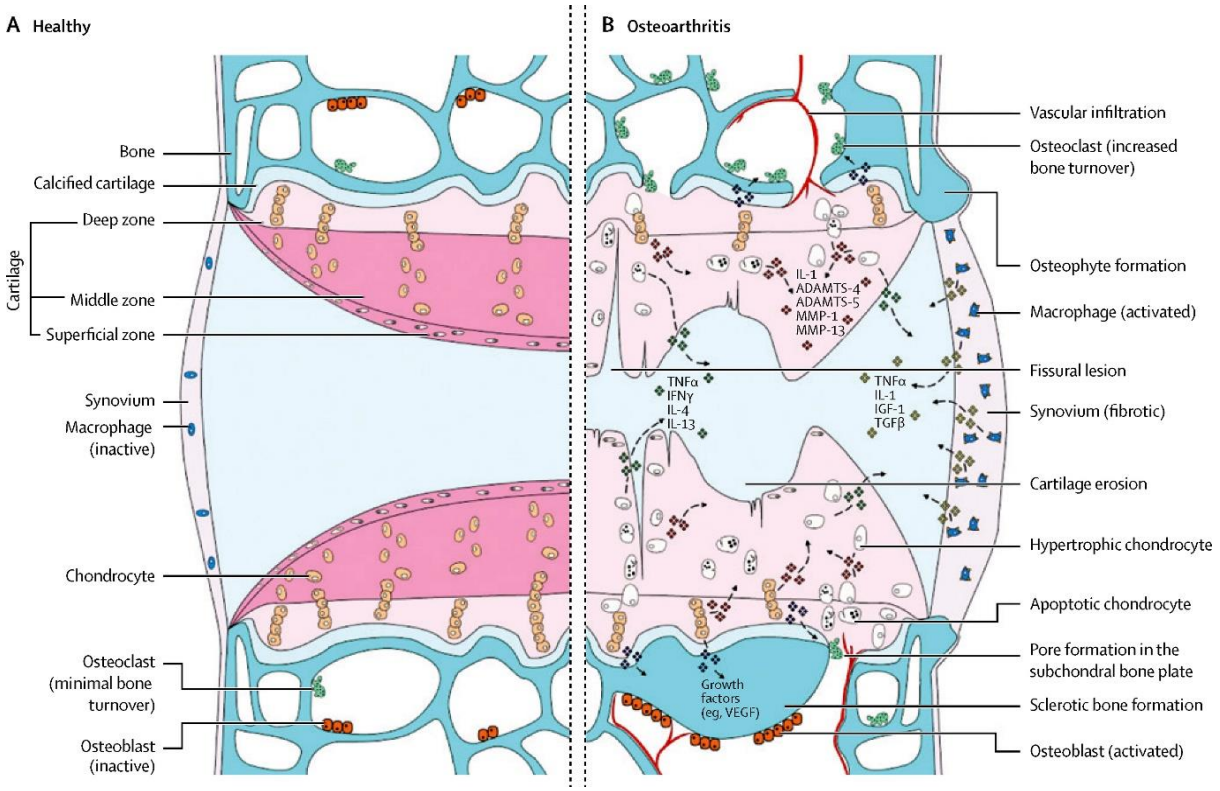

Figure 5. Schematic representation of healthy articular cartilage and OA cartilage. The figure shows on the left (A) the healthy articular cartilage and on the right **(B)** the structural changes and the signaling pathways associated with the development of osteoarthritis. Adapted from S. Glyn-Jones et al., 2015 [44]

In OA, the progressive and irreversible destruction of cartilage ECM together with the other pathological changes, cause joint pain, stiffness, tenderness, loss of mobility, and disability, greatly impacting patients' life quality and even leading to mortality. The pain is considered the major source of distress and disability in OA patients.

Osteoarthritis is currently incurable, and its etiology is largely unknown, due in part to a limited understanding of OA as a whole-joint disease. The commonly used non-surgical OA treatment modalities include intra-articular injections of soluble materials such as corticosteroids or hyaluronate, autologous blood products, joint realignment, nonsteroidal anti-inflammatory drugs (NSAIDs), and weight loss. Surgical treatments, like joint replacement, are usually indicated in the late phase of the disease. These procedures improve OA symptoms to a certain degree but do not completely heal the progressive loss of joint functions. [45]

Since cartilage differentiation and maintenance of homeostasis are finely tuned by a complex network of signaling molecules and biophysical factors, shedding light on these mechanisms appears to be extremely relevant for both the identification of pathogenic key factors, as specific therapeutic targets, and the development of biological approaches for cartilage regeneration. [45]

A topic that is currently subject of intense investigations, is the role of non-coding RNAs, particularly microRNAs, in the onset and progression of OA. Many investigators have used miRNA expression profiling arrays to identify specific miRNAs that are differentially expressed in OA. [46]

1.3.2 Metabolic disorders and diseases related to impaired osteogenesis

Bone is a dynamic organ capable of replacing old or damaged tissue through a remodeling process. The key cells involved in bone turnover are osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively. Osteoblasts specifically originate from the osteogenic differentiation of MSCs by multi-step process. Even small dysfunctions in MSCs osteogenic commitment or differentiation toward the osteogenic lineage, result in demineralization or bone loss in different pathological settings. [47], [48]

The multipotential MSCs' ability to differentiate into different lineages appears to change with age. In general, during aging, MSC shifts the balance in favor of adipocyte differentiation against osteoblast differentiation, resulting in decreased bone formation and an increased risk for osteogenesis associated disorders, such as osteoporosis.

Gene expression analysis consistently demonstrated an age-related down-regulation of osteoblast-specific genes such as CBFA1, Dlx5, RUNX2, collagen, and osteocalcin, whereas up-regulation occurs in adipocyte-specific genes such as peroxisome proliferator-activated receptor-g (PPAR-g) and the adipocyte fatty acid-binding protein (aP2). This age related shift in differentiation potential from osteoblast towards adipocyte is associated with osteoporosis, a common disease in the elderly that raises the risk of fracture and mortality. [48]

M.T. Valenti et al. discovered that, in osteoporotic patients, ox-PAPCs (modified lipoproteins derived from the oxidation of arachidonate-containing phospholipids) influence osteogenesis by increasing MSCs adipogenesis. [49]

They also discovered that bone loss and increased bone marrow adiposity are associated with glucocorticoid-induced osteoporosis (GIOP), a prevalent type of secondary osteoporosis,

caused by the imbalance between osteogenesis and adipogenesis due to glucocorticoid treatment. [49], [50]

Osteopenia is found also in chronic kidney diseases, where various bone loss patterns can be recognized, either characterized by higher bone turnover and osteoclastic activity or by decreased turnover associated with lower osteoblast differentiation.

Consequently, in aging-associated osteoporosis, in glucocorticoid-induced osteoporosis, and in chronic kidney diseases, bone loss is caused not only by an increased osteoclastic activity, but also by an impairment of MSCs commitment toward the osteogenic lineage, in favor of adipogenesis.

Multiple disruptions of the complex molecular pathways that promote MSC proliferation and their differentiation to osteogenic lineage can result in heritable bone diseases. These diseases are determined by mutations that affect the expression of important genes involved in the regulation of bone development. A few examples of heritable bone diseases are discussed below to illustrate the pathological consequences of disruption in different stages of osteogenic commitment, osteoblast maturation, and matrix mineralization. [51]

Fibrodysplasia ossificans progressiva (FOP) (OMIM 135100) is a rare autosomal dominant genetic skeletal disorder, manifesting progressive heterotopic ossification (HO) in the skeletal muscles, fascia, tendons and ligaments, and congenital deformity of the great toe. The majority of cases are sporadic, due to a recurrent de novo point mutation (c.617G>A) in the glycine-serine (GS) activation domain of activin A type 1 receptor, a bone morphogenetic protein (BMP) type I receptor. As a result of the consequent single amino acid substitution (R206H), which causes a gain-of-function effect, the mutant receptor becomes more sensitive to BMPs. [52] In vitro disease models, i.e., induced pluripotent stem cells (iPSC) derived from FOP patient fibroblasts, have shown that the ACVR1 R206H mutation confers an enhanced propensity towards cartilage formation and mineralization, with a transient increase in the expression of osteogenic markers. [53] Interestingly, recent studies in the cellular FOP model revealed that treating cells with a soluble recombinant fusion protein, ACVR1-Fc, containing the extracellular domain of wild-type ACVR1 and the Fc portion of human IgG1, resulted in a significant downregulation of the aberrant BMP signaling and inhibition of chondro-osteo differentiation. [54]

Cleidocranial Dysplasia (CCD) (OMIM 119600) is a rare autosomal dominant skeletal disorder characterized by short stature, absent/hypoplastic clavicles, persistently open or

delayed closure of sutures and fontanelles, abnormal dentition, and other skeletal changes. CCD is caused by various mutations (mostly resulting in loss of function) in CBFA1/RUNX2, the master gene for MSC osteogenic commitment. It has been shown that Runx2-null mice do not form bone and die soon after birth, whereas heterozygous Runx2 deficient mice (+/−) have specific CCD features and lower alkaline phosphatase (ALP) activity. MSCs derived from a CCD patient who was heterozygous for a RUNX2 mutation (+/−), showed a reduced proliferative capacity and a lower ability to differentiate into osteoblasts. [51]

Osteogenesis imperfecta (OI) comprises a heterogeneous group of diseases characterized by fragile, deformed bones and osteopenia, resulting in high incidence of bone fractures. OI was first described in 1788 and was long considered to be a collagen disorder. In fact, 90% of OI cases are actually due to dominant mutations in COL1A1 and COL1A2 genes, which encode the major component of type 1 collagen. Collagen type 1 is the most abundant protein in the bone matrix. The remaining 10% could be due to causative recessive variants in the 8 genes known to be related to collagen biosynthesis, as well as other genes that are currently unknown. Quantitative and qualitative defects in Collagen type 1 produce different clinical types of OI. [55]

Impaired collagen biosynthesis and secretion, as well as the extracellular matrix deposition, jeopardizes proper bone mineralization. Recent molecular findings of causative mutations for rare recessive forms of OI have revealed specific defects in the osteogenic commitment of progenitors. Glorieux and colleagues first described autosomal recessive-type VI OI (OMIM 613982) as having unusual histological features revealing defects in the mineralization process. Patients with type VI OI have homozygous or compound heterozygous null mutations in SERPINF1, the gene coding for PEDF (Pigment epithelium-derived factor). Children with type VI OI appear normal at birth, but they begin fracturing between the ages of 8 and 12 months, and then develop a severe progressively deforming bone dysplasia, resulting in the loss of autonomous walking. The bone histology of type VI patients is peculiar, with increased amounts of unmineralized osteoid and abnormalities lamellae orientation. Other recessive OI forms have been linked to mutations in two key genes that control osteoblast differentiation: SP7/Osterix (OI type XII, OMIM 613849) and WNT1 (OI type XV, OMIM 615220). Studies on the rare conditions described above contributed further knowledge about the major actors in bone development and mineralization. [55]; [56]

Hypophosphatasia (HPP, OMIM 241500) is a genetic condition that results from mutations in the ALPL gene, which encodes for the tissue-nonspecific isoenzyme of alkaline phosphatase (TNSALP). TNSALP is a homodimeric cell-surface phosphohydrolase that is abundant in the

skeleton, liver, kidney and developing teeth. In hypophosphatasia, extracellular accumulation of TNSALP natural substrates includes inorganic pyrophosphate, an inhibitor of mineralization, which explains the dento-osseous and arthritic complications such as tooth loss, rickets or osteomalacia, and calcific arthopathies.

Different ALPL loss-of-function mutations can lead to the production of defective/inactive TNSALP. [57] As a result, the concentration of inorganic pyrophosphate (PPi) in the bone matrix increases, impairing bone mineralization and disrupting calcium and PPi homeostasis. The pathogenetic mechanisms causing bone hypomineralization in HPP have been studied fairly well. Biomedical research has primarily focused on enzyme replacement therapy. Nonetheless, results were unsatisfactory. For the future, thanks to current biotechnological innovations, more innovative therapeutic approaches can be developed. The employment of MSCs could be a viable option. In infants with severe HPP who were treated with ex vivo expanded allogeneic MSCs, skeletal mineralization results were encouraging. [58]

All these findings highlight the pivotal role of MSCs in the pathogenesis of systemic and heritable bone diseases. Disruption of MSC differentiation is an interesting area of study, and MSCs represent important targets for skeletal regenerative therapy.

1.4 Post-transcriptional regulation in chondrogenesis and OA

1.4.1 microRNAs biogenesis, localization and regulation

Micro ribonucleic acids (miRNAs or miRs) are important post-transcriptional regulators of genes that define cellular identity and regulate cell differentiation. It is well known that miRNAs play a crucial role in MSCs differentiation. Indeed, their regulation is involved in the fine modulation of gene expression during MSCs osteogenic and chondrogenic differentiation. MiRNAs dysregulation is involved in the development and pathogenesis of bone and cartilage diseases, as well as in malignancies. [59]

- **Biogenesis**

MiRNAs are a class of endogenous, single-strand, small non-coding RNA molecules of about 18-22 nucleotides, that were first identified in C. elegans in the 1993 from studies carried out by Victor Ambros et al. MiRNAs genes are one of the most abundant gene families, and are widely distributed in animals, plants, protists and viruses.
MiRNAs negatively regulate gene expression by base-pairing with a sequence located in the 3'-untranslated region (3'-UTR) of a specific messenger RNA (mRNA). This regulatory mechanism promotes the degradation of mRNA and/or its translational repression. [60] MiRNAs sequences can be found in a broad range of genomic contexts: the majority of canonical miRNAs are encoded by introns of non-coding or coding transcripts and exonic regions. miRNA genes that reside in the introns of protein-coding genes, share the promoter of the host gene. [61]

Most miRNA genes are transcribed by RNA polymerase II (Pol II) as long primary miRNAs (pri-miRNAs), characterized by a stem-loop structure, which undergoes several steps of maturation. In the nucleus, pri-miRNAs are processed by RNase III endonuclease, Drosha, to form a miRNA precursor (pre-miRNA) of 70-100 nucleotides length. Pre-miRNAs are exported, through Exportin 5, from the nucleus to the cytoplasm where a further maturation step, mediated by Dicer RNase, generates a double stranded miRNA (miRNA duplex) of about 18-22 nucleotides. The miRNA duplex is subsequently loaded into an Argonaute protein (Ago) to form an effector complex RISC (RNA-Induced Silencing Complex), in which guide strand remains linked to Ago and constitutes the mature miRNA that will associate to the 3'UTR of its target mRNA, while passenger strand is subjected to degradation. [60]

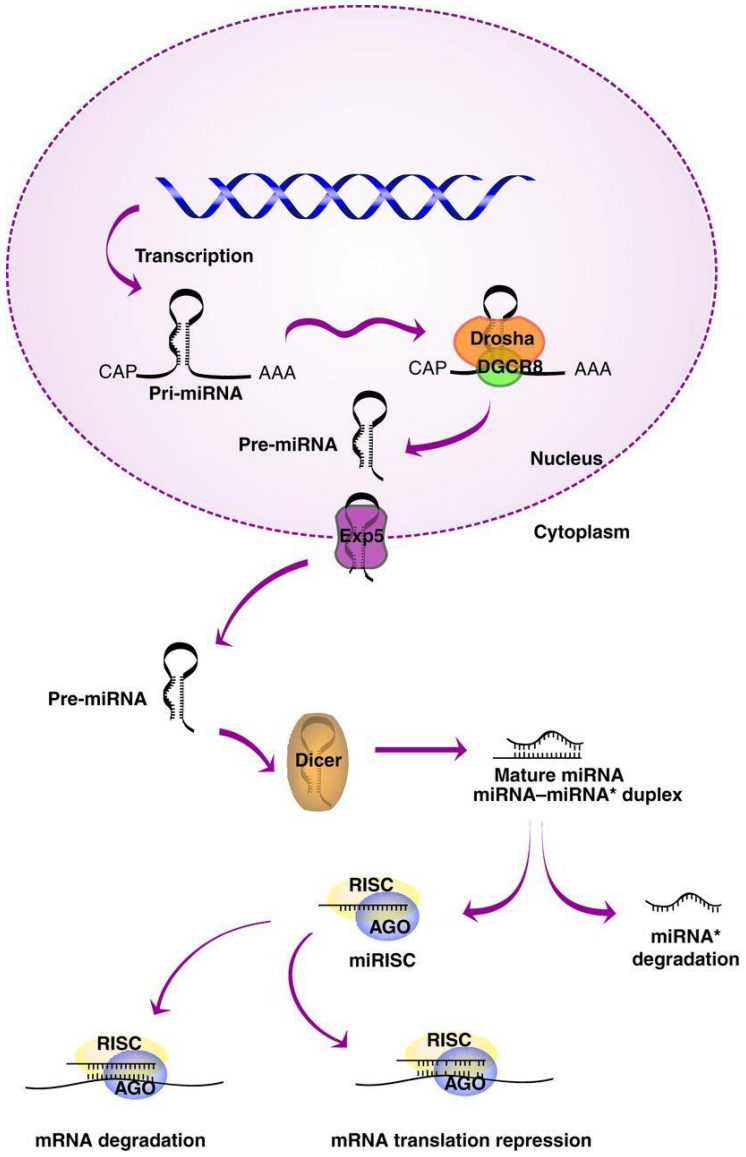

Figure 6. microRNA biogenesis and mechanisms of action. Schematic representation of canonical pathway of microRNA transcription and processing. Adapted from B. Gamez et al., 2014 [59]

- **Localization**

MiRNAs can localize to different subcellular compartments like the mitochondria, endoplasmic reticulum, processing bodies (P-bodies), nucleus, and nucleolus. Additionally, mature miRNAs are secreted from cells via exosomes and can be detected in plasma and other body fluids. Processing bodies (P-bodies) are sites of mRNA surveillance that are enriched with AGO family members and other RNA decay factors. MiRNAs and their targets localize at P-bodies, but it is not yet known if they play a function in disease etiology.

Localization and distribution of miRNAs in subcellular compartments and exosom can be affected by numerous factors. These include the availability of AGO family members and

other non-AGO miRNA-binding proteins, as well as protein modifications based on cellular conditions. The presence of AGOs is essential for miRNA stability, as the overexpression of Ago genes promotes mature miRNAs accumulation.
Secreted miRNAs are of particular interest since they have the ability to modulate the gene expression of neighboring cells as well as in a variety of cell types, including those in the immune system, neuronal synapses and cancer. [46]

- **Regulation**

MiRNAs are specifically expressed in a variety of tissues and biological fluids, and specific miRNA patterns are associated with different cell types and functions. Furthermore, they participate in essential biological activities and cellular processes, such as the regulation of cell cycle, proliferation, differentiation, development and apoptosis. [61]

MiRNA expression is a strictly regulated process that may be monitored at many levels related to miRNA synthesis and processing. These comprise proteins and molecules associated to miRNA transcriptional regulation (RNA PolII), nuclear processing (Drosha and DGCR8), nuclear export (Exportin-5), and pre-miRNA processing (RISC complex). Modifications of these molecules can occur through many mechanisms, including DNA methylation, histone deacetylation, gene mutation, and DNA copy alteration.
MiRNA expression may also be subject to intrinsic regulation, which comprises alterations to RNA sequence and/or structure that have an effect on mature miRNA maturation and turnover. These modifications include single nucleotide polymorphisms, miRNA tailing, RNA editing, and RNA methylation. [46]
Changes in miRNAs expression have been linked to a variety of diseases, including obesity, cardiovascular diseases, and cancer. Because of their stability and ease of detection in body fluids, miRNAs have prompted a lot of interest as biomarkers for disease diagnosis, monitoring and treatment. [46]

1.4.2 miRNAs involved in cartilage homeostasis and OA

Numerous miRNAs play significant roles in cartilage development and homeostasis, and they may influence both chondroblast differentiation and maturation. MiRNAs, which key regulators of chondrogenesis, are upregulated in OA chondrocytes and target key signaling mediators involved in OA pathogenesis. [45], [46]
Differential miRNAs expression patterns have been discovered in OA cartilage and chondrocytes when compared to normal. This resulted in the identification of several key miRNAs that target OA-associated genes that encodes for matrix-degrading enzymes, pro-inflammatory cytokines, proteins involved in chondrocytes apoptosis and autophagy. [46]

Among miRNAs that regulate chondrogenesis, miR-140 is the most extensively studied. miR-140 is known to be a cartilage-specific miRNA in zebrafish and mouse embryos, and a key regulator of cartilage development and homeostasis. [62] miR-140 targets Sp1 in order to maintain chondrocyte proliferation. During chondrogenesis, miR-140-3p and miR-140-5p are up-regulated in human MSCs and target genes involved in terminal hypertrophic differentiation. miR-140-5p specifically targets the 3'UTR of Ras-related protein Ral-A (RALA), promoting SOX9 and ultimately, ACAN expression. It was discovered that miR-140 expression is downregulated in human OA cartilage. [45], [63]
The miR-140 primary transcript is an intron-retained RNA, which is co-expressed with Wwp2 gene and is directly induced by Sox9 during chondrogenesis. Nakamura et al. discovered that miR-140 has numerous binding sites for SOX) within the Wwp2 gene in humans, mice, and zebrafish, and that miR-140 is downstream of the transcription factor Sox9 during zebrafish embryogenesis and in mammalian cells. Indeed, miR-140 expression is reduced when Sox9 is deleted. [45]

Another important miRNA is miR-455. MiR-455, located in the intron 10 of COL27A1, is expressed in chondrogenesis cell culture models (along with miR-140). Specifically, miR-455-3p acts as an activator of early chondrogenic differentiation of MSCs by boosting the expression of the cartilage-specific genes Col2a1 and Comp and by directly targeting and inhibiting RUNX2. Indeed, miR-455-3p regulates OA and MSCs chondrogenic differentiation.

By inhibiting the expression of histone deacetylase 2 (HDAC2) and HDAC8, miR-455-3p encourages chondrogenic differentiation by maintaining a suitable level of histone H3 acetylation at the COL2A1 promoter to promote the synthesis of collagen type II.

According to some studies, miR-455-3p directly targets P21-activated kinase (PAK2) to prevent cartilage degradation and regulates TGFβ signaling by blocking the Smad2/3 pathway.

In miR-455-3p global KO mice, at six months of age, thinner cartilage thickness was observed compared to WT mice. Moreover, these mice displayed an OA-like phenotype, because they had cartilage disruption in their knee joints at five months of age, supports the idea that miR-455-3p is an essential regulator of cartilage homeostasis.

Among miR-455 targets, was found the endothelial PAS domain protein 1 (EPAS1), which encodes hypoxia inducible factor (HIF)-2α. HIF-2α is known as a catabolic transcription factor for cartilage homeostasis. Y. Ito et al. revealed that both miR-455-5p and -3p directly control EPAS1 expression. This suggest that both miR-455s have anti-inflammatory properties and defend against cartilage damage in OA. In comparison to the effects of the injection of both miR-455-5p and -3p mimics into the knee joints of surgical DMM model of OA, dramatically prevented cartilage breakdown. [45]

The deterioration of the cartilage matrix can be associated, in part, to the lower number of surviving chondrocytes. Previous research has found that chondrocyte apoptosis is increased in aging and OA. In chondrocytes, a multitude of miRNAs target apoptotic genes. [46] For example, miR-34a is overexpressed in human OA cartilage and promotes the advancement of OA via altering delta-like protein 1 (DLL1) and PI3K/AKT pathways, triggering cell death and senescence. A study conducted by S. Yan et al., shown that miR-34a was upregulated in human OA chondrocytes, and it induce cell apoptosis via targeting SIRT1/p53 signaling pathway. [64], [65] miR-146a was found to be highly upregulated in IL-1β treated chondrocytes and it directly targets the 3'-UTR of SMAD4 mRNA resulting in an increase of VEGF and chondrocyte apoptosis. Apoptosis and autophagy in chondrocytes are also induced by miR-195, which target the transcription factor hypoxia-inducible factor-1-α (HIF-1α). This miRNA was found in high concentrations in OA samples. [46]

Two other miRNAs that regulate chondrocyte homeostasis include miR-137, which targets RUNX2, and miR-483-5p, which targets MAPK family members. It is interesting to note that RUNX2 promotes articular cartilage degeneration by inhibiting chondrocyte proliferation and directly controlling the expression of MMP13 and ADAMTS5 to digest collagen or aggrecan, respectively. RUNX2 was discovered to be highly expressed in both murine OA models and OA patients. Therefore, miR-137 and miR-483-5p have a protective effect against OA progression. [46], [66]

miRNAs clearly play a wide range of important roles in regulating chondrocyte and cartilage homeostasis, but their short half-life, degradation susceptibility, and high mismatch rate limit clinical applications of targeting miRNA. [45], [46]

1.5 miR-204

1.5.1 miRNA-204 genomic organization

The human gene *MIR204*, is located within the intron 9 of TRPM3, and encodes a 110 bp pre-miR-204 stem loop. TRPM3 gene is one of the largest genes situated on the long-arm of human chromosome 9 (9q21.12-q21.13) and exhibit extensive alternative splicing and genetic variations. Similarly, *Mir204* gene in mouse, which encodes a 68 bp pre-miR-204 stem loop, is located in an intron of Trpm3. [67]

MIR204 human gene is co-transcribed in the same direction as its host gene TRPM3 and they are co-regulated. miR-204-5p is the major strand of mature miR-204, whereas miR-204-3p is the minor strand, and it is present at much lower amount than miR-204-5p. The sequence of mature miR-204 is highly preserved across human, mouse and rat with an identical seed region (Figure 5). Notably, miR-204-5p has the same seed region as miR-211-5p; this implies that they can target the same mRNAs and have been classified as one microRNA family. [68]

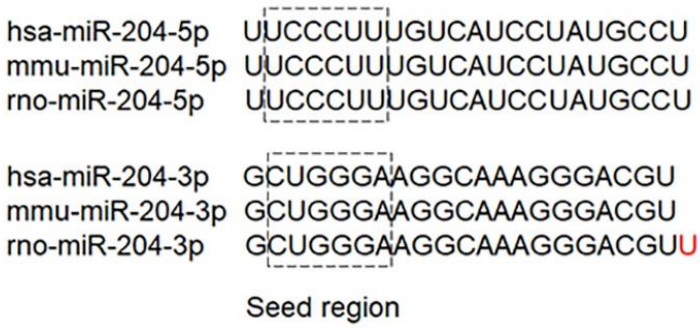

Figure 7. Seed region sequence of miR-204. The illustration shows the mature sequences of miR-204-5p and -3p, which are highly conserved in human (has-), mouse (mmu-), and rat (rno-) and possess identical seed regions. Adapted from J. Liu et al., 2021 [68]

1.5.2 miR-204 expression and regulation

MiR-204 exhibits a highly tissue-specific expression pattern. An analysis of human tissues, has revealed that miR-204-5p expression is very abundant in various neural tissues including arachnoid mater, dura mater, brain and spinal cord. Moreover, high miR-204-5p expression levels were detected in kidney, epididymis, and testis and measurable levels of miR-204-5p were found in artery. Additionally, mir-204-5p has been identified as one of the most abundant miRNAs in human retinal pigment epithelial cells. miR-204-3p exhibits a similar tissue pattern but at much lower abundance. [69], [70]

The regulation of miR-204 expression can occur by genomic deletion and epigenetic silencing. This occurs especially in malignancies, where miR-204 is a well-known tumor-suppressor miRNA and its expression is downregulated by genomic deletion in a variety of cancers. Ying et al. demonstrated that the downregulation of miR-204 in glioma specimens was associated with hypermethylation in CpG island inside the TRPM3/miR-204 promoter. The demethylation through the inhibition of DNA methyltransferase in several glioma cells with low levels of miR-204 significantly promoted the expression of miR-204. [68]

The expression of miR-204 can also be regulated by transcription factors. STAT3 is a key transcription factor that administrates a variety of biological processes. When phosphorylated, cytoplasmic STAT3 dimerize and translocate into the nucleus, regulating gene expression including that of miR-204. In a study conducted by Courboulin et al., has been shown that activated STAT3 bound directly to the regulatory regions of TRPM3 and diminished the expression of miR-204 in pulmonary artery smooth muscle cells (PASMCs). Transcriptional repression of miR-204 by activated STAT3 has also been observed in cancers. [68]

Interestingly, miR-204 expression can be managed by a regulatory circuit between miR-204 and its targets. In a study conducted by Modar K. et al., in a contest of endoplasmic reticulum (ER) stress, it has been demonstrated that miR-204 is upregulated. Upregulated miR-204 promoted endothelial vasodilation and dysfunction by targeting Sirtuin1 (Sirt1). Sirt1 is an NAD-dependent lysine deacetylase. Sirt1 could downregulate miR-204, suppressing ER stress and preserving vasorelaxation. In this miR-204/Sirt1 feedback loop, the decrease of vascular Sirt1 by miR-204 during ER stress contributes to amplification of miR-204, and endothelial dysfunction is attributed to the further downregulation of Sirt1. [68], [71]

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with limited or no protein coding potential but acting as potent regulators in diverse biological processes. Yu et al.

revealed that lncRNA TUG1 (Taurine Upregulated Gene 1) bounded miR-204, thus preventing its function. In particular, lncRNA TUG1 interfered with the binding of miR-204 to its target RUNX2, promoting osteogenic differentiation in calcific aortic valve disease. [68]

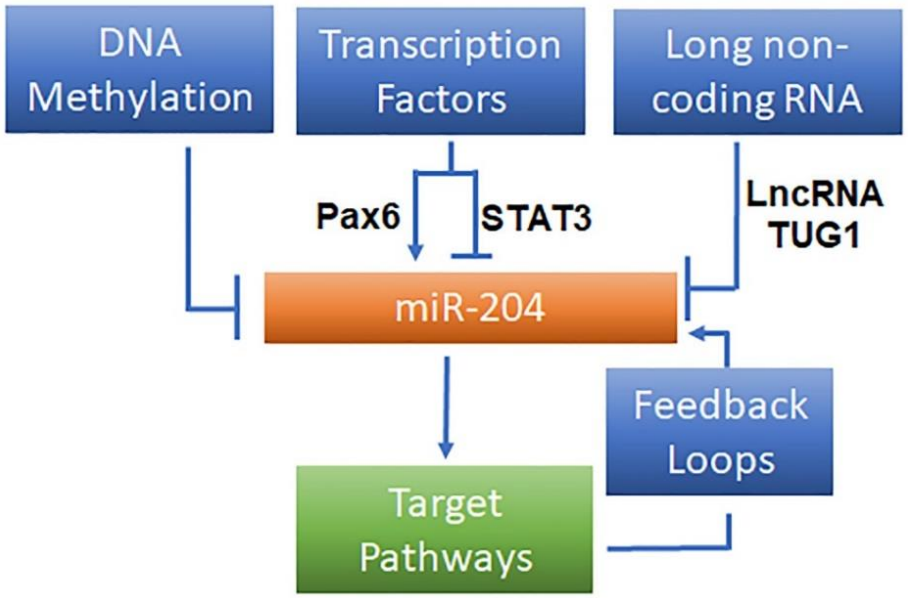

Figure 8. Mechanisms that regulate miR-204 expression. MiR-204 expression can be regulated by DNA hypermethylation in the TRPM3/miR-204 promoter, transcription factors activity (like Pax6 and STAT3), long non-coding RNAs and a feedback loop between miR-204 and its targets. Adapted from J. Liu et al., 2021 [68]

1.5.3 Role of miRNA-204 in joint homeostasis and OA

As previously mentioned, since miRNAs regulate different and specific cellular processes, they are critical determinants of many biological functions and individual miRNAs have been found to be involved in more than one setting. [72]

MiR-204 is expressed in a wide range of tissues and exhibits a highly tissue-specific expression profile. Its abundance is controlled by several transcriptional and post-transcriptional mechanisms. MiR-204 can target important biological processes and has a well-established role in maintaining the normal function of the retinal pigment epithelium, suppressing tumors, and influencing the development of osteoarthritis. [68], [69]
Huang J. et al, demonstrated that miR-204 and its homolog miR-211 act as significant endogenous negative regulators of RUNX2 protein expression in mesenchymal progenitor

cells (MPCs) and bone marrow stromal cells (BMSCs). RUNX2 is a key osteogenesis regulator, and miR-204 can bind to multiple sites in the 3'-UTR of RUNX2. When miR-204/-211 is overexpressed in these cells, RUNX2 protein levels decreased and osteoblast differentiation is inhibited, whereas adipocyte differentiation is promoted. Conversely, miR-204 inhibition significantly elevated RUNX2 protein levels, promoting osteogenesis and impairing adipocyte formation. [73], [74]

MiR-204 expression is also important for maintaining joint homeostasis and preventing the pathogenesis of OA. [75] Huang J. et al. specifically demonstrated that genetically modified mice with miR-204/-211 deficiency in MPCs (dKO mice) had structural deterioration in the knee joints involving multiple types of tissues within the joint. These dKO mice developed severe synovial hyperplasia, subchondral bone thickening, excessive osteophyte formation, and meniscus calcification, reflecting the pathological condition of human OA disease.
Additionally, Huang J. et al. showed in a different study that mir-204/211-RUNX2 axis is pivotal for maintaining joint tissue homeostasis and the dysregulation of this signaling pathway results in the multifactorial pathogenesis of OA. In fact, miR-204/-211 deficiency in MPCs leads to RUNX2 accumulation in a variety of joint cells and tissues, such as articular cartilage, meniscus, and synovium. It also results in the induction of proteases, such as MMP13, Adamts5 and Adamts4, which stimulate articular cartilage destruction and contribute to OA pathogenesis. Importantly, the mir-204/-211-RUNX2 axis is confirmed by the fact that OA induced by mir-204/-211 deficiency is largely rescued by RUNX2 insufficiency. [39]
Furthermore, Huang J. et al. evidenced that RUNX2 accumulation induced by miR-204/-211 ablation enhances NGF expression (nerve growth factor, fibroblasts-ECM organization), which in turn hyper-activates Akt signaling in mesenchymal tissues and promotes MPCs proliferation, recapitulating all non-cartilaginous OA conditions observed in the dKO mice. [76], [39] For instance, osteophyte outgrowth is significantly contributed by excessive MPCs' proliferation. Moreover, NGF overexpression in MPCs may constitute a key mechanistic cause of improper nerve growth inside joints and the resulting OA pain hypersensitivity. [39]

There is a growing body of evidence that both ageing and aberrant mechanical stress promote accumulation of oxidative stress in chondrocytes, which in turn can activate multiple downstream pathways resulting in cellular senescence, dedifferentiation, and apoptosis. Chondrocyte senescence is a critical cellular event that contributes to matrix metabolism imbalance during OA development. [77] In a recent study, D. Kang et al. discovered that in the context of cellular senescence, miR-204 is markedly up-regulated in OA-affected human and mouse cartilage and barely detectable in undamaged regions of arthritic cartilage. [78]

It has been demonstrated that several senescence-inducing stimuli, including protracted exposure to H_2O_2, ionizing radiation, and chemical agents that cause DNA damage (such as bleomycin or doxorubicin) significantly increase miR-204 transcription. In response to senescence signals, miR-204 is induced by transcription factors GATA4 and NF-kB. Up-regulated miR-204 concurrently targets multiple components of the sulfated proteoglycan (PG) synthesis pathway in chondrocytes, thereby effectively restraining PGs anabolism and impairing the cartilage matrix deposition. The diminished capacity to synthesize cartilage matrix is one of the main symptoms of OA chondrocytes.

Intra-articular delivery of miR-204 to mouse knee cartilage considerably decreased the extracellular release of PGs in chondrocytes and induced spontaneous cartilage demolition. Immunohistochemistry analysis revealed increased MMP13 expression in articular chondrocytes of miR-204-injected mice, indicating a cartilage degeneration process.

Gene set enrichment analysis (GSEA) disclosed that the predicted target genes of miR-204 were negatively enriched within the whole transcriptomes of OA cartilage, suggesting that miR-204 up-regulation acts to suppress its target genes under OA conditions. [78]

In summary, the role of miR-204 in OA development and pathogenesis appears to be controversial. According to studies conducted by Huang J. et al., miR-204/-211 regulate MPCs proliferation and differentiation in the entire joint and are essential for preserving a healthy joint tissue metabolism and effectively counteract OA pathogenesis. Additionally, intraarticular administration of adeno-associated viruses that express miR-204 significantly slows the progression of OA. However, the recent study by D. Kang et al. showed that the ectopic expression of miR-204 in joints triggers cartilage decline and OA development, whereas miR-204 inhibition improves experimental OA in mice, with concurrent recovery of PG synthesis and eradication of inflammatory senescence-associated manifestations in cartilage.[78]

1.6 Bioactive compounds

1.6.1 Role of bioactive molecules in osteogenesis and chondrogenesis

Nutraceuticals are defined as bioactive substances, originally derived from food sources, aimed to provide medical or health benefits, including the prevention and treatment of diseases (like cardiovascular disease, diabetes, cancer, etc.). In recent years, nutraceutical compounds have received considerable interest due to their potential nutritional, safety and therapeutic effects. [79]

Nutraceuticals are heterogeneous compounds that act through different but complementary mechanisms to reduce inflammation, oxidative stress, pain, and joint stiffness while also improving cartilage formation. Indeed, many nutraceuticals can promote chondrocyte proliferation, stabilize chondrocyte phenotype, and promote mesenchymal stem cell chondrogenic differentiation. To accomplish this, nutraceuticals mainly act on the Wnt, MAPK, Akt, and Ihh pathways. They activate the synthesis of matrix protein genes, such as COL2A1, aggrecan, and glycosaminoglycans (GAGs), that promote the development of cartilage matrix and cartilage formation. [80], [81]

Nutraceutical supplementation has been demonstrated to be a relevant adjuvant strategy in the management of OA pathogenesis for its help with pain, inflammation and oxidative stress. This was supported by a wealth of information collected from cellular OA models, animal studies, and random clinical trials in people (RCTs).

Among the most popular nutraceuticals for OA, chondroitin sulphate, glucosamine sulphate, collagen, hyaluronic acid, and methylsulfonylmethane (MSM) have been proven to be effective in reducing inflammatory indices and improving clinical symptoms in OA patients.

An important point with regard to the nutraceutical supplements is the low bioavailability of the more promising compounds, which inevitably reduces the treatments' overall effectiveness. So, choosing the right bionutraceutical formulation is essential for the success of a nutraceutical supplementation.

The high expense of obtaining titrated and standardized extracts is another important restriction on the use of nutraceuticals in the treatment of OA. The development of inexpensive, high-quality extracts, obtained with low-cost methods is not always possible using the conventional procedures that are already utilized by industries. The problem of cheap but low-quality nutraceutical extracts is a serious, underestimated, and potentially dangerous one for people's health, not only due to the absence of specific active ingredients or

the low-quality of un-titrated extracts, but also, and most importantly, due to the presence of contaminants. [80]

1.6.2 Methylsulfonylmethane (MSM)

Methylsulfonylmethane (MSM), also known as dimethyl sulfone, is an organic sulphur molecule suggested for relieving joint/muscle pain, thanks to its anti-inflammatory properties. For this reason, it has also been linked to the prevention of the onset and progression of OA. MSM is naturally present in a variety of plants and foods. [80], [82]

Studies conducted *in vitro* demonstrated the MSM's capacity to function as an anti-inflammatory agent by inhibiting NF-κB transcription factor as well as p65 phosphorylation, and so reducing the expression of interleukins (IL-1, IL-6, IL-1β) and TNF-α, all of which are associated with an inflammatory response. In addition, MSM reduces oxidative stress by inhibiting the expression of cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS), lowering reactive oxygen species (ROS) formation. MSM regulates the balance of ROS and antioxidant enzymes by modulating at least four different transcription factors including NF-κB, STAT, p53, and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). [80]

Recent research has proven that MSM also encourage osteogenic differentiation of mesenchymal stem cells (MSCs). Indeed, MSM acts through the Jak2/STAT5b pathway and induces the upregulation of the osteogenic master gene RUNX2, which transactivates its downstream partner SP7 (Osterix) and thus the expression of osteogenic genes (e.g. COL1A1, COL1A2, BGLAP (osteocalcin), SPARC (osteonectin), and SSP1 (osteopontin)). These molecular events stimulate MSCs differentiation into osteoblasts. [80] Additionally, according to a study by Kim et al., the osteogenic differentiation operated by MSM is aided by the expression of bone morphogenetic protein 2 (BMP2). [83]

According to RCTs, MSM is effective at reducing pain, as indicated by the WOMAC and other pain subscales, as well as improving swelling and stiffness in arthritic patients. Similar findings have been reported in other RCTs that used MSM in combination with other treatments. In this regard, in a double-blind RCT including 147 people with knee OA, patients were randomly assigned to receive a placebo, 1500 mg of glucosamine + 1200 mg of chondroitin sulphate or 1500 mg of glucosamine + 1200 mg of chondroitin sulphate + 500 mg of MSM for 3 months. At the end of the treatments, the MSM treated group had statistically significant WOMAC ($p = 0.01$) and VAS scores ($p < 0.001$) compared with the control and the

glucosamine and chondroitin group. The combination of glucosamine, chondroitin, and MSM has also been shown to improve functional ability and joint mobility. To determine MSM effectiveness, particularly when compared to chondroitin and glucosamine, larger and long-term clinical trials are required. [80]

1.6.3 Flavonoids and Fisetin

Flavonoids are a kind of polyphenolic compounds widely present in vegetables and fruits, which possess biological activities and are characterized by different pharmacological properties. Various flavonoids have been identified and they are commonly classified into the following subclasses: flavanones, flavonols, flavonones, and isoflavones, anthocyanins, flavanols.

In terms of chemical structure, flavonoids generally refer to a series of hydroxylated phenolic molecules with a C6-C3-C6 structure, containing two benzene rings (A and B) connected by a three carbon heterocyclic ring (C). [84]

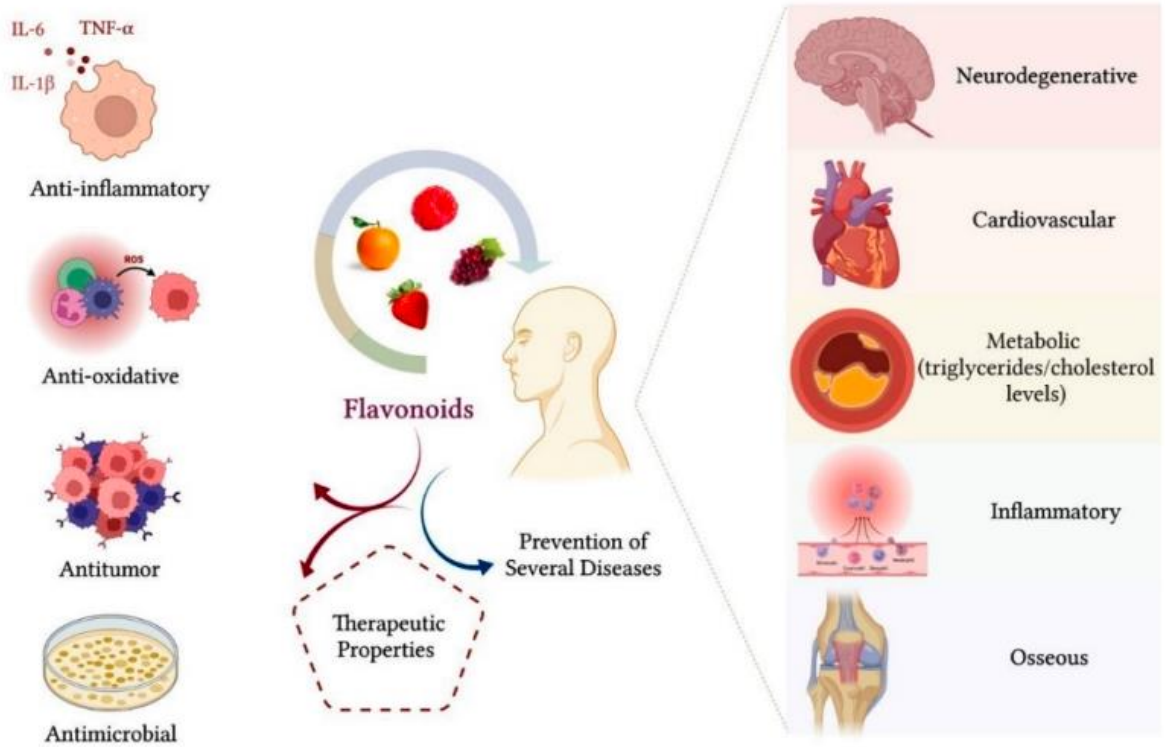

Figure 9. Therapeutic effects of flavonoids. Flavonoids' main health benefits are related to their anti-inflammatory, antioxidant, antitumor, and antimicrobial properties. Consumption of a diet containing flavonoids can also help lower triglyceride and cholesterol levels in the plasma, as well as help prevent neurodegenerative, cardiovascular, metabolic, inflammatory and bone diseases, such as osteoarthritis and osteoporosis. Adapted from A. de Cassia Ortiz et al., 2022 [85]

Plant extracts containing flavonoids compounds have been extensively employed in traditional medicines for centuries, and nowadays numerous flavonoids have been extracted or synthesized for use in the treatment of various diseases, among them osteoporosis. [86], [85]
Flavonoids possess a wide range of pharmacological activities including anti-inflammatory, anti-oxidative, anti-microbial, and anti-tumor properties. All these properties may be beneficial to bone regeneration. Furthermore, both *in vitro* and *in vivo* studies have shown that flavonoids have a direct influence on bone regeneration by promoting osteogenic differentiation of mesenchymal stem cells (MSCs) and inhibiting osteoclast-mediated bone resorption. [87], [88]

Firstly, many flavonoids, including Baicalin and Kaempferol, exert their anti-inflammatory properties by preventing the activation of the NF-κB pathway, which is strongly linked with inflammation.
Secondly, flavonoids reduce the production of free radicals, lower lipid peroxidation, and stimulate antioxidant enzymes activity, resulting in free radical elimination. Some of flavonoids have been used in clinical treatments, such as in central retinitis, thrombophlebitis, and ischemic cerebrovascular diseases.
Thirdly, flavonoids have been discovered to have anti-tumor actions via reducing tumor cell proliferation and metastasis, preventing tumor invasion, and triggering autophagy or apoptosis in tumor cells.
Finally, recent studies have revealed that flavonoids can influence MSCs' ability for self-renewal and osteogenic differentiation. MSCs' capacity to differentiate into osteoblasts may become impaired in certain pathophysiological conditions characterized by oxidative stress and inflammation, as well as in degenerative bone diseases like osteoporosis. For this reason, in recent years, the regulatory effects of flavonoids on MSCs differentiation have received increased attention. Some studies have shown that flavonoids control the self-renewal and osteogenic differentiation potential of MSCs by targeting multiple signal pathways such as Wnt/β-catenin pathway, ERK pathway, PI3K/Akt pathway, and regulating the bone-specific markers and transcription factors including ALP, RUNX2, BMP-2, Cbfa1, Osx (Figure 4).

Despite the fact that the majority of studies discovered that flavonoids promoted MSC osteogenic differentiation, some reports revealed that flavonoids occasionally inhibited this process while promoting adipogenesis. To better understand this issue, further studies are required. Moreover, as most flavonoid compounds lack high solubility and hydrophilicity, delivery systems, such as nanocarriers, including flavonoids are promising strategies that need to be evaluated in order to improve cell uptake efficiency. Additionally, using MSCs in

combination with biological scaffold materials that contain flavonoids is an excellent way to use flavonoids in the field of bone tissue engineering. [88]

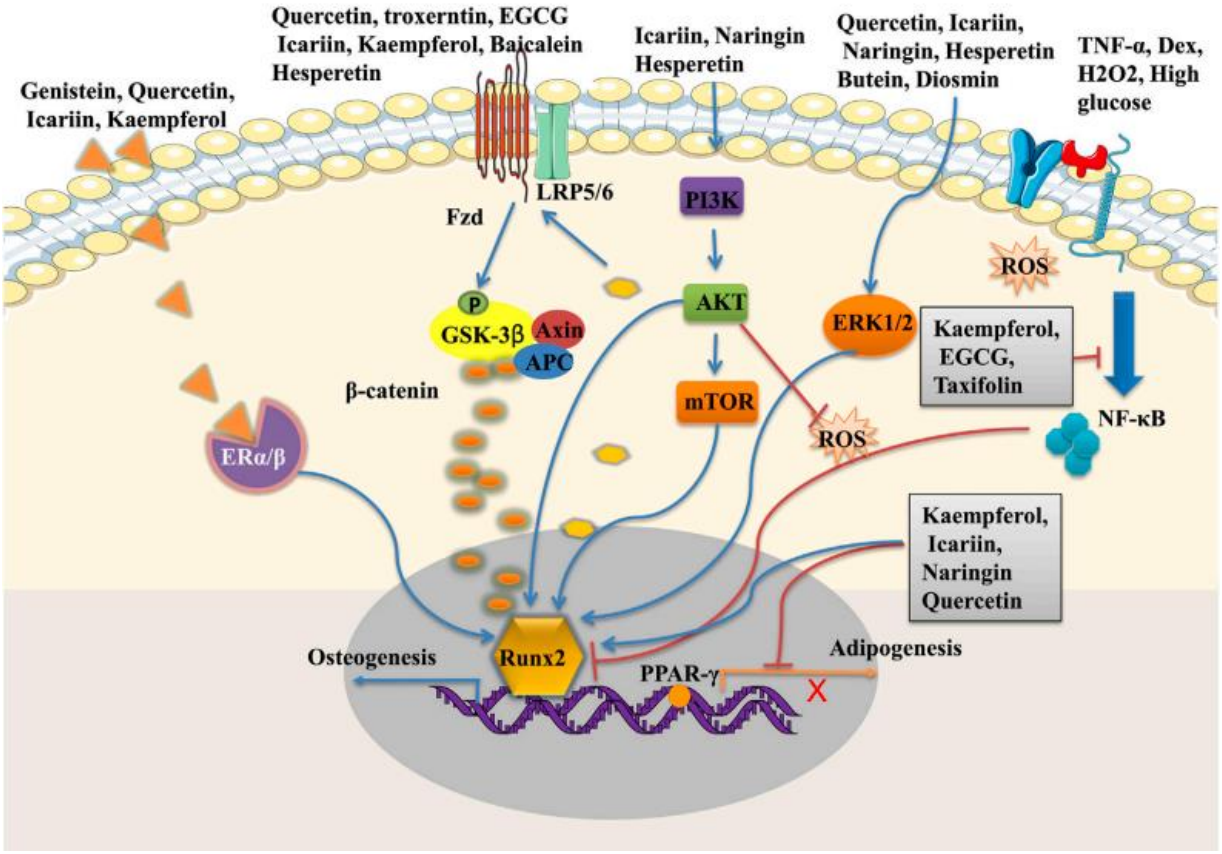

Figura 10. Signaling pathways activated by flavonoids during MSCs osteogenic differentiation. Adapted from J. Zhang et al., 2022 [88]

- **Fisetin**

Among flavonoids, fisetin belong to flavonol subclass, and is present in fruits and vegetables including apples, grapes, persimmons, and strawberries. Fisetin has been associated with several powerful biological effects, and is known to counteract tumor growth, osteoarthritis, and rheumatoid arthritis. [89]

Moreover, fisetin protects against inflammation-induced bone loss by inhibiting osteoclastogenesis and promoting osteoblastogenesis.

Molagoda I. et al. demonstrated that Fisetin inhibits RANKL-induced osteoclast differentiation by suppressing the NFATc1 signaling pathway, while increasing RUNX2 expression to boost primary osteoblastic activity, thereby enhancing the mineralization process. Moreover, many molecular mechanisms have recently been hypothesized to support

fisetin effects on osteogenesis, such as GSK-3β phosphorylation and subsequent β-catenin activation, or the activation of the PI3K-AKT signaling pathway.

Fisetin in vivo administration is challenging due to its poor water solubility and extensive in vivo metabolism. In addition, fisetin may lose its functional properties when submitted to some processing conditions such as adverse temperatures, pH variations, and exposure to oxygen and light. Therefore, protective measures such as encapsulation strategies using biological matrices could be an excellent strategy to improve its bioavailability. [90]

Since fisetin promotes osteoblast differentiation and osteogenesis, it could be an useful tool to treat bone resorption disorders, such as osteoporosis. However, no integrated approach to fisetin employment in osteogenesis promotion has been taken. [89], [91]

2. AIM OF THE STUDY

In aging-related diseases, cartilage and bone are the tissues most commonly damaged. Chondrocytes and osteocytes, the cells responsible for cartilage and bone formation, originate from mesenchymal stem cells (MSCs) following differentiation.

Epigenetic factors, such as microRNAs, play a crucial role in regulating MSCs differentiation fate, and consequently, the generation of mature chondrocytes and osteocytes. Numerous studies have revealed that miR-204 plays a role in MSCs fate and differentiation. It has been reported that miR-204 targets RUNX2 and it can inhibit osteogenic differentiation. Instead, contradictory data has been published on its role in chondrocyte differentiation, as well as in promoting or counteracting osteoarthritic pathology.

Methylsulfonylmethane (MSM) and Fisetin, are nutraceutical and bioactive compounds with anti-oxidant and anti-inflammatory properties. MSM has been demonstrated to be effective in treating age-related diseases associated to inflammation, such as osteoarthritis, while Fisetin can boost bone mineral density and modulate osteogenesis. Few studies have investigated MSM's role in the differentiation of MSCs, and none have assessed the MSM's overall impact on both osteogenic and chondrogenic differentiation.

The aim of this study is to investigate more thoroughly the functional role of miR-204 in MSCs commitment and chondrogenic and osteogenic differentiation, and also the effects of two bioactive compounds in counteracting inflammation and improve chondrogenesis and osteogenesis.

The primary purpose was to investigate *in vitro* (MSCs) and *in vivo* (zebrafish model) the expression profile of miR-204 at the early, middle and late MSCs chondrogenic and osteogenic differentiation phases, and during development *in vivo*. Moreover, by using an anti-miR-204 inhibitor, it was investigated the impact of miR-204 silencing on the expression of downstream genes and transcription factors involved in MSC chondrogenic commitment and differentiation.

The second purpose was to investigate *in vitro* and *in vivo* (zebrafish larvae and adults) the effects of two bioactive compounds, MSM and fisetin, on chondrogenic and osteogenic differentiation of MSCs. To assessed this, it was evaluated the expression of proteins and genes related to chondrogenesis and osteogenesis, as well as cartilage and bone deposition by performing Alcian blue and Alizarin Red staining on zebrafish. Moreover, it was evaluated the

capacity of MSM to revert the effects of IL-1β on chondrocytes and MSCs during chondrogenic differentiation. In addition, it was investigated the ability of fisetin to induce osteogenic maturation in cells from pediatric patients carrying RUNX2 mutations.

Since fisetin in vivo administration is challenging due to its poor water solubility and extensive in vivo metabolism, poly lactic-co-glycolic acid (PLGA) nanoparticles encapsulating fisetin were generated to prevent the degradation and improve fisetin internalization into cells. The effects of PLGA-(Fis) formulation versus free fisetin on MSCs during osteogenic differentiation were compared. Successively, it was evaluated the ability of PLGA-(Fis) to cross the intestinal epithelium performing a microfluidic system containing human intestinal epithelial tissue.

3. MATERIALS AND METHODS

3.1 Cell cultures

Bone Marrow-derived human Mesenchymal Stem Cells (hBM-MSCs, Promocell, Heidelberg Germany) were cultured in MesenPRO RS Basal Medium (Gibco, Life Technologies Corporation, Catalog Number 12747-010) supplemented with 2% of MesenPRO RS Growth Supplement (Gibco, Cat. N. 12748), 2 mM L-glutamine (Lonza), and 100 U/ml Penicillin, 100 µg/ml Streptomycin and 0,25 µg/ml Amphotericin B (PSA, Biological Industries, Cat. N. 03-033-1B). Cultured hBM-MSCs were induced to chondrogenic or osteogenic differentiation using StemPRO Chondrocyte/Osteocyte Differentiation Basal Medium (Gibco, Life Technologies Corporation, Cat. N. A10069-01) supplemented with 10% of Chondrocyte/Osteocyte Supplement (Gibco, Cat. N. A10064; A10066) and PSA antibiotic mix, as reported above. During MSC's differentiation, the medium was changed every 2–3 days after initial plating.

Human chondrocytes (HCH, Promocell, Heidelberg, Germany) were cultured with Chondrocytes Growth Medium (Promocell, C-27101) plus its supplement and PSA antibiotic mix at the concentration reported above. To simulate OA condition, IL-1β was added to the HCH cell medium at the concentration of 10 ng/ml. Three independent experiments were performed for each condition.

Human dermal fibroblast cells were isolated from skin biopsies taken from patient P1 [mutation: c.897T>G->p (Tyr299*), male, 8 years old], P2 [c.1019del-> p (Ser340*), female, 10 years old], and a healthy age-matched donor, with appropriate consent. Skin biopsies were immediately rinsed with 1X PBS containing 1% PSA antibiotics mix, cut into small pieces and plated in culture flasks containing DMEM-GlutaMAX™ (Gibco, Cat. N. 10566016) medium with 20% FBS (Gibco, 10270-106) and 1% PSA under sterile conditions. After 2 weeks, fibroblasts grown out of the tissues were collected and plated in new culture flasks containing DMEM-GlutaMAX™ medium with 10% FBS and allowed to grow until they reached 80% confluency, adding fresh new medium every other day.

All cell lines were propagated in culture according to standardized methods: split using Accutase (Biowest, Cat. N. L0950-100) or 1X Trypsin/EDTA (Lonza) solution, and cultured at 37°C in a humidified atmosphere with 5% CO_2.

3.2 NC4000 participants enrolled

NorthCape4000 (NC4000) is the most participated ultra-endurance unsupported cycling adventure. The 4th edition began July, 24th, 2021. It covered 4,400 km and an elevation gain of 40,000 m and consisted of an unsupported, non-drifting race, where participants arrived to North-Kapp (NRW), starting from Rovereto (ITA), passing through 4 mandatory checkpoints (Lake Balaton, Krakow, Riga, Rovaniemi). Organizers set a completion time limit of 22 days. Monitored temperature fuctuations were between 32° and 7 °C.

Eight healthy male amateur Caucasian cyclists (47.5±13.5 years old) who participated in the NC4000 4th edition were contacted via social media and underwent clinical evaluation and venipuncture for blood samples collection, before the preparation period (BPP; from 21st December 2020 to 2nd March 2021), the week before NC4000 (BN) and up to 10 days after NC4000 (AN). The study was approved by the ethical committee of Azienda Ospedaliera Universitaria Integrata of Verona, Italy (number 1538; Dec. 3, 2012; local ethical committee of Azienda Ospedaliera Integrata di Verona). The methods and study design adhere to the Helsinki Declaration. Before the procedures, cyclists provided their voluntarily written consent.

3.3 Blood samples and circulating progenitor cells collection

Blood samples were collected in the morning BPP, BN, and AN. Circulating progenitor cells (CPCs) were isolated from heparinized blood. In particular, CPCs were isolated from 50 ml of heparinized blood using a depletion method, performed by two Ficoll procedures. First, peripheral blood mononuclear cells (PBMCs) were isolated by a gradient centrifugation at 800×g for 30 min at 20°C (first Ficoll procedure). Then, we removed the unwanted hematopoietic cells using a RosetteSep antibody cocktail (Stemcell Technologies Inc., Vancouver, Canada) and 5 ml of whole blood mixed with the PBMCs obtained from the first Ficoll. The antibody cocktail (against CD3, CD14, CD19, CD38, and CD66b-positive cells), at dilution of 50 µl/ml, was incubated with samples for 20 min at room temperature. Finally, a second Ficoll procedure was performed to remove the unwanted cells linked to antibody cocktail and crosslinked to red blood cells (glycophorin A). Collected cells were then washed in phosphate-buffered saline (PBS).

3.4 Cell transfection

Before transfection, hBM-MSCs were seeded into 6-well plates or T25 flasks and differentiated to the chondrogenic or osteogenic lineage for 3, 7 and 10 days. Cell transfection was performed at the end of the 3th, 7th and 10th day of differentiation and cells were collected after 48 hours.

When cell reached the confluency of 60–70%, transfection was carried out according to the manufacturer's instructions using Lipofectamine 3000 Reagent (L3000-008, Invitrogen by Thermo Fisher Scientific). MSCs were transfected with anti-miR^{TM}-204-5p Inhibitor (Cat#: AM17000, ID: AM11116; Ambion by Thermo Fisher Scientific) and anti-miR^{TM} Inhibitor Negative Control (Cat#: AM17010, Ambion by Thermo Fisher Scientific) or just with Lipofectamine reagents (scramble). Six hours after transfection, supernatant was removed with fresh complete differentiation medium. 48 hours after transfection, cells were harvested, and the cell pellet was processed for RNA and protein extraction.

3.5 CCK-8 assay

Colorimetric Cell Counting Kit-8 (CCK-8/ WST-8; Immunological Sciences, Cat. N. IK-11133) is a colorimetric assay used for the sensitive quantification of viable cells in proliferation and for the evaluation of their sensibility to cytotoxic substances. The assay was performed by seeding 2,5 x 10^3 MSCs per well, in a 96-well plate in complete growth medium one day before the application of any treatment. Then cells were treated for 24 hours with and without Fisetin at concentrations ranging from 0 to 5 µM. After 24 hours, 10 µl of CCK-8 solution were added to each well of the plate. The 96-well plate was incubated in the incubator and the absorbance, proportional to viable cells, was measured at 450 nm after 1, 2, and 3 hours using a microplate reader. Six replicates in three independent experiments were tested.

3.6 Metylsulfonylmethane cell treatment

Metylsulfonylmethane (MSM) is a dietary supplement, suggested for relieving joint/muscle pain thanks to its anti-inflammatory properties. MSM was purchased from Artrosulfur; Laborest S.r.l, Assago, MI, Italy.

hMSCs were seeded at a number of 7,5 x 10^4 cells/well in 6-well plates in complete growth medium. The day after, the medium was changed with chondrogenic differentiation medium supplemented with or not with MSM at the concentration of 20 mM. The cells were allowed to

differentiate for 3, 7, and 14 days. Then cells were harvested, and the cell pellet was processed for RNA extraction.

MSM at the same concentration (20 mM) was also used to treat HCH cells. HCH cells were treated in four different conditions: control; MSM 20 mM; IL-1β 10 ng/ml; MSM + IL-1β. The cells were cultured for 7 days in chondrocyte growth medium; then cells were harvested, and the cell pellet was processed for RNA and protein extraction.

3.7 Fisetin and PLGA nanoparticles analyses

Fisetin (Santa Cruz Biotechnology, SC-276440) stability tests were carried out by the team of Prof. Massimiliano Perduca (*Biocrystallography and Nanostructure laboratory, University of Verona).* They evaluated fisetin stability in complete culture medium (DMEM with 10% FBS and 1% PSA) at different experimental time points (from 0 to 6 hours). Starting from a 10 mM stock solution of fisetin in DMSO, they obtained a 100 µM solution, which was analyzed by HPLC at time T_0 and after 6 hours. In particular, the measurements in HPLC were performed at time T_0 and after 30 min, 1, 2, 4, and 6 hours. The solution was kept at 37°C and away from direct light to simulate experimental conditions.

Poly lactic-co-glycolic acid nanoparticles (PLGA-NPs; 140 nm size; 50:50 lactic to co-glycolic acid ratio) were synthesized by the team of Prof. Massimiliano Perduca (*Biocrystallography and Nanostructure laboratory, University of Verona).* Their obtained empty and fisetin (2.5 µM) loaded PLGA-NPs. The protocol followed to produce PLGA nanoparticles was based on a single emulsion evaporation method, under sterile conditions at 20°C. [92] The purified nanoparticles were resuspended in 1 ml of phosphate-buffered saline (PBS) solution pH 7.4 (or NaCl 0.9%) for the subsequent analysis and storage at 4°C, otherwise freeze-dried. Empty nanoparticles were prepared with the same protocol avoiding the addition of fisetin to the reaction. Furthermore, to perform an internalization study and visualize nanoparticles inside cells, PLGA NPs co-delivering fisetin and fluorescein isothiocyanate (FITC) [PLGA (Fis-FITC)] were prepared. The molar ratio between fisetin and FITC is 1:1.

The Lab of Prof. Massimiliano Perduca estimated the size and ζ-potential of PLGA nanoparticles at 25° C by dynamic light scattering (DLS) (Nano Zeta Sizer ZS, ZEN3600, Malvern Instruments, Malvern, Worcestershire, United Kingdom).
To support DLS data, a Nanosight tracking analysis was performed on PLGA (Fis) and empty NPs (Malvern NanoSight NS300). Due to the high concentration, each sample was diluted

10,000 or 5,000 times; 1,498 frames divided into 3 runs of 60 s were recorded at a camera level of 13, and the analysis was performed with a detection threshold in the range 5–7. Finally, the number of particles/ml was estimated as well.

The performed atomic force microscopy (AFM) analysis using an NT-MDT Solver Pro atomic force microscope with NT-MDT NSG01 golden coated silicon tip in semi-contact mode with different scanning frequencies (3–1 Hz). Images were processed with the Scanning Probe Image Processor (SPIP™) program (Friis Jan, 2009), and a statistical study was performed to compare results to DLS and Nanosight data.

To assess the presence of fisetin inside nanoparticles and the co-presence of fisetin and FITC, the team of Prof. Massimiliano Perduca measured the absorbance pattern of each sample suspended in PBS or water using a Thermo Fisher Evolution 201 UV-Visible Spectrophotometer in the range 250–600 nm. Moreover, the emission pattern was recorded upon excitation at 360 and 495 nm (excitation wavelengths for fisetin and FITC, respectively) by using a Jasco Spectrofluorometer FP-8200.

To quantify the amount of fisetin entrapped, PLGA (Fis) NPs were dissolved in DMSO and analyzed using the calibration line. Encapsulation efficiency (EE) and drug loading (DL) were estimated using the following equations:

$$EE\ (\%) = \frac{Fisetin_{loaded}}{Fisetin_{fed}}\ x\ 100$$

$$DL\ (\%) = \frac{mg\ of\ Fisetin_{loaded}}{mg\ of\ PLGA}\ x\ 100$$

To assess the capacity of the nanoparticles to retain entrapped fisetin over time, the Prof. Perduca's team performed a first release study in a total volume of 1 ml, at different temperatures (4 and 37° C) and different media (water, PBS NaCl 0.9%, and citric acid pH 5). Dialysis was used to carry out the in vitro drug release studies increasing the final volume. PLGA (Fis) NPs were introduced into the dialysis bag (14,000 Da molecular weight cutoff, Sigma Aldrich, D9777-100FT) and placed in 100 ml of PBS pH 7.4 containing 0.1% v/v Tween 80 as the release media and stirred at 100 rpm. Samples were collected at different time intervals and replaced with an equal volume of media to maintain the sink condition. The released fisetin was quantified using a calibration curve obtained by UV–visible spectroscopy at 360 nm.

3.8 Fisetin and PLGA nanoparticles cell treatment

Fisetin is a dietary flavonoid with anti-oxidant and anti-inflammatory effects that is abundant in many vegetables and fruits.

hMSCs were seeded at a number of 7,5 x 10^4 cells/well in 6-well plates in complete growth medium. The day after the medium was changed with osteogenic differentiation medium supplemented with or without fisetin at different concentrations (0.625; 1.25; 2.5; 5 µM). The cells were allowed to differentiate for 3, 7, or 14 days, then cells were harvested, and the cell pellet was processed for RNA extraction.

Fisetin at the concentration of 2.5 µM was also used to treat the fibroblast-like cells derived from a healthy donor and from two unrelated pediatric patients affected by CCD, who carried mutations in exon 7 of one RUNX2 allele. The fibroblast-like cells were first treated with ascorbic acid (50 µg/ml) for 24 hours before being cultured for 1 week in the presence or absence of fisetin (2,5 µM). Ascorbic acid (also known as vitamin C) was used to induce osteogenic differentiation, because it enhances the development of collagen matrix, the activation of integrin signaling, and the expression of osteoblastic-markers. After 7 days, cells were harvested and the dried pellet was processed for RNA and extraction.

hMSCs were seeded in 6-well plates (7,5 x 10^4 cells/well) or T25 flaks (200000 cells) in complete growth medium. The day after, hMSCs were induced to osteogenic differentiation and treated in four different conditions: control; fisetin 2.5 µM; fisetin (2.5 µM)-PLGA nanoparticles; bare PLGA nanoparticles. Cells were cultured for 7 days and the exhausted medium was changed with fresh medium plus treatments one time during the experiment. At day 7, cells were harvested and the dried pellet was processed for RNA and protein extraction.

3.9 3D fluid-dynamic intestinal model resembling systemic administration for PLGA-(Fis) nanoparticles

A compartmental fluidic device (MIVO React4life S.r.l., IT) was used to perform in vitro drug efficacy tests. The 3D fluidic model was performed as follows: 1) 24-well size inserts containing human intestinal tissue (EpiIntestinal by Mattek) were placed and cultured within the device, forming two fluidically independent chambers: the donor and the receiver; 2) both chambers were filled with culture medium; and 3) the receiver chamber was connected to the peristaltic pump to form a closed-loop fluidic circuit containing 3.8 ml medium circulating at a rate of 0.3 cm/s, to simulate the capillary flow rate. Therefore, the medium containing PLGA-

(Fis) nanoparticles was added to the donor chamber. The concentration of fisetin encapsulated was 2,5 µM.

3.10 *In vivo* experiments with Zebrafish model

Zebrafish experiments were performed at the CIRSAL (Interdepartmental Centre of Experimental Research Service) of the University of Verona, Italy, under ethical authorization n. 662/2019-PR of 16/09/2019. Zebrafish embryos were obtained from natural spawning of Nacre adults (ZFIN database ID: ZDB-ALT-990423-22), according to standard procedures and staged according to Kimmel. Zebrafish embryos were grown at 33°C in water until 3, 7, and 14 days post fertilization (dpf). At the experimental endpoint, zebrafish embryos or adults were euthanized and collected for molecular analyses. From adult zebrafish, tails and scales were collected. Imaging was performed using Leica M205FA fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

For the experiments with MSM, zebrafish embryos were grown at 33°C in water containing 20 mM MSM from their 2nd day post fertilization (dpf). Zebrafish embryos were treated with MSM up to 1 week (experimental endpoint, 9 dpf) or 2 weeks (experimental endpoint, 16 dpf). Adult zebrafish (15–20 months) were grown in water containing 20 mM of MSM for 14 weeks. At the end of the treatment, both embryos and adult zebrafish were euthanized and collected for molecular analyses or staining as described below.

For the experiments with Fisetin, zebrafish embryos were grown at 33°C in water containing 15 µM Fisetin starting from the 2nd dpf. The zebrafish embryos were supplemented with Fisetin up to 1 week (experimental endpoint, 9 dpf). Also adult zebrafish (15–20 months) were grown in water in the presence of 15 µM Fisetin for 14 days (for 1 week of supplementation, no effect on the modulation of osteogenic gene expression was observed). At the end of the treatment, both embryos and adult zebrafish were euthanized and collected for performing staining procedure and molecular analyses.

3.11 Zebrafish staining

To evaluate bone development, we performed calcein staining in zebrafish larvae. To make the Immersion solution (0.2%), 2 g of calcein powder (Sigma Chemical, St. Louis, MO) were dissolved in 1 liter of deionized water. Due to the strong acidifying effects of calcein, a suitable quantity of NaOH (0.5 N) was added to the solution to return the pH to its original value. Zebrafish embryos were netted and dipped into the Immersion solution in petri dishes.

Depending on the size of the embryos, the immersion times varied from 3 to 10 min. After the immersion, the embryos were rinsed with fresh water several times, and then left to stand for 10 min to allow the excess, unbound calcein to diffuse out of the tissues. After being euthanized in tricaine–methanesulfonate (MS 222), the embryos were mounted on glass slides with methyl-cellulose (3%). Images were captured with a Leica M205FA microscope equipped with a green fluorescence filter set (Leica Microsystems, Wetzlar, Germany). ImageJ software was used to quantify the stained areas.

Bone and cartilage staining on adult zebrafish (>1 year old) was performed as described in the paper of Sakata-Haga et al. [93], strictly following their "RAP System" protocol.
Briefly, euthanized fishes were immersed into the fixative solution (5% formalin, 5% Triton X-100, 1% potassium hydroxide (KOH)) and gently rocked for 48 h at room temperature (RT). Then, we proceeded either to the cartilage staining step for cartilage-only staining or directly to the bone staining step for bone-only staining. For cartilage staining, the specimens were immersed into C-Staining Solution (70% ethanol, 20% acetate, 0.015–0.02% Alcian Blue) overnight at 20°C, then washed in C-Staining Medium (containing 1% Triton X-100) overnight at 20°C and subsequently washed with 50–70% ethanol. For cartilage-only staining, the protocol proceeded directly to clearing and stock steps. For cartilage and bone double staining, the protocol proceeded to the bone staining step. The specimens were immersed in B-Staining Medium (20% ethylene glycol and 1% KOH) and then in B-Staining Solution (0.05% Alizarin Red S, 20% ethylene glycol, 1% KOH) overnight at 20°C. The specimens were then washed with clearing solution (20% Tween 20, 1% KOH) with gentle rocking for 12h, and the stocking was performed in glycerol 100%. All needed solutions were prepared as indicated in Sakata-Haga et al. paper. The stained area was quantified by using the ImageJ software.

3.12 Alizarin red staining

To evaluate calcium deposition in differentiating osteogenic cells, we performed Alizarin red staining. 3 x 10^4 hMSCs were seeded in a 12-well plate, in osteogenic differentiating medium with the supplementation or not of fisetin at the concentrations of 0.625 µM, 1.25 µM and 2.5 µM. After 21 days of culture in osteogenic medium, cells were fixed with 70% ethanol and washed with water. Then, cells were stained with 40 mM Alizarin red S for 5 min at pH 4.1 and rinsed for 15 min with 1x PBS. The stained area was quantified by using ImageJ software (NIH, Bethesda, MD, USA). Six independent experiments were performed.

3.13 Immunofluorescence

hMSCs were plated at a density of 6 x 10^3 cells/well in a 8-culture chamber slide. The cells were treated adding in the medium FITC-PLGA-(Fis) nanoparticles for 6 hours. The concentration of embedded fisetin was 2.5 μM. At the time point of 4 and 6 hours, the cells were fixed using 2% paraformaldehyde solution in PBS 1X for 10 minutes at room temperature. Then, cells were washed with PBS 1X twice and cellular permeabilization was performed using a PBS-TritonX 0,3% solution, for 2 minutes at room temperature. Two PBS 1X washes were repeated. Then, the slides were incubated overnight at 4° C with β-actin primary antibody. Subsequently, the slides were incubated with the Alexa Fluor® 488 anti-mouse secondary antibody, and nuclear staining was performed by using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). The slides were mounted with Entellan mounting solution. Images were captured using a Leica M205FA fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

hMSCs were plated at a density of 6 x 10^3 cells/well in a 8-culture chamber slide. The day after, the cells were induced to differentiate towards osteogenic lineage and treated with or without 20 mM MSM for 3 and 14 days. Cells were fixed and processed as described above. Then, the slides were incubated overnight at 4° C with RUNX2 and osteocalcin primary antibodies (Cat. N. sc74495, Santa Cruz, Dallas, Texas, USA). Slides were then incubated with the secondary antibody goat or mouse fluorescein conjugated (Cat. N. Ap124f, Millipore, Burlington, Massachusetts, USA). Nuclear staining was performed by using ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). The staining was analyzed using a Leica (Wetzlar, Germany) TCS SP5 AOBS microscope. To express data in a semiquantitative way, six different fields were analyzed for each sample, in three independent experiments with about 80–100 total cells.

3.14 RNA extraction and reverse transcription PCR

The total RNA and miRNAs were extracted from cell dry pellets or zebrafish samples using the RNeasy Protect and miRNeasy Protect Mini Kits (Quiagen) following the manufacturer's protocol.

The concentration and purity of extracted RNA and miRNAs were measured through a NanoDrop™ instrument (ThermoFisher). To assess RNA purity, 260/280 and 260/230 absorbance ratios were considered to evaluate respectively protein and carbohydrate or phenol

(solvents) contamination. For pure RNA, the first ratio is considered as ~ 2.0 and the second is considered as ranging from 1.8 to 2.2.

Total RNA and mature miRNAs were reverse-transcribed to obtain cDNA using Applied Biosystems Reverse Transcription kits (Cat. N. 4368814 and 4366596). Total RNA was reverse-transcribed using random hexamers primers, whereas miRNAs and the house-keeping snRNA U6, were reverse-transcribed using specific primers (looped RT primers), that allow to reverse-transcribed only mature miRNA and snRNA and not their precursors, which have a complex secondary structure.

The RT-PCR reaction was performed on a total volume of 20 µl for total RNA (1 µg RNA) and of 15 µl (10 ng RNA) for mature miRNAs. The reaction was set up as indicated in table 1 and 2 and run in a thermocycler (SimpliAmp by Applied Biosystem) as indicated in table 3 and 4.

COMPONENT	VOLUME/SAMPLE
RNA sample	1 µg
10X RT Buffer	2 µl
10X RT Random Primers	2 µl
25X dNTP Mix	0,8 µl
MultiScribe™ Reverse Transcriptase	1 µl
RNase free water	up to 20 µl

Table 1: RT-PCR mix composition for total RNA

COMPONENT	VOLUME/SAMPLE
RNA sample	10 ng (Vf = 5 µl)
10X RT Buffer	1,5 µl
RNase Inhibitor 20 U/µl	0,19 µl
25X dNTP Mix	0,15 µl
MultiScribe™ Reverse Transcriptase	1 µl
RNase free water	4,16 µl
5X RT Primer	3 µl

Table 2: RT-PCR mix composition for miRNAs

DURATION	TEMPERATURE
10 minutes	25°C
120 minutes	37°C
5 minutes	85°C
Infinite hold	4°C

Table 3: thermocycler program for total RNA RT-PCR

DURATION	TEMPERATURE
30 minutes	16°C
30 minutes	42°C
5 minutes	85°C
Infinite hold	4°C

Table 4: Thermocycler program for miRNA RT-PCR

3.15 TaqMan qRT-PCR

Following RT, all cDNA samples were diluted in nuclease free water to a final volume of 20 µl for miRNAs retro-transcript and 30 µl for total RNA retro-transcript.

The TaqMan Real-Time PCR was performed using the TaqMan® Universal PCR Master Mix 2X (Applied Biosystems, Ref: 4304437) and TaqMan® pre-designed probes (Applied Biosystems). β-actin and snRNA U6 were used as housekeeping. The reaction components and their relative volume are reported in table 5.

REAGENT	VOLUME/SAMPLE	
	qRT-PCR total RNA	**qRT-PCR miRNA**
Master Mix 2X TaqMan	10 µl	10 µl
qRT-PCR probes target gene/miRNA	1 µl	1 µl
qRT-PCR probes housekeeping	1 µl	/
water	6 µl	7 µl

Table 5: qRT-PCR mix composition for total RNA and miRNAs analysis.

The reaction was performed in a 96-well plate. Each well contained 18 µl of reaction mix and 2 µl of cDNA. To ensure the absence of non-specific amplification products derived from the contamination of reagents, a negative qRT-PCR control (blank) was used. The blank sample

consists of 18 µl of reaction mixture and 2 µl of nuclease free water instead of cDNA. qRT-PCR was carried out both in multiplex and in singleplex using U6 and β-actin as internal references. All samples and negative controls were analyzed in duplicate or triplicate and the amplification was performed using LineGene 9600 Real-Time PCR System (BIOER). The PCR stage was repeated for 40-45 cycles and each thermal cycle consisted of three steps: denaturation, primers annealing and extension as reported in table 6.

STEP	TIME AND DURATION	CYCLE NUMBER
Initial denaturation	10 mins at 95°C	1
Denaturation	15 sec at 95°C	
Annealing and extension	1 min at 60°C	40-45

Table 6: Thermocycler program for qRT-PCR with TaqMan system.

A relative quantitative analysis was performed using $2^{-\Delta\Delta Ct}$ method. For each sample, Ct (threshold cycle) of specific miRNAs and genes were normalized with those of U6 snRNA and β-actin, respectively. At least three independent analyses were performed and each analysis was performed in duplicate, and Ct values were averaged.

3.16 Sybr Green qRT-PCR

The Sybr Green Real time PCR was performed using the SyBr Green PCR Master Mix Kit (Applied Biosystems, Ref: 4309155) and Sybr Green® forward and reverse primers (Sigma Aldrich). β-actin was used as housekeeping. The reaction components and their relative volume are reported in table 7.

REAGENT	VOLUME/SAMPLE
SyBr Green PCR master mix	10 µl
Primer reverse	0,6 µl
Primer forward	0,6 µl
water	6,8 µl

Table 7: SyBr Green qRT-PCR mix composition.

Sybr Green qRT-PCR reaction was performed in a 96-well plate, in which were loaded 18 µl of reaction mix and 2 µl of cDNA per single well. The same procedure as described for

TaqMan qRT-PCR was used. The amplification was performed using LineGene 9600 Real-Time PCR System (BIOER) with reactions step reported in table 8. The data were analyzed as described above.

STEP	TEMPERATURE	TIME
Step 1	95°C	3 minutes
Step 2	95°C	10 seconds
Step 3	55°C	10 seconds
Step 4	72°C	30 seconds
Step 5	95°C	10 seconds
Step 6	50°C	5 seconds
Step 7	95°C	15 seconds

Table 8:Tthermocycler program for SyBr Green qRT-PCR.

3.17 Protein extraction and quantification

For protein analysis of whole cell lysate, cell medium was removed and cells were washed twice in ice-cold PBS 1X. Then, cells were detached using 1X Trypsin/EDTA and the pellet was dried. Cellular pellets were lysate using an appropriate volume of cold RIPA buffer 1X (ThermoFisher Scientific), supplemented with protease inhibitor cocktail (Complete^{TM}, Roche, 25X) and incubated for 40 minutes on ice, vortexing every 20 minutes. Afterward, cell lysates were centrifugated at 16000 g for 40 minutes at 4°C and supernatants, containing clear total cell extracts, were recovered and transferred into clean tubes.

Protein quantification was evaluated with Bicinchoninic Acid (BCA) Protein Assay Kit (Quantum Protein, Bicinchoninic Protein Assay, Euroclone). The standard curve was prepared using 8 dilutions of BSA (Bovine Serum Albumin) at the following concentrations 2000, 1500, 1000, 750, 500, 250, 125, 25 µg/ml. To 200 µl of BCA solution, 10 µl of standard solution or 10 µl of cell lysate were added into wells of 96-well plate to measure absorbance at 562 nm using a spectrophotometer (VICTOR, Perkin Elmer).

3.18 Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis

For electrophoretic analysis, 20 to 30 µg of total protein lysate of each sample were taken to the same volume with RIPA buffer 1X, and then, Loading Buffer 4X with β-mercaptoethanol was added in the quantity of 1/3 of the final volume. Then samples were boiled at 99°C for 5 minutes. After this step, denatured protein samples were loaded and separated on 4-20% Mini-Protean TGX precast gel (Bio-Rad) in order to perform a sodium sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretic run was performed at 135 V, in denaturing condition, using Running Buffer 1X for required time. During the electrophoretic run, protein were separated depending on their molecular weight. BenchMark™ Pre-stained Protein Ladder 180-6 kDa molecular weight (Cat. N. 10748010; Life Technologies) was used to estimate relative protein molecular weight.

After electrophoresis, proteins were transferred from polyacrylamide gel to a PVDF membrane (0.45 µm thickness, Immobilon-P, Transfer membrane) by assembling a "sandwich". Sandwich was assembled as follow: a sponge, 1 sheet of 3MM paper, polyacrylamide gel, PVDF membrane (previously activated using Et-OH absolute), 1 sheet of 3MM paper and a sponge. Prior to assembly the sandwich, all sandwich's components were wetted with Transfer Buffer 1X solution. Sandwich was located into Bio-Rad transfer apparatus, immersed in Transfer buffer 1X, placed in ice to maintain 4°C and the transfer was performed at 100 V for 1 hour and 30 minutes.

After the transfer, the membrane was washed two times with MilliQ water and stained with Red Ponceau S solution (Cat. N. 141194-25G; Merck) for 15 minutes, to verify quality of transfer and protein loaded. Then, the membrane was destained with subsequent MilliQ water washes and equilibrate in TBS/0.1% Tween-20.

Afterward, membrane was blocked with 5% Non-Fat Dried Milk (NFDM) in Tris-Buffered Saline (TBS) plus 0.1% Tween-20 for 1 hour at RT. Next, PVDF membrane was incubated overnight at 4°C with primary antibody. As housekeeping protein was used β-actin.

After incubation with primary antibody, membrane was washed in TBS/0.1% Tween-20 three times for 10 minutes to remove the excess of primary antibody and then the membrane was incubated with specific secondary antibody for 1 hour at RT. Secondary HRP-conjugated antibodies were used for Enhanced ChemiLuminescence (ECL) detection; they were diluted 1:1000 or 1:2000 in 5% NFDM in TBS/0.1% Tween-20 solution. Signals were detected using the ECL™ Western Blotting Detection Reagent Kit (Amersham, Cytiva) and the UVITEC

Imager. Image-Quant software (GE Healthcare, Little Chalfont, UK) was used to perform densitometric analyses. Proteins optical density was normalized to β-actin.

To reuse the membrane, Strip-A-Blot solution (Euroclone) was used to remove primary and secondary antibody from membrane. Membrane was incubated with stripping solution for 15 minutes at RT. Then, membrane was washed two times in TBS/0.1% Tween-20 for 10 minutes. To re-probe, membrane was blocked and incubated with primary and secondary antibodies respectively as described before.

3.19 Statistical analysis

The results were expressed as the mean ± SD. The statistical analysis was assessed by Student's t-test or Student's paired t-test, which were used to compare the variation of a variable between each sample to the control. In all experiments, differences were considered statistically significant with $p^* < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.005$. For *in vitro* data, statistical analyses were carried out in three independent experiments performed in triplicate. For *in vivo* data, statistical analyses were applied to experiments carried out at least two times.

Statistical analyses were performed using GraphPad Prism software program (version 8.3; GraphPad Software) or R software (version 2.14.2). All western blots bands were quantified using ImageQuant ImageJ software (GE Healthcare, Little Chalfont, UK) to perform densitometric analyses.

4. RESULTS

4.1 miR-204-5p modulation during chondrogenesis *in vitro* and *in vivo*

In order to assess the modulation of miR-204-5p during chondrogenic differentiation, we conducted *in vitro* experiments and analyzed miR-204-5p expression as well as the expression of genes linked to chondrogenesis in MSCs cultured in the presence of chondrogenic differentiation medium for 3, 7 and 14 days.

As shown in Figure 1A, the expression of miR-204-5p and chondrogenic maturation-related genes (SOX9, COMP, COL2A1) increased during differentiation. The expression of the chondrogenic transcription factor SOX9 increased and reached a peak on the 7th day of differentiation before declining on the 14th day.

Since it has been reported that miR-204 targets RUNX2 [73], we investigated RUNX2 gene expression and protein levels. Although RUNX2 gene expression did not appear to be altered during differentiation, the amount of RUNX2 protein was significantly reduced during chondrogenesis (Figure 1B). This observation suggests that miR-204-5p inhibits RUNX2 at post-transcriptional level.

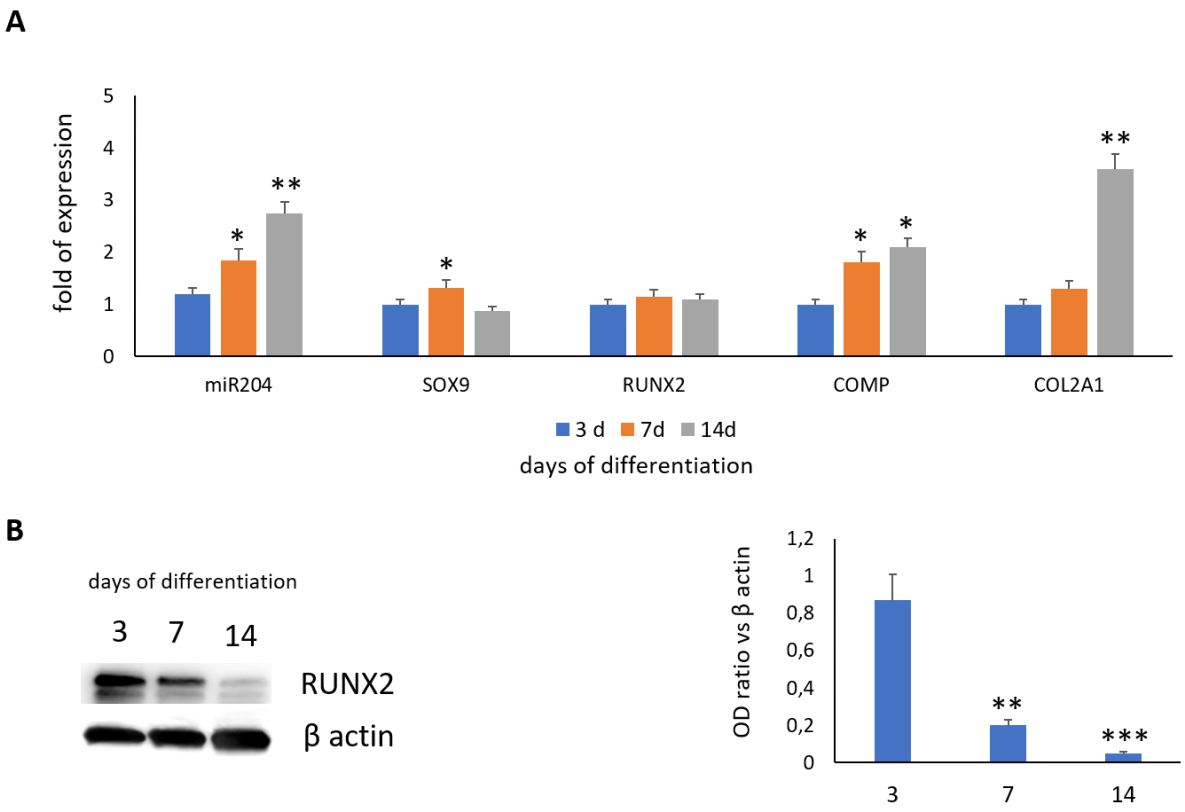

Figure 1. miR-204-5p and chondrogenic maturation-related genes expression during *in vitro* chondrogenesis at different time points. (A) miR-204-5p and SOX9, RUNX2, COMP and COL2A1 genes expression analyzed by qRT-PCR in MSCs cultured for 3, 7 and 14 days in chondrogenic differentiation medium. **(B)** Western blot analysis of RUNX2 expression level (left side) in MSCs after 3, 7, and 14 days of chondrogenic differentiation and quantification as a relative optical density (OD) ratio vs. β-actin (right side). All graphs report the mean (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ vs. expression levels relative to 3 days of differentiation.

Since β-catenin plays a significant role in chondrogenic differentiation and its degradation is promoted by SOX9, we analyzed β-catenin protein levels during chondrogenic differentiation *in vitro*. As illustrated in Figure 2A, β-catenin protein levels gradually decrease during chondrogenic differentiation.

We also looked at ERK and p-ERK protein levels, since it has been established that the extracellular signal-regulated kinase (ERK) pathway is involved in chondrogenesis [94]. We observed decreased p-ERK levels during chondrogenesis, especially on the 7th day of chondrogenic differentiation (Figure 2B).

To explore the miR-204-5p modulation during growth and aging processes, we conducted *in vivo* analyses on larvae and adult zebrafish (1-2 years old). We observed that miR-204-5p expression increased in larvae during development at 3, 7 and 14 days post fertilization (dpf) (Figure 3A). Intriguingly, miR-204-5p expression was high until six months of growth, then it drastically decreased in adult zebrafish (Figure 3B). Western blot analysis had revealed that during the aging process, ERK phosphorylation increased and aggrecan levels decreased (Figure 3C).

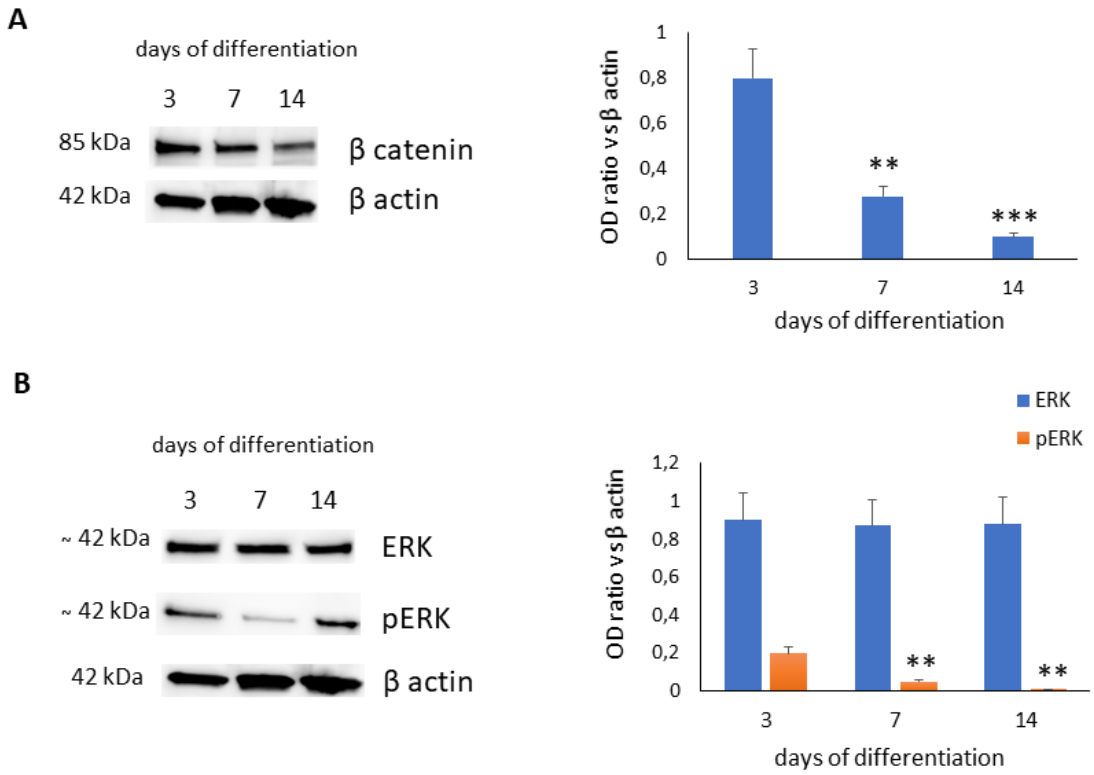

Figure 2. β-catenin, ERK, and p-ERK protein modulation during *in vitro* chondrogenesis. Western blot analysis of β-catenin levels **(A)** and ERK and p-ERK levels (**B**) in MSCs during chondrogenic differentiation at different time points. Bands quantification as relative OD ratio vs. β-actin (right side). Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at **p < 0.01; ***p < 0.005 vs. expression levels relative to 3 days of differentiation.

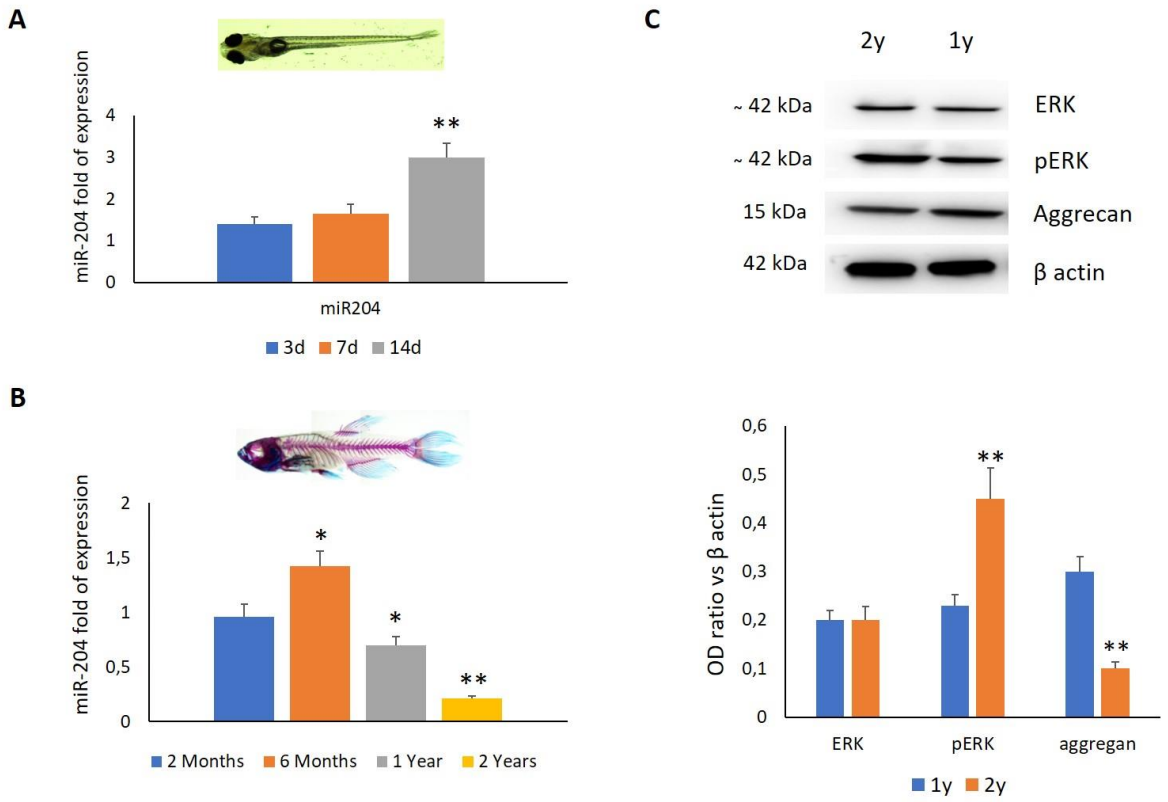

Figure 3. miR-204-5p modulation during development and aging *in vivo*. The graph reports qRT-PCR analyses of miR-204-5p expression normalized for U6 in **(A)** zebrafish larvae (3, 7, 14 days post fertilization (dpf)) and in **(B)** adult zebrafish (2 and 6 months, 1 and 2 years old). **(C)** Western blot analyses of ERK, p-ERK and aggrecan protein expression in 1 and 2 years old zebrafish (top) and quantification as relative OD ratio vs. β-actin (below). The graphs report the mean (± SD) of two or three independent experiments. Statistical significance calculated with Student's t-test, was set at *p < 0.05; **p < 0.01 vs. expression levels relative to 3 days of differentiation or vs. two months.

4.2 miR-204-5p influences MSCs commitment and osteogenic/chondrogenic differentiation

Since miR-204 appears to be crucial during the MSCs chondrogenic differentiation process, we wanted to investigate if miR-204 may also influence MSC commitment to the osteo-chondrogenic lineage and differentiation phases. In order to address this hypothesis, we transfected MSC with a miR-204 inhibitor or a negative anti-miR control (scramble) during their chondrogenic and osteogenic differentiation.

When miR-204-5p was silenced during the early chondrogenic differentiation phase (3th day of differentiation), the expression of SOX9, COL2A1 and COMP increased compared to

scrambles. Notably, we observed that the expression of SOX9 and COL2A1 was 1.5- and 2.2-fold higher, respectively, when compared to scrambles (Figure 4A).

We also assessed the effects of miR-204-5p silencing during the early osteogenic differentiation phase (3th day of differentiation). We noticed the upregulation of COL1A1 (a RUNX2 downstream gene), although RUNX2 expression was only slightly affected when compared to scrambles (Figure 4B).

Furthermore, we observed that miR-204-5p downmodulation had a significant impact on chondrocytes and osteocytes maturation. Indeed, the silencing of miR-204-5p during the middle chondrogenic differentiation phase (7th day of differentiation), resulted in a 0.67 and 0.7-fold decrease in SOX9 and COL2A1 expression compared to scrambles (Figure 4C). Instead, RUNX2 expression increased in miR-204 silenced cells compared to scrambles.

On the contrary, the miR-204-5p silencing during the middle osteogenic differentiation phase (7th day of differentiation) showed positive effects. Indeed, we observed an increase in the expression of osteogenic-related genes, such as RUNX2, IBSP, BGLAP and SPP1, in miR-204-5p-silenced cells compared to scrambles (Figure 4D).

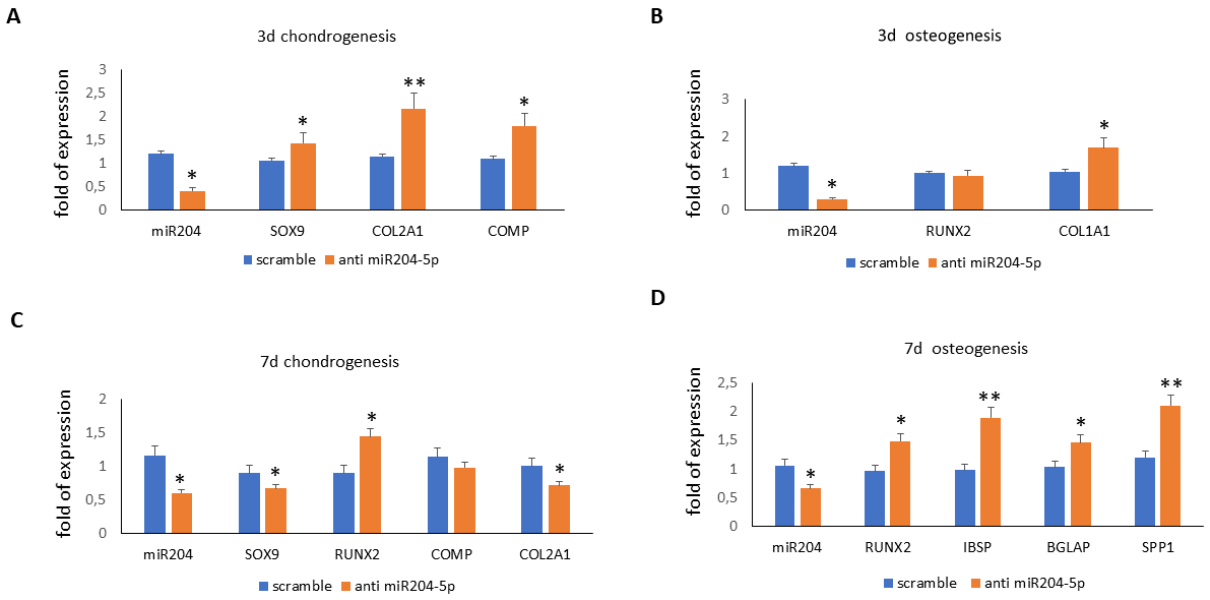

Figure 4. miR-204-5p silencing impacts on gene expression during the early and the middle chondrogenic/osteogenic differentiation phases. qRT-PCR analyses of miR-204-5p, chondrogenic maturation (SOX9, COL2A1, COMP, RUNX2) and osteogenic maturation (RUNX2, COL1A1, IBSP, BGLAP, SPP1) related genes expression during the early (**(A, B)** 3 days) and the middle (**(C, D)** 7 days) MSCs differentiation phase in control (scramble) and in miR-204-5p-silenced MSCs. Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$; **$p < 0.01$ vs. scramble.

However, when we analyzed cells transfected with anti-miR-204-5p after 10 days of osteogenic differentiation, we noticed a decrease in the expression of genes associated to osteogenic maturation (Figure 5).

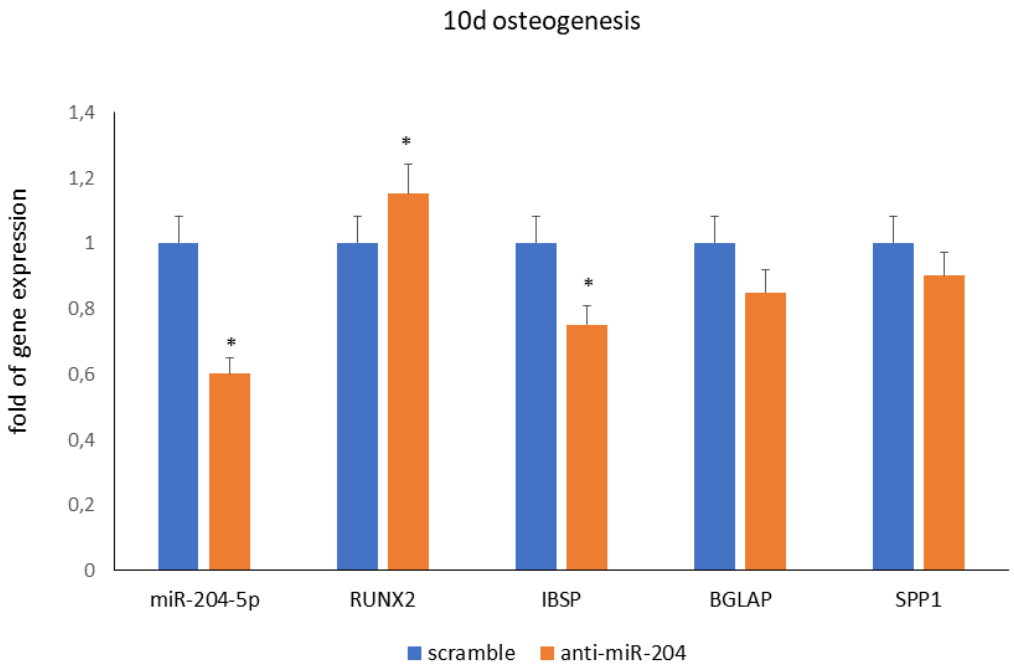

Figure 5. miR-204-5p downregulation influence osteogenic-related genes expression during the late osteogenic differentiation phase. qRT-PCR analyses of miR-204-5p, RUNX2, IBSP, BGLAP, SPP1 expression normalized for U6 and β-actin in scramble and miR-204-5p-silenced MSCs after 10 days of osteogenic differentiation. Data are expressed as mean value (± SD) of three independent experiments performed in triplicate. Statistical significance was calculated with Student's t-test and set at *$p < 0.05$ vs. scramble.

4.3 Inflammation and bioactive molecules influence miR-204-5p and SOX9 expression

Since inflammation state can affect osteo-chondroprogenitor cell proliferation and chondrocytes maturation, we examined the modulation of miR-204-5p in response to an inflammatory stimulus, such as that induced by interleukin 1 beta (IL-1β). We assessed miR-204 modulation in both mature chondrocytes and MSCs undergoing chondrogenic differentiation.

As illustrated in Figure 6A, we observed lower levels of miR-204-5p as well as SOX9 in MSCs during chondrogenesis (after 7 days of differentiation) and in chondrocytes treated with IL-1β.

Then we decided to compare the efficacy of two bioactive molecules (methylsulphonylmethane and clodronate), which are known to have anti-inflammatory and anti-oxidant properties, to counteract the effect induced by IL-1β. We first examined how methylsulphonylmethane (MSM) affected the expression of miR-204-5p and SOX9 in MSCs during chondrogenic differentiation treated or not with IL-1β. As shown in Figure 6B, we found that MSM alone increased the expression of miR-204-5p and SOX9 and counteracted the effects of IL-1β.

Secondly, we analyzed the effects of clodronate on MSCs during chondrogenic differentiation treated or not with IL-1β. Clodronate is a non-aminobisphosphonate, used in the management of osteoarthritis. We saw no difference in miR-204-5p modulation between MSCs treated with clodronate and the control, (Figure 6C). However, clodronate was able to increase SOX9 expression and prevent miR-204 and SOX9 downregulation induced by IL-1β treatment (Figure 6C).

To corroborate the role of clodronate in the prevention of IL-1β effects, we evaluated cyclooxygenase-2 (COX2) expression in MCSs during chondrogenesis and in chondrocytes after clodronate supplementation. As shown in Figure 6D, clodronate was able to prevent the upregulation of COX2 in IL-1β treated MSCs and chondrocytes (Figure 6D).

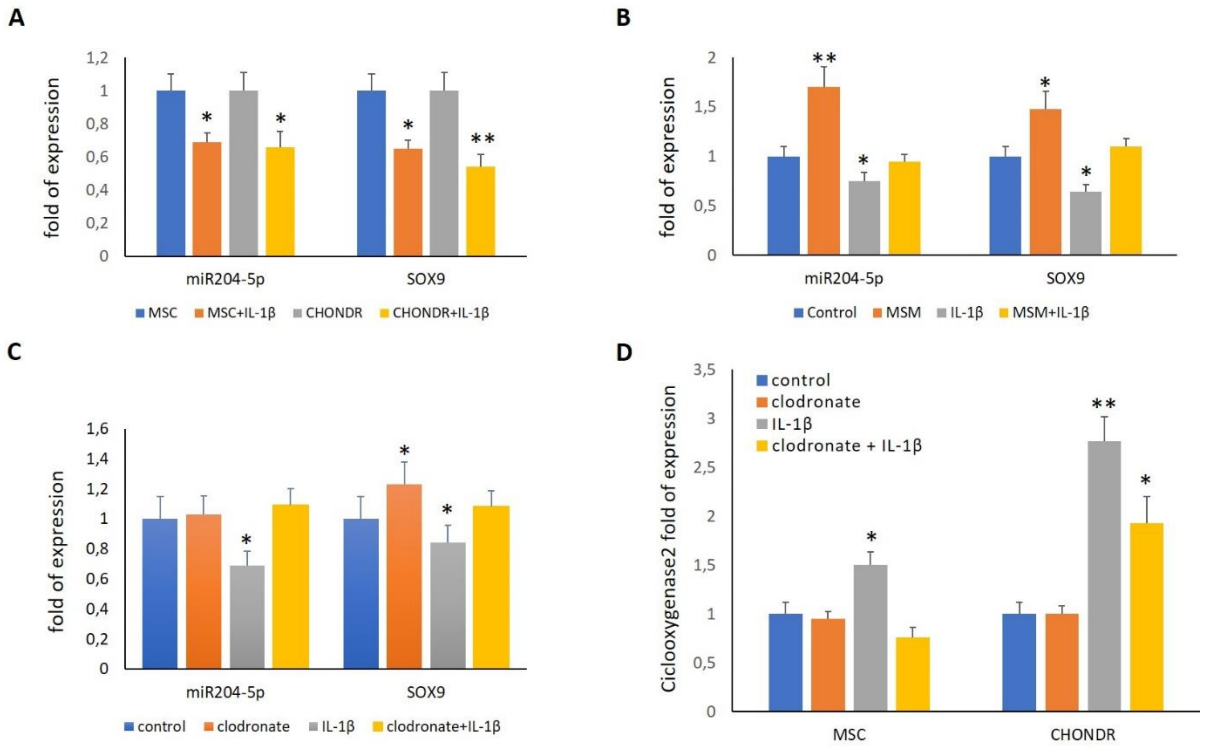

Figure 6. IL-1β and bioactive molecules impinge on miR-204-5p and SOX9 expression during chondrogenesis. (A) The graph reports qRT-PCR analyses of miR-204-5p and SOX9 expression in MSCs during chondrogenesis (7 days of differentiation) and in chondrocytes, both treated with or without IL-1β. **(B)** qRT-PCR analyses of miR-204-5p and SOX9 expression in MSCs during chondrogensis, supplemented with MSM and **(C)** clodronate, with and without IL-1β treatment. **(D)** qRT-PCR analyses of cyclooxygenase 2 expression in MSCs during chondrogenesis and in chondrocytes treated with or without IL-1β. All graphs report the mean (± SD) of three independent experiments. Statistical significance was calculated with Student's t-test and set at *$p < 0.05$; **$p < 0.01$ vs. control.

4.4 miR-204-5p affects MSC commitment and differentiation by targeting RUNX2

As illustrated in the previous Figures 4 and 5, miR-204-5p silencing resulted in an increase in RUNX2 gene expression levels after 7 days of chondrogenic and osteogenic of MSCs, as well as after 10 days of osteogenic differentiation. When we looked at RUNX2 protein levels, we noticed an increase of RUNX2 in MSCs transfected with anti-miR-204-5p inhibitor after 7 days in growth culture condition (Figure 7). These findings confirm the previous reports that miR-204 targets RUNX2. Moreover, we found that as miR-204-5p expression increased during chondrogenesis, RUNX2 gene expression levels remained constant while protein levels decreased (Figure 1), suggesting a post-transcriptional regulation of RUNX2 by miR-204-5p. Therefore, our data indicate that miR-204-5p may influence MSC osteo-chondrogenic commitment and promote osteoblasts' and chondroblasts' maturation by modulating RUNX2 expression (Figure 8).

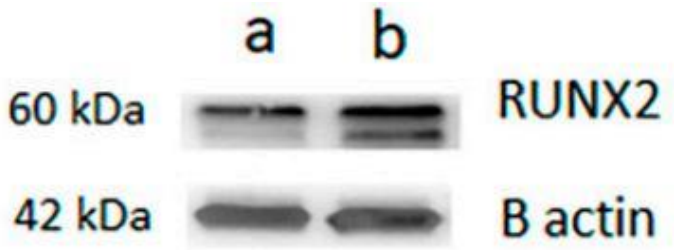

Figure 7. RUNX2 protein expression in MSCs. Western blot analysis of RUNX2 protein expression in **(a)** control and in **(b)** miR-204-5p-silenced MSCs after 7 days of culture in growth medium. RUNX2 protein levels increased when miR-204-5p is silenced. The two RUNX2 bands indicate the splicing isoforms RUNX2-II and RUNX2-I, which are under the control of a distal promoter (P1) and a proximal promoter (P2), respectively.

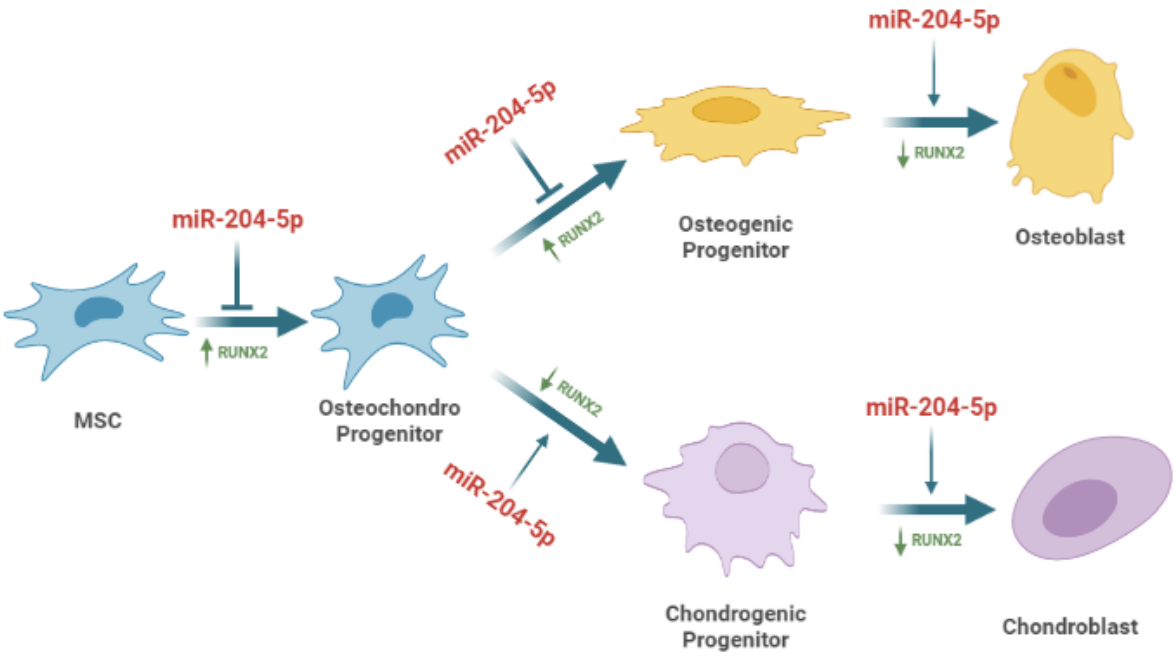

Figure 8. miR-204-5p impinges on MSCs' commitment and differentiation by targeting RUNX2. miR-204-5p negatively regulates the osteochondrogenic commitment of MSCs, while it positively regulates the maturation of osteoblasts and chondroblasts by modulating RUNX2 expression. The levels of RUNX2 gene expression during MSCs differentiation are shown in green. (Created with BioRender.com)

4.5 MSM promotes chondrogenesis both *in vitro* and *in vivo*

To assess the impact of MSM on chondrogenesis, we cultured mesenchymal stem cells with and without MSM during chondrogenic differentiation. We observed that the addition of MSM to the medium had no effect on cells number during the differentiation period (Fig. 9A). We noticed an increase in the expression of chondrogenic transcription factor SOX9 (Fig. 9B) and COL2A1 (collagen type II) (Fig. 9B) after 3, 7, and 14 days of chondrogenic differentiation. Additionally, chondrocytes cultured for 7 days with MSM showed increased SOX9 expression (Fig. 9C). MSM supplementation reversed the negative effects of IL-1β on chondrocytes. Indeed, MSM counteracted SOX9 downregulation as well as Sestrin 1 (SESN1) and COMP downmodulation induced by IL-1β (Fig. 9C). Beyond the modulation of chondrogenic genes induced by MSM treatment, we observed aggrecan protein levels. Aggrecan is a cartilage specific proteoglycan protein. We observed that aggrecan protein expression increased in chondrocytes in the presence of MSM, and this effect was noticeable even in chondrocytes that had received IL-1β treatment (Fig. 9D). In *in vivo* experiments, we discovered that

chondrogenic related genes were upregulated in zebrafish larvae after 7 (9 dpf) (Fig. 10A) and 14 (16 dpf) (Fig. 10B) days of MSM treatment.

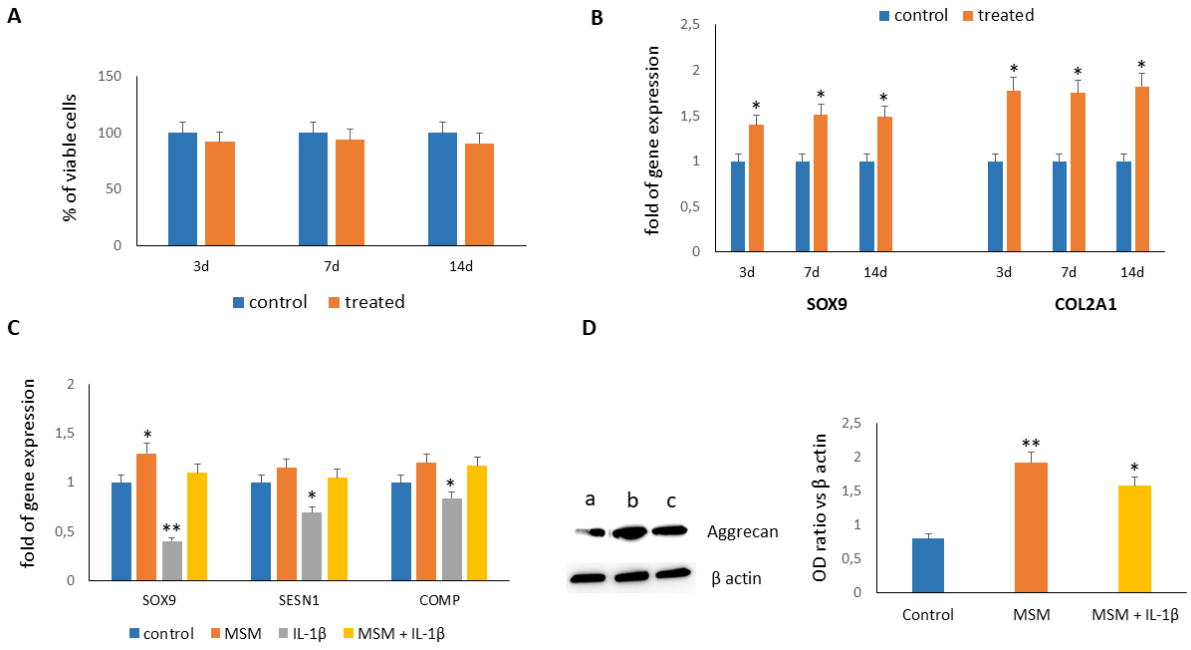

Figure 9. MSM boosts chondrogenesis and impinges on IL-1b effect. (A) MSM addition had no effect on cell viability during chondrogenesis. (**B**) MSM increased the chondrogenic transcription factor SOX9 and COL2A1 expression during MSC chondrogenic differentiation. **(C)** In chondrocytes, MSM improved SOX9 expression and restored the negative effects of IL-1β. **(D)** MSM supplementation increased aggrecan protein levels in chondrocytes, even in the presence of IL-1β. a) control; b) MSM treated; c) MSM treated + IL-1β. Data are expressed as mean value (± SD) of three independent experiments, performed in triplicate for qRT-PCR. Statistical significance calculated with Student's t-test, was set at *$p<0.05$; **$p<0.01$ vs. control.

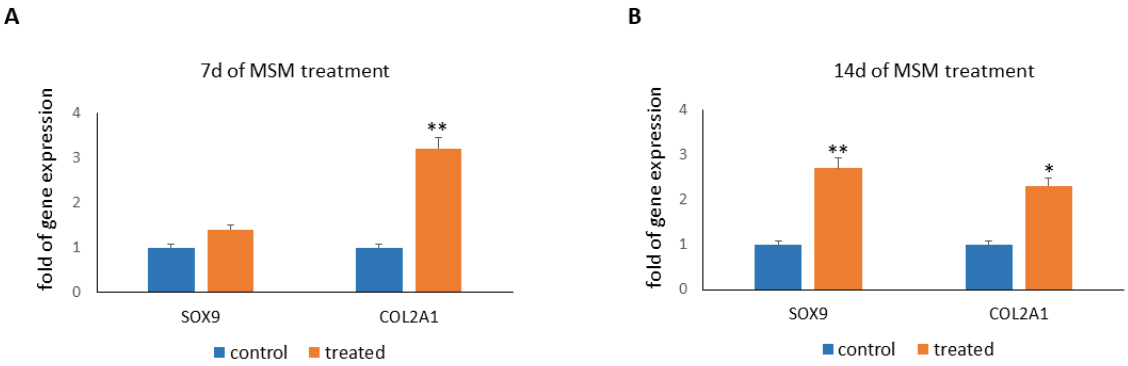

Figure 10. MSM improves chondrogenesis in zebrafish larvae. MSM increased the expression of chondrogenic maturation-related genes in zebrafish larvae after **(A)** 7 (9 dpf) and **(B)** 14 (16 dpf) days of treatment. Data are expressed as mean value (± SD) of two independent experiments performed in triplicate. Statistical significance calculated with Student's t-test, was set at *p<0.05; **p<0.01 vs. control.

We next examined the effects of MSM treatment on middle-aged zebrafish (15-20 months corresponding to 36-48 years of human age) and found that treated zebrafish had a more intense chondrocyte-specific Alcian blue staining than control zebrafish (Fig. 11A). Moreover, we observed that zebrafish that had received MSM treatment had higher SOX9 and COL2A1 expression in fins (Fig. 11B) and in scales (Fig. 11C).

As aggrecan is important in cartilage maintenance, we next investigated MSM's effects on aggrecan production. In particular, looking at protein levels in fins, we found that MSM-treated zebrafish produced more aggrecan protein than the control (Fig. 11D). On the contrary, MSM treatment did not result in significantly increase of aggrecan in zebrafish scale (Fig. 11D).

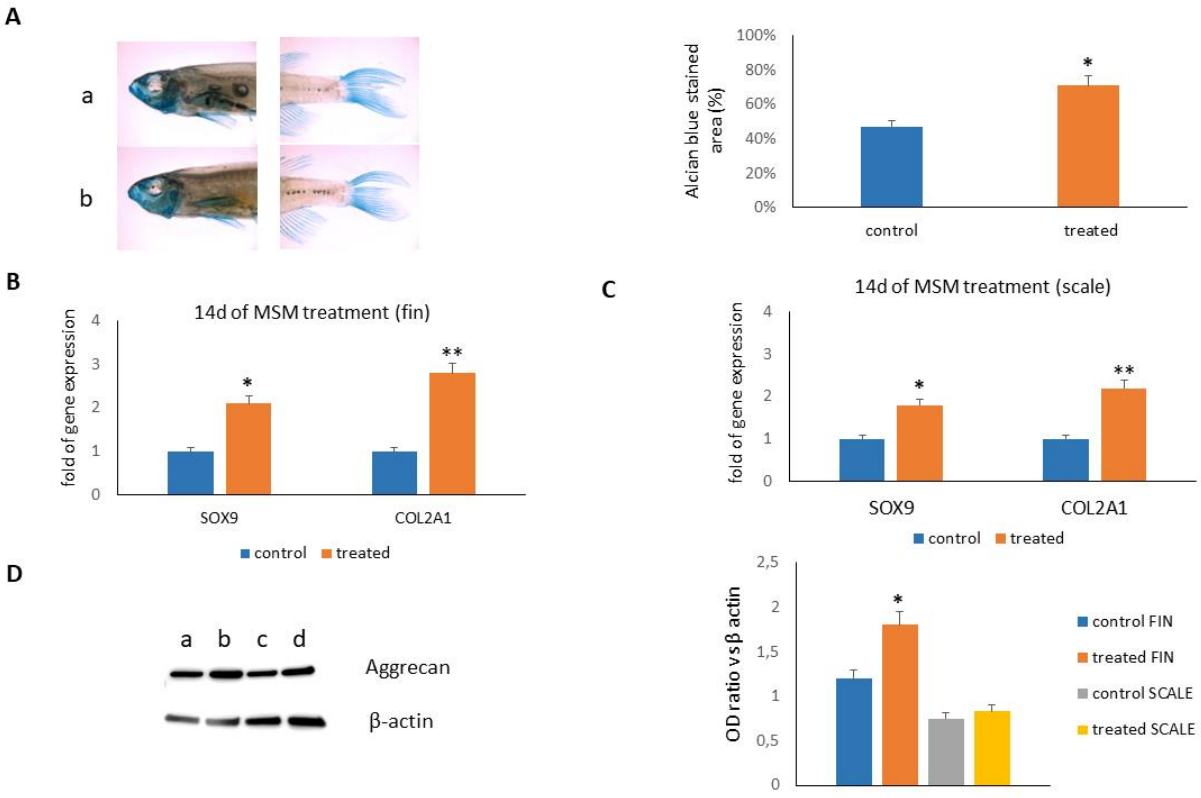

Figure 11. MSM promotes chondrogenesis in middle-aged zebrafish (15-20 months old). (A) Alcian blue staining was higher in MSM-treated zebrafish compared to untreated zebrafish: a) control; b) MSM treated. The expression of chondrogenic maturation-related genes increased in fin **(B)** and in scales **(C)** after 14 days of MSM treatment. **(D)** Aggrecan protein levels increased in fin of MSM-treated zebrafish, while there was no difference in scale: a) control fin; b) treated fin; c) control scale; d) treated scale. Results are expressed as mean value (± SD) of two or three independent experiments. Statistical significance calculated with Student's t-test, was set at *p<0.05; **p<0.01 vs. control.

4.6 MSM stimulates osteoblasts' maturation

Considering that chondrogenesis and osteogenesis are intertwined processes, we looked into the effects of MSM on osteogenic differentiation of MSCs. As shown in Fig. 12A, after 3 and 7 days of osteogenic differentiation, we observed a significantly reduction in the number of differentiating cells. Therefore, we analyzed the expression of the osteogenic transcription factor RUNX2 and its downstream partner SP7. According to Fig. 12B, we observed the upregulation of SP7 in MSM-treated cells, whereas RUNX2 expression levels remained unchanged after 3 days of osteogenic differentiation. Moreover, after 7 days of differentiation, we noticed that RUNX2 gene expression was reduced in MSM-treated cells, while SP7 expression remained high.

RUNX2 protein levels were consistently unaltered after 3 days of osteogenic differentiation (Fig. 12C), whereas RUNX2 protein levels were significantly reduced after 7 days (Fig. 12D) in MSM-treated cell nuclei.

MSM effects on osteogenic differentiation persisted also after 14 and 21 days. As shown in Fig. 13A, after 14 days of osteogenic differentiation, the expression of RUNX2 was reduced, instead the expression of osteoblast maturation related genes (e.g., SPARC and SPP1) as well as the number of osteocalcin-positive cells were increased in MSM-treated cells (Fig. 13B). Additionally, after 21 days of osteogenic differentiation, we observed increased calcium deposition, evaluated by alizarin red staining, in cells treated with MSM (Fig. 13C).

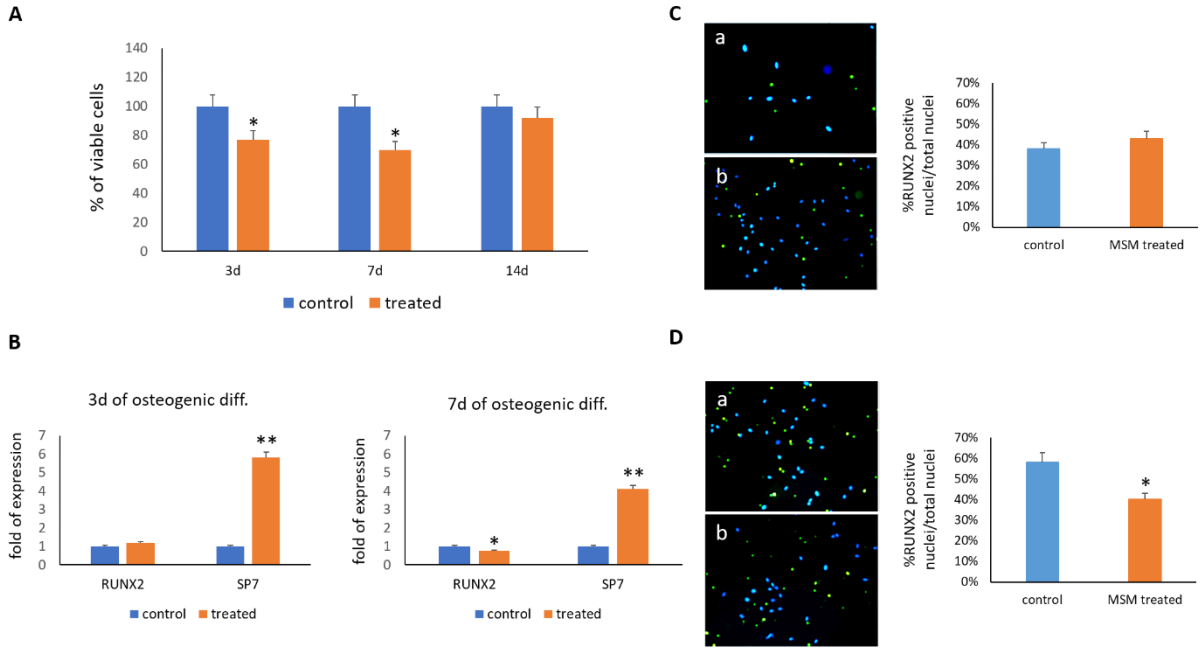

Figure 12. MSM affects the early and middle phase of osteogenic differentiation. (A) MSM reduced the number of differentiating cells after 3 and 7 days of osteogenic differentiation. **(B)** SP7 gene expression increased after 3 and 7 days of differentiation in MSM-treated cells, whereas RUNX2 gene expression decreased after 7 days of differentiation. **(C, D)** Immunofluorescence of RUNX2 protein (green color), showed that RUNX2 levels were similar after 3 days of osteogenic differentiation **(C)** and significantly lower after 7 days of differentiation **(D)** in nuclei of MSM-treated cells. a) control; b) MSM-treated cells. All graphs report the mean (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *p<0.05; **p<0.01 vs. control.

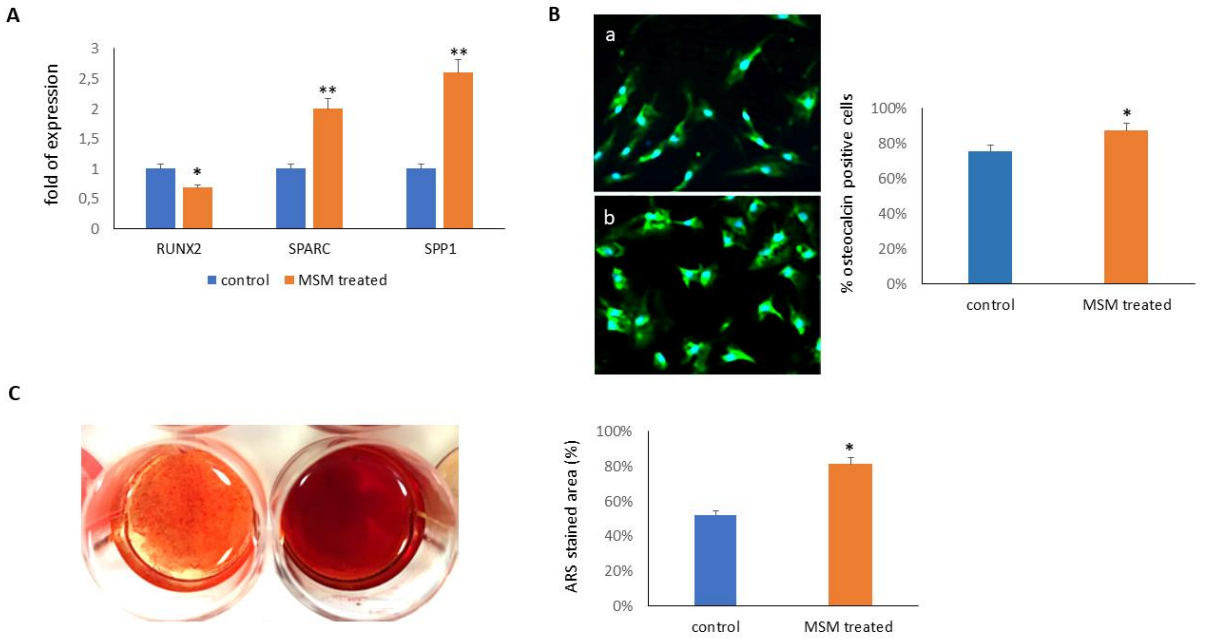

Figure 13. Effects of MSM on osteogenic maturation *in vitro*. (A) After 14 days of MSC osteogenic differentiation, RUNX2 gene expression was reduced, while SPARC and SPP1 gene expression, as well the number of osteocalcin positive cells **(B)** increased in MSM-treated cells. **(C)** After 21 days of osteogenic differentiation, calcium deposition was higher in MSM-treated cells than in control cells, as measured by alizarin red staining (ARS). Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *p<0.05; **p<0.01 vs. control.

4.7 MSM increases runx2b gene expression in zebrafish larvae

In order to confirm the impact of MSM on osteogenic maturation, we examined osteogenic markers in zebrafish larvae. Starting from the 2nd day post-fertilization (dpf), zebrafish larvae were treated with and without 20 mM MSM for 7 or 14 days. After 7 days of treatment, we detected increased expression of runx2b and sp7 genes and decreased expression of tnfrsf1b (homolog of human RANK) gene (Fig. 14A). After 14 days of treatment, increased expression of runx2b and sp7 and reduced expression of tnfrsf1b were confirmed (Fig. 14C). In addition, we also found an increase of col1a1 gene expression.

To assess the direct effects of MSM on bone formation, we measured bone mineralization using calcein staining. The fluorescence density was measured under microscope and analyzed digitally. As illustrated in Fig. 14B-D, after 7 and 14 days of MSM treatment, MSM-treated larvae had higher intensity of calcein staining than untreated larvae.

Then, we also evaluated bone mineralization in adult zebrafish treated with MSM for 14 days by alizarin red staining (ARS) (Fig. 15A). As shown in Fig. 15B, MSM-treated zebrafish displayed a more intense ARS and a higher proportion of AR positive areas than untreated zebrafish.

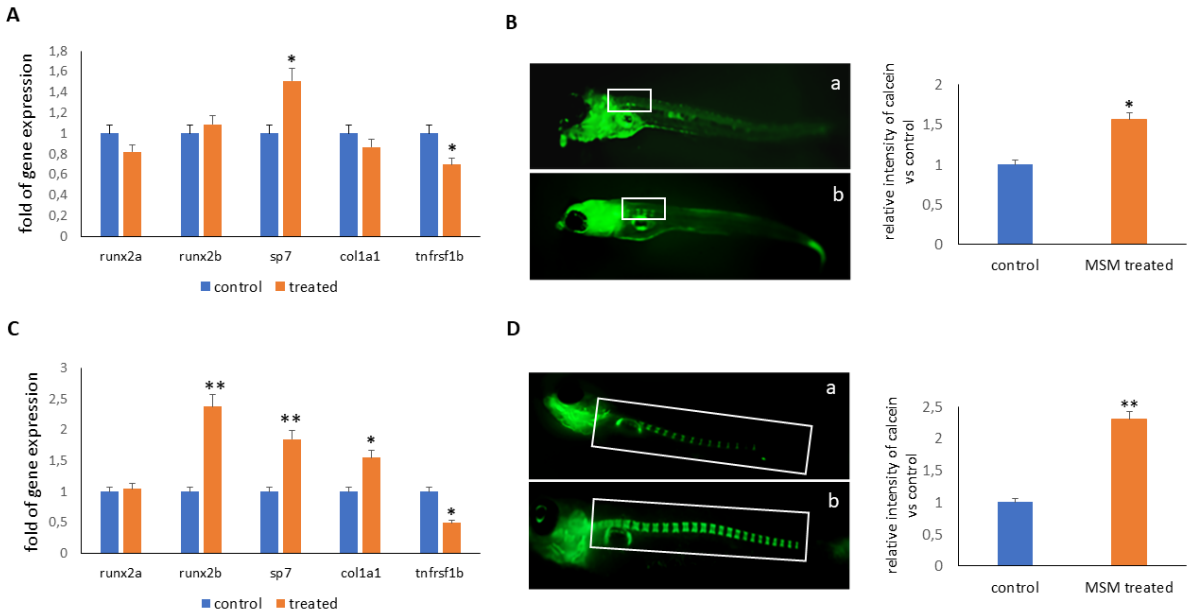

Figure 14. MSM modulates gene expression in zebrafish larvae. (A) Increased sp7 and reduced tnfrsf1b gene expression in larvae after 7 days of MSM treatment at 20 mM. **(B)** After 7 days of treatment, the fluorescence density produced by calcein staining was higher in MSM-treated larvae compared to untreated larvae: a) control; b) MSM-treated. **(C)** Increased runx2b, sp7 and col1a1 gene expression, and reduced tnfrsf1b gene expression after 14 days of MSM treatment. **(D)** After 14 days of treatment, the fluorescence density produced by calcein was higher in MSM-treated larvae compared to untreated larvae: a) control; b) MSM treated. Results are expressed as mean value (± SD) of two or three independent experiments. Statistical significance calculated with Student's t-test, was set at *p<0.05; **p<0.01 vs. control.

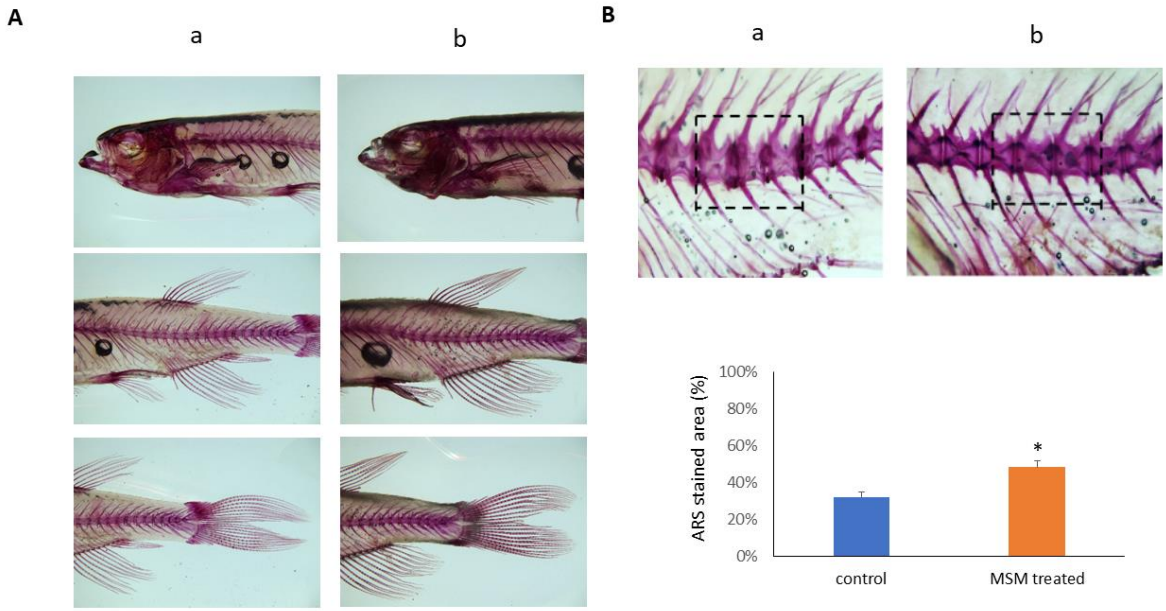

Figure 15. MSM increases mineral deposition in middle-aged zebrafish (15-20 months old). **(A)** Alizarin red staining (ARS) in **(a)** untreated and **(b)** MSM-treated adult zebrafish after 14 days. Magnification 4X. **(B)** Higher percentage of ARS positive areas in **(b)** MSM-treated zebrafish compared to **(a)** untreated zebrafish. Magnification 20X. Results are expressed as mean value (± SD) of two independent experiments. Statistical significance calculated with Student's t-test, was set at *$p<0.05$ vs. control.

We also looked at osteogenic gene expression in adult zebrafish and discovered a distinct gene modulation in fins and scales. We discovered that runx2a was downregulated in the fins of adult MSM-treated zebrafish (Fig. 16A). On the other hand, we observed increased runx2b and decreased tnfrsf1b gene expression in scales of MSM-treated zebrafish (Fig. 16B). To better understand the different osteogenic genes modulation, we assayed Alcian blue and Alizarin Red staining for the evaluation of cartilage and calcium deposition, respectively. As shown in Fig. 16C, the fin of MSM-treated zebrafish had more pronounced Alcian blue stained areas than the control. This result is in line with the elevated aggrecan protein levels previously noted in fins of MSM-treated zebrafish. We also noticed that the fin of MSM-treated zebrafish had more intense Alizarin red stained areas than the control. On the contrary, in the scales of MSM-treated zebrafish the Alizarin red staining was higher than the control (Fig. 16E), while Alcian blue staining was the same in both control and treated zebrafish (Fig. 16D).

In order to investigate a potential MSM-epigenetic effect, we explored the modulation of miR-146b, which has been observed to be involved in human mesenchymal stem cells differentiation. As shown in Fig. 17A, miR-146b expression was decreased in both fin and scale treated with MSM. In addition, as extracellular signal-regulated kinase (ERK) is involved in both osteogenic and chondrogenic commitment, we assessed the ERK activation pathway in fins and scales of MSM-treated zebrafish. As shown in Fig. 17B, we noticed elevated ERK levels and a reduction of pERK levels as well as of pERK/ERK ratio in both fins and scales of MSM-treated zebrafish.

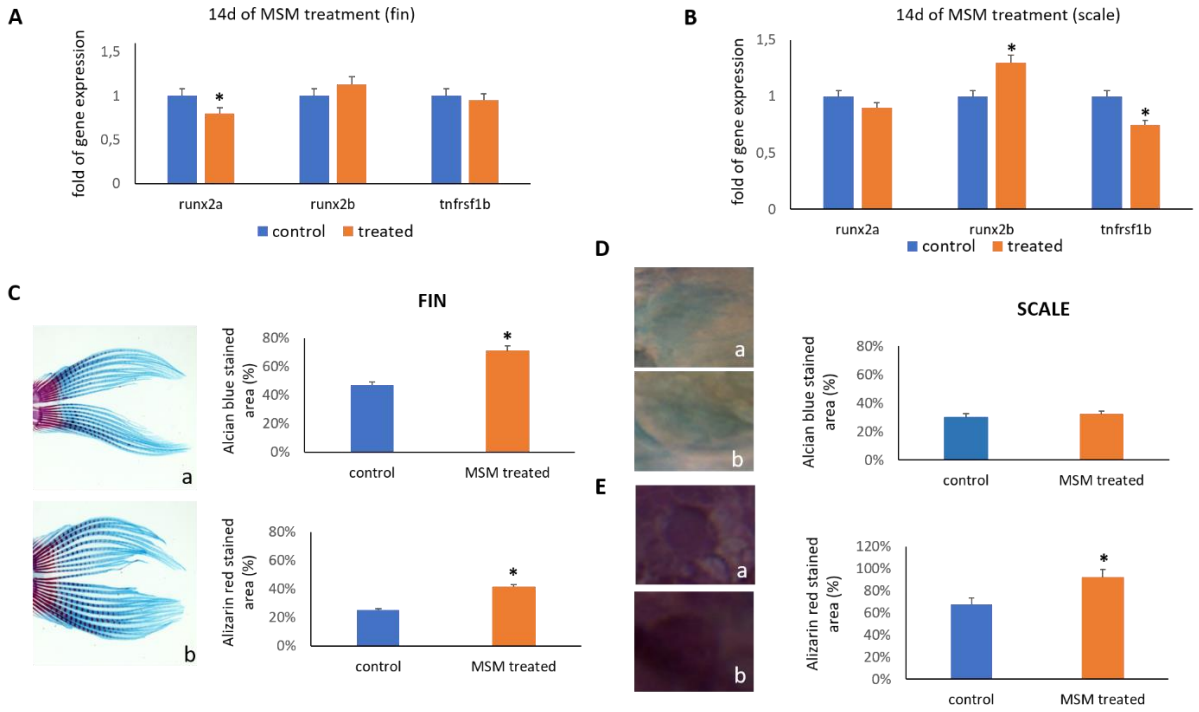

Figure 16. MSM modulates gene expression in middle-aged zebrafish. (A) Reduced runx2a gene expression was observed in fins of MSM-treated adult zebrafish and **(B)** increased runx2b and reduced tnfrsf1b gene expression was observed in scales of MSM-treated adult zebrafish compared to controls. **(C)** A more intense alcian blue **(a)** and alizarin red **(b)** staining areas were observed in the fins of MSM-treated zebrafish compared to control. Magnification 4X. **(D)** Weak alcian blue staining was observed in scales of both control **(a)** and treated **(b)** zebrafish, **(E)** whereas a stronger alizarin red staining areas were observed in scales of MSM-treated zebrafish **(b)**. Magnification 40X. Results are expressed as mean value (± SD) of two or three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p<0.05$ vs. control.

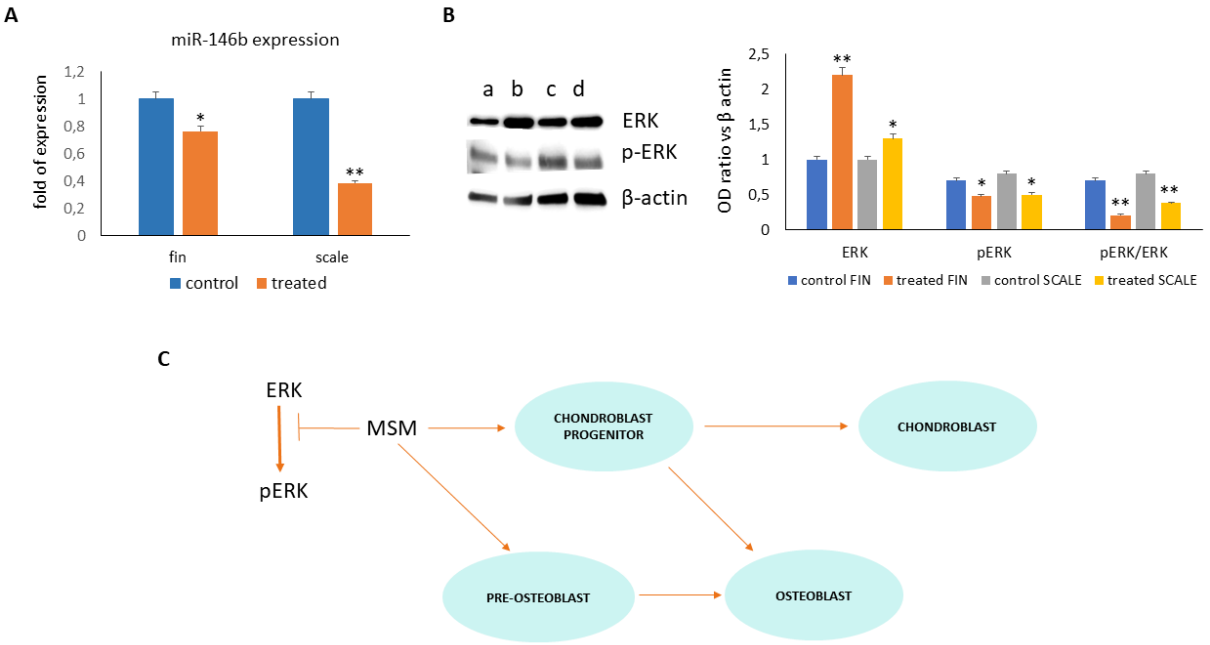

Figure 17. MSM influences miR-146b expression and ERK signaling. (A) MiR-146b expression was reduced in the fins and scales of MSM-treated adult zebrafish. **(B)** Increased ERK levels, reduced p-ERK levels, and a lower p-ERK/ERK ratio were observed in fins and scales of MSM-treated zebrafish: a) control fin; b) treated fin; c) control scale; d) treated scale. Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$; **$p < 0.01$ vs. control. **(C)** A schematic illustration showing how MSM promotes chondroblast progenitor cells proliferation and pre-osteoblasts maturation by decreasing ERK phosphorylation.

4.8 Fisetin promotes osteogenic differentiation *in vitro*

In order to assess fisetin's toxicity, we examined the viability of MSCs treated with fisetin for 24 hours at concentrations ranging from 0 to 5 µM. As shown in Figure 18, fisetin had no effect on cell viability at any tested concentration.

We then decided to evaluate fisetin's impact on MSCs during osteogenic differentiation. We treated MSCs without and with fisetin at the four previously used concentrations, and we found that fisetin promoted osteogenic differentiation (Figure 19). Interestingly, the upregulation of osteogenic genes (BGLAP, SPP1, SPARC, and COL1A1) was much more evident when fisetin was added to MSCs at the concentration of 2.5 µM (Figure 19A). By treating MSCs with fisetin at the concentration of 2.5 µM, we also evaluated the expression of the osteogenic master gene RUNX2. We discovered that RUNX2 expression in treated cells generated the same modulation generally observed during osteogenic differentiation. [95]

RUNX2 expression was indeed upregulated until the 7TH day of differentiation; on the 14TH day, RUNX2 expression was downregulated (Figure 19B).

Oxidative stress has been shown to impair MSCs' ability to differentiate into osteoblasts. Previously in the Lab, we measured ROS levels during osteogenic differentiation to assess the beneficial antioxidative properties of fisetin. As shown in Figure 19C, we observed that whereas fisetin supplementation had no effect on ROS levels during the early and middle phases of differentiation, but it significantly decreases ROS levels in cells treated with fisetin during the late phase of differentiation (14 days of differentiation).

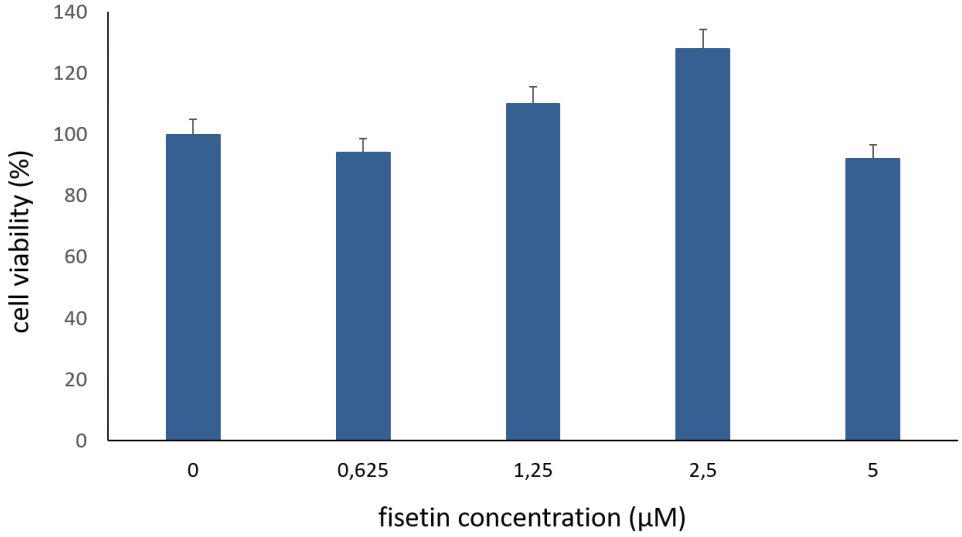

Figure 18. MSC cell viability under fisetin treatment. Cells were treated with fisetin at the indicated concentrations (from 0 to 5 µM) for 7 days in growth medium. After 7 days cell viability was assessed using the CCK-8 assay. Fisetin had no statistically significant impact on cell viability. Data are expressed as mean value (± SD) of three independent experiments. Statistical significance was calculated with Student's t-test.

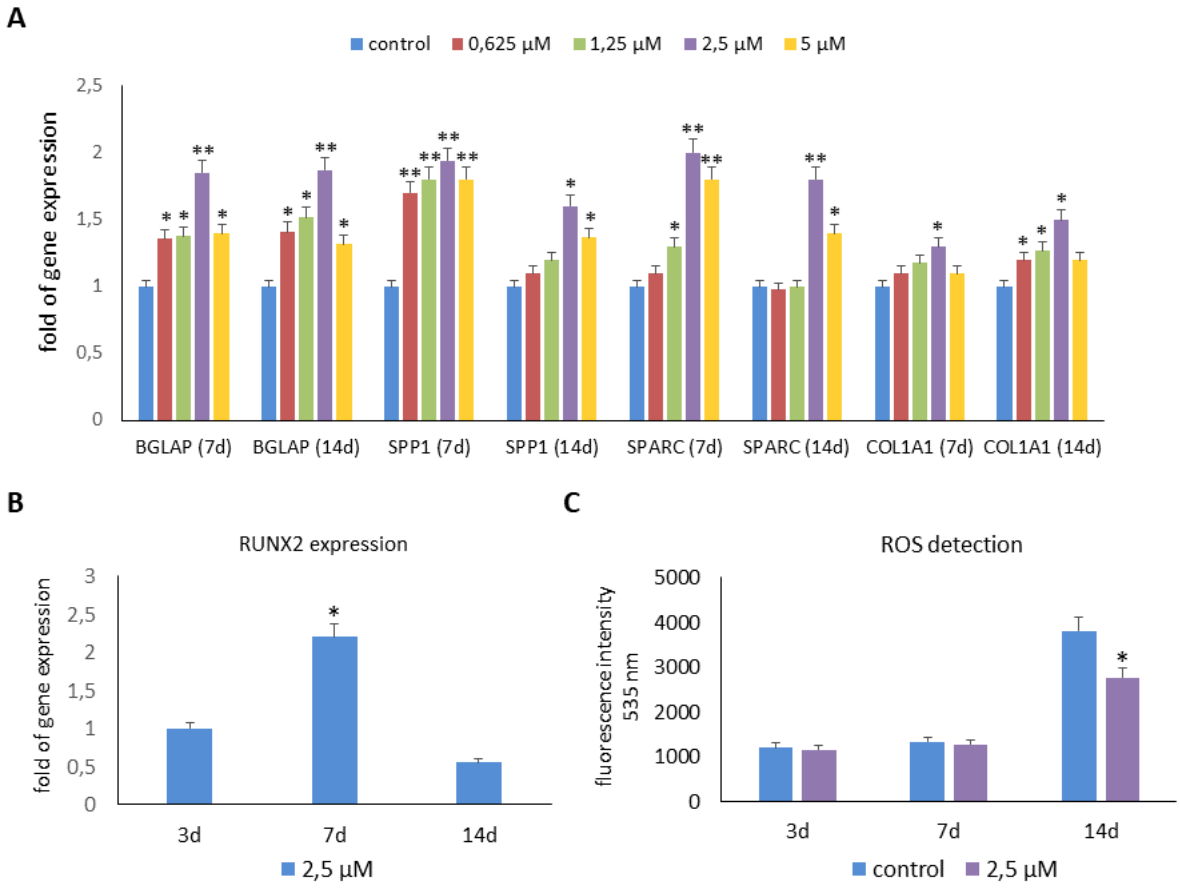

Figure 19. Fisetin supplementation impacts on osteogenic differentiation. (A) The graph shows the expression of some osteogenic maturation-related genes in MSCs after 7 and 14 days of osteogenic differentiation and treatment with fisetin at the indicated concentrations. It was observed that fisetin at 2.5 µM induced a more pronounced upregulation of osteogenic genes. **(B)** RUNX2 expression in fisetin-treated cells increased at 7 days of differentiation and then decreased. **(C)** Fisetin supplementation affected ROS levels during the late osteogenic differentiation phase (14 days of differentiation). All graphs report the mean (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$; **$p < 0.01$ vs control or 3 days of differentiation.

We also decided to look into the effects of fisetin on the late phase of osteogenic differentiation, so we cultured MSCs in osteogenic medium with or without fisetin for 21 days. MSCs were treated with fisetin concentrations ranging from 0 to 5 µM, including 2.5 µM. Figure 20 shows that fisetin supplementation increases the expression levels of RUNX2-downstream genes related to the late osteogenic phase (A) as well as mineral deposition (B), which was evaluated by Alizarin Red staining.

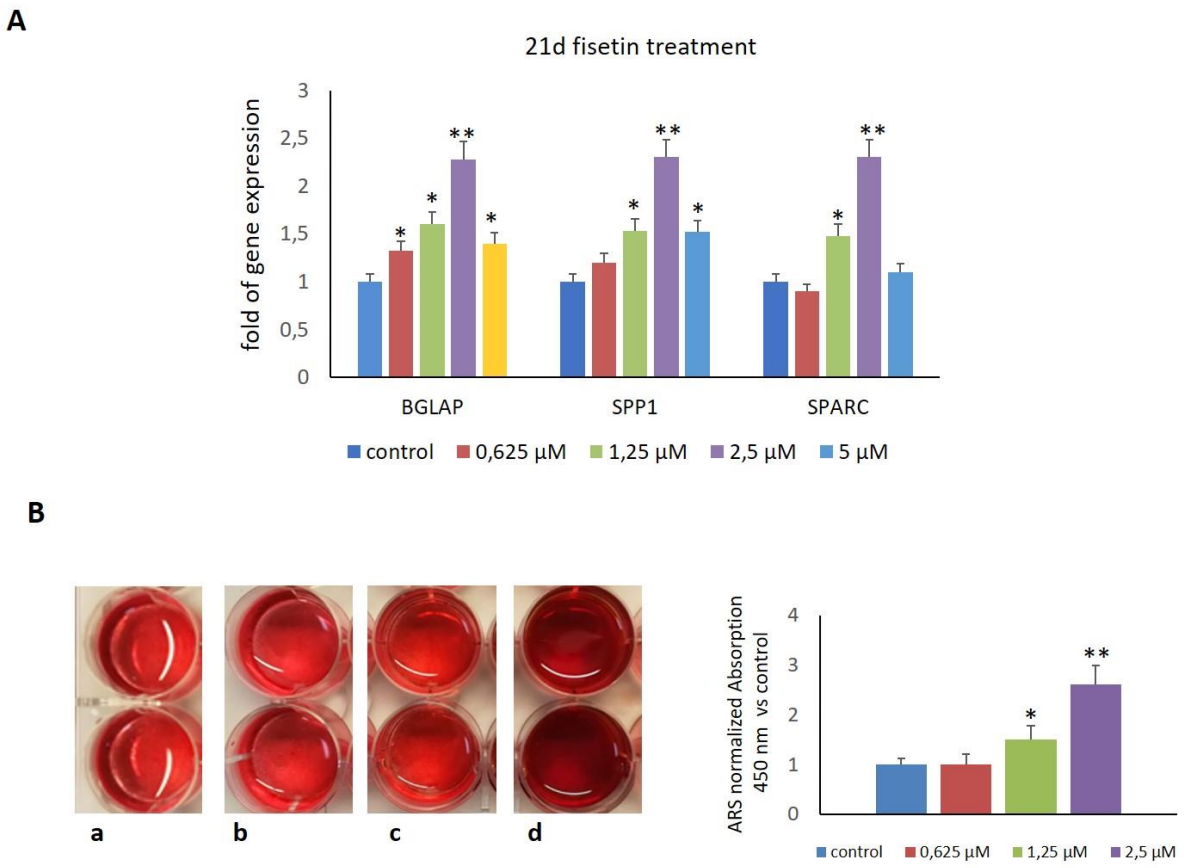

Figure 20. Effects of fisetin supplementation on the late phase of the osteogenic differentiation. **(A)** Fisetin increased the expression levels of osteogenic maturation related genes and **(B)** it increased also the mineral deposition after 21 days of osteogenic differentiation (mineralization phase). **(B)** The imagines on the left shows the Alizarin Red (AR) staining for the evaluation of mineral deposition: a) control; b) fisetin 0.625 µM; c) fisetin 1.25 µM; d) fisetin 2.5 µM. Magnification 4X. The graph on the right shows the quantification of AR staining measured by absorbance at 450 nm. Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$; **$p < 0.01$ vs. control.

4.9 Fisetin's effects are comparable to those of known pro-osteogenic molecules

The effects of fisetin were then compared with those of other supplemental molecules known to promote osteogenesis and improve the quality of bone tissue. Among these molecules, ascorbic acid (AsA) regulates collagen deposition and promotes the differentiation of MSCs to osteoblasts and chondrocytes [96], and the pigment epithelium-derived factor (PEDF) inhibits adipogenesis and promotes osteogenesis of human MSCs. [97]

Therefore, we compared the effects of fisetin supplementation on MSC during osteogenic differentiation with those of AsA and PEDF supplementation. As shown in Figure 21, fisetin induced the upregulation of all examined osteogenic genes after 7 (A) and 14 (B) days of differentiation. The gene modulation induced by fisetin treatment was comparable to that observed when cells were treated with PEDF and AsA. After 14 days of differentiation, fisetin supplementation induced a more pronounced COL1A1 and SPARC gene expression than PEDF and AsA treatment.

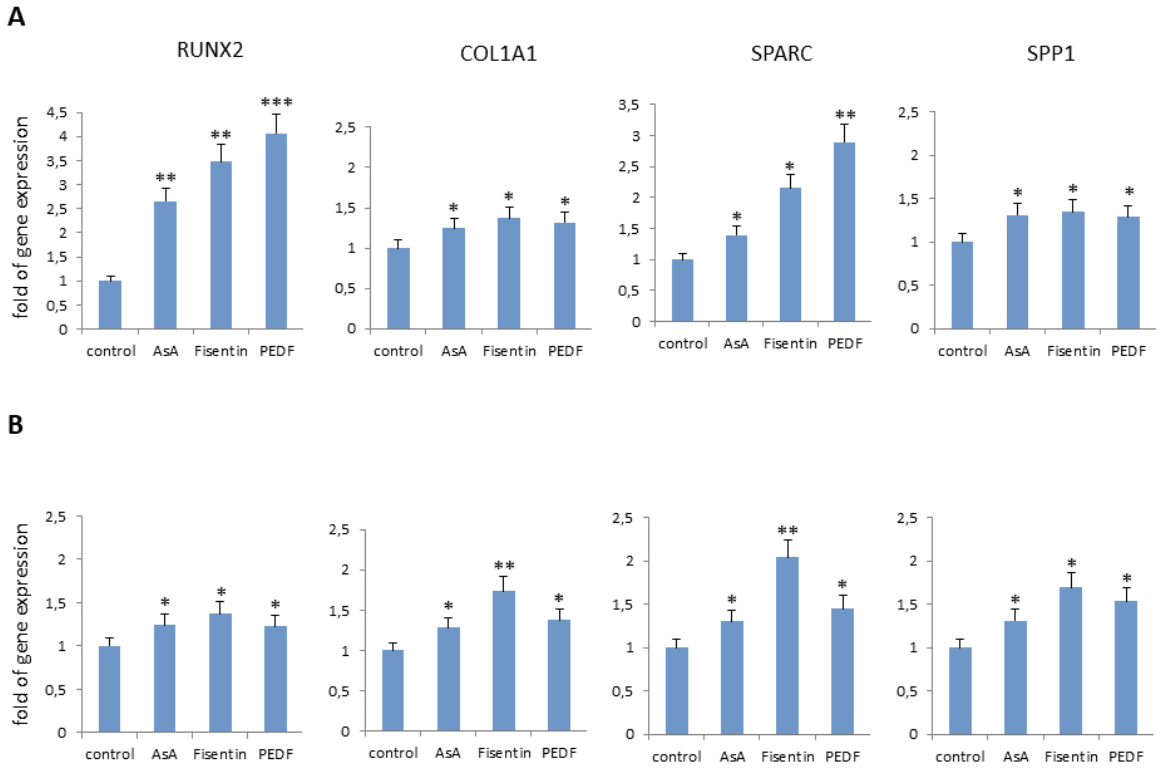

Figure 21. Fisetin, PEDF and AsA supplementation affect the expression of osteogenic-related genes. Fisetin, PEDF and AsA supplementation were able to increase the expression of osteogenic related genes in MSCs after 7 **(A)** and 14 **(B)** days of osteogenic differentiation. All the graphs report the mean (± SD) of three independent experiments performed in triplicate. Statistical significance calculated with Student's t-test, was set at *p < 0.05; **p < 0.01, **p < 0.005 vs. control.

4.10 Fisetin promotes osteogenesis and bone mineralization in zebrafish larvae and adults

We employed the Danio Rerio (zebrafish) model to assess the effects of fisetin *in vivo*. Zebrafish larvae were grown in water at 33°C and in presence or absence of fisetin from the second days post-fertilization (dpf) Preliminary tests at 2.5, 5 and 10 µM revealed no difference in the expression of osteogenic genes when compared to untreated zebrafish. However, we noticed increased osteogenic gene expression in zebrafish treated with 15 µM fisetin. Thereafter, we conducted all the experiments using fisetin at 15 µM.

After 7 (9 dpf) days of treatment, zebrafish were euthanized and collected to perform staining or gene expression analyses on tails and scales. As illustrated in Figure 22A, fisetin-supplemented zebrafish larvae displayed an increase in the expression of osteogenic genes.

In order to record the direct effects of fisetin on bone mineralization, we measured the vertebral area using calcein staining. We observed calcein fluorescence density under microscope and we analyzed fluorescence levels using digital methods.

Calcein staining revealed that larvae treated with fisetin for 7 days had an increased vertebral area than untreated larvae (Figure 22C). Interestingly, the upregulation of osteogenic genes was also observed in adult zebrafish (15–20 months) supplemented with fisetin for 14 days (Figure 22B). Moreover, we observed increased bone mineralization, evaluated by Alizarin Red staining (ARS), in adult zebrafish treated with fisetin for 14 days. Indeed, fisetin-treated adult zebrafish had more intense ARS and a higher percentage of positive ARS areas than untreated zebrafish (Figure 22D).

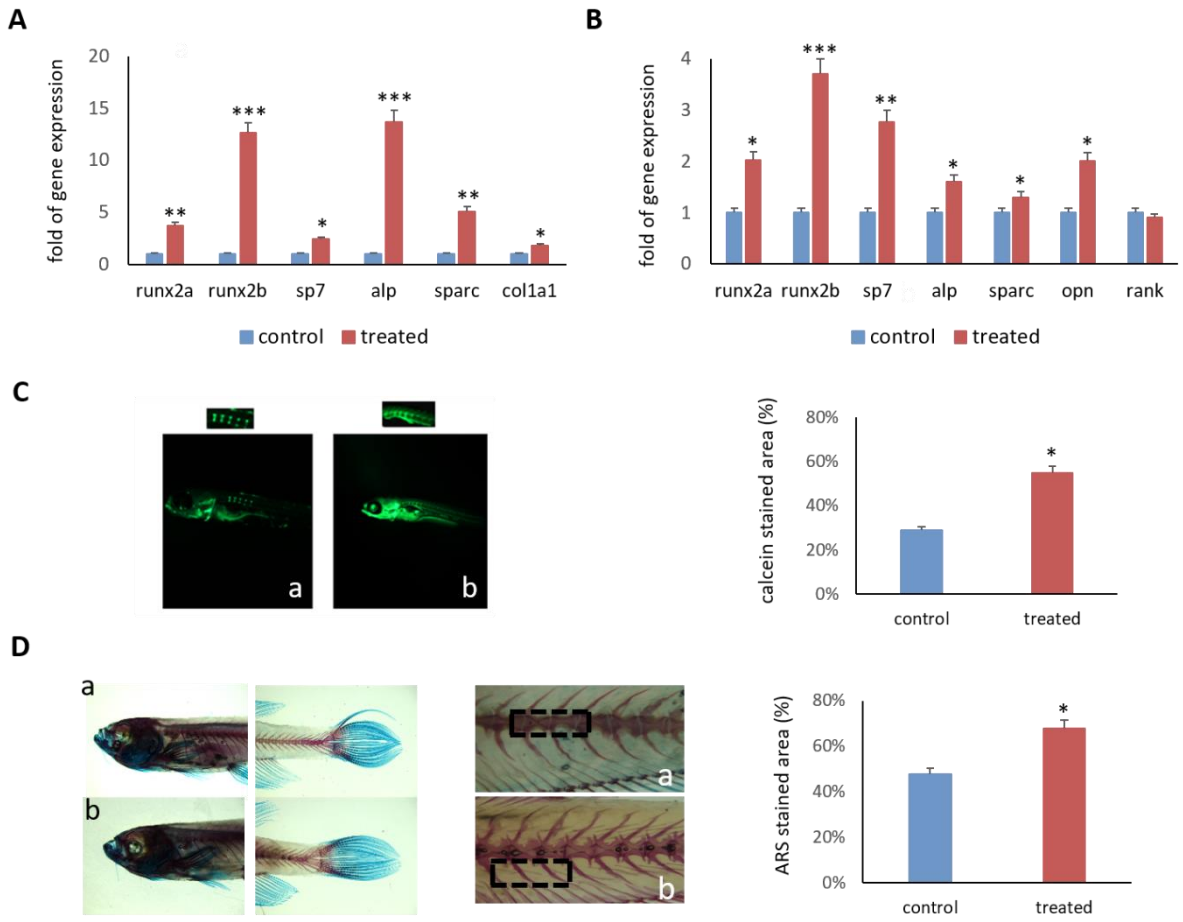

Figure 22. Effects of fisetin *in vivo* on zebrafish model. (A) After 7 days of treatment, fisetin increased the expression of osteogenic maturation related genes in zebrafish larvae (9 dpf). **(B)** Also in adult zebrafish, fisetin supplementation increased osteogenic genes expression after 14 days of treatment. (**C**) The fluorescence density produced by calcein staining revealed an increased vertebral area in larvae after 7 days of fisetin treatment. **(D)** In adult zebrafish, 14 days of fisetin supplementation increased bone mineralization, evaluated by Alizarin Red Staining (ARS). Data are expressed as mean value (± SD) of two or three independent experiments. Statistical significance calculated with Student's t-test, was set at *p < 0.05; **p < 0.01, ***p < 0.005 vs. control.

4.11 Fisetin induces osteogenic maturation in cells of CCD pediatric patients carrying RUNX2 mutations

Given the positive effects of fisetin on osteogenic differentiation, we made assumptions about its potential good effects in the context of skeletal diseases. As a result, we look into the impact of fisetin in *ex vivo* models of cleidocranial dysplasia (CCD, OMIM#119600), a dominantly inherited skeletal disease. In particular, as we previously reported, we isolated fibroblast-like cells from two unrelated pediatric patients affected by CCD, who carried mutations in exon 7 of one RUNX2 allele. [98]

In that previous study, we discovered that RUNX2 gene expression levels in both patients were comparable to those in the healthy control, whereas SPARC gene expression levels were lower in both patients than the control. [98] However, when the fibroblast-like cells were cultured in the presence of fisetin, we observed SPARC upregulation compared to untreated patients' cells, while RUNX2 levels remained unaltered (Figure 23). This indicated fisetin's ability to restore osteogenic maturation in this *ex vivo* skeletal disorder model (Figure 23).

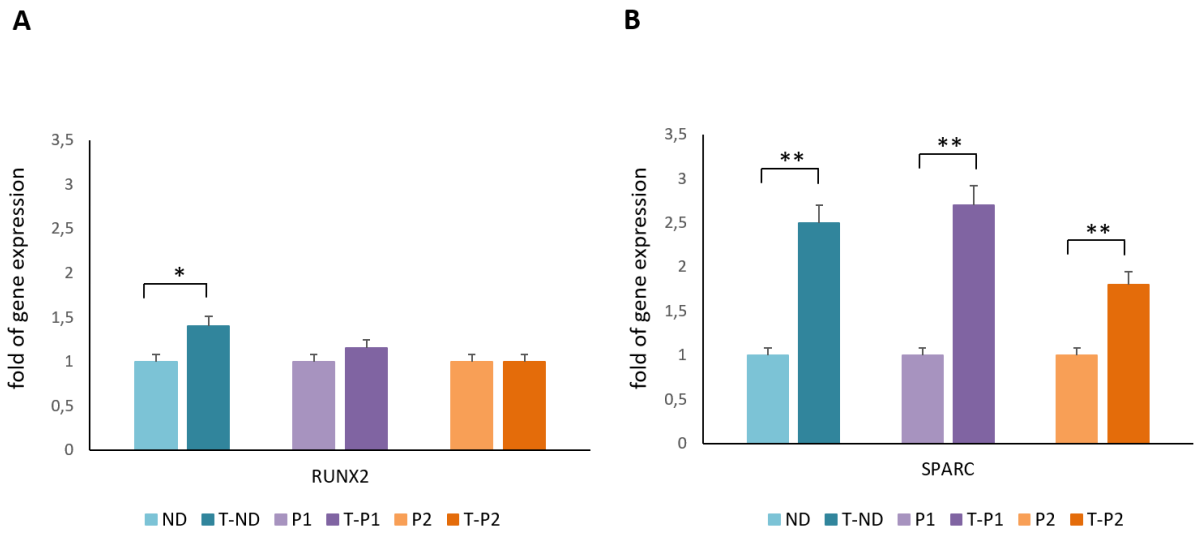

Figure 23. Fisetin promotes osteogenic maturation in cells of CCD patients carrying RUNX2 mutations. The figure shows RUNX2 and SPARC gene expression in fibroblast-like cells isolated from a healthy donor (ND) and two unrelated CCD pediatric patients (P1; P2). Cells were treated with ascorbic acid (50 µg/ml) for 24 hours, then cultured for 1 week in the presence/absence of fisetin (2,5 µM). Fisetin had no effect on RUNX2 expression in CCD patients **(A)**, while its supplementation significantly increased the expression of SPARC in healthy donor as well as in both RUNX2 mutated patients **(B)**. Statistical analysis was carried out in three independent experiments performed in triplicate and evaluated by the Student's paired t-test, comparing the treated sample to the untreated sample for each individual. Data are expressed as mean value (± SD). Statistical significance was set at *$p < 0.05$ and **$p < 0.01$ vs. untreated samples. ND: Normal Donor; T-ND: Treated Normal Donor; P1: Patient 1; T-P1: Treated Patient 1; P2: Patient 2; T-P2: Treated Patient 2.

4.12 Fisetin-encapsulated nanoparticles PLGA-(Fis) retain fisetin from degrading

In order to develop an effective fisetin supplementation for the enhancement of bone quality, we examined the properties of this molecule. First, to verify fisetin's stability, we evaluated its concentration in the culture medium at various time points. We assessed fisetin degradation upon time lapse observation, as shown in Figure 24. In particular, fisetin was nearly completely degraded after 6 h. Consequently, we produced fisetin containing PLGA nanoparticles [PLGA-(Fis)] to prevent fisetin degradation and make the molecule available for human use. We compared the information about our nanoparticles' formulation with data of fisetin encapsulation into PLGA previously published by Sechi et al., Kadari et al.

Our nanoparticles' formulation has better colloidal stability than that reported by Kadari et al. [99], because it has a higher negative ζ-potential. Furthermore, we obtained a higher drug loading (DL) value, corresponding to 23.51%, which is 6 to 8 times higher than the result reported by Sechi et al. [100] With the exception of those made by Kadari et al., our PLGA-(Fis) nanoparticles exhibit a smaller particle size than all the other previously reported. Moreover, ours PLGA-(Fis) nanoparticles exhibit a better mono-dispersity, as evidenced by the lower polydispersity index (PDI) value.

Nano-tracking and atomic force microscopy (AFM) analysis were also carried out to further study the size distribution of nanoparticles. The nanoparticles shape is quite spherical; data from the three different methods are in agreement and span in the same order of magnitude, even if the techniques are based on different physicochemical properties. Additionally, freeze-drying has no negative effects on the size of the samples after resuspension and the co-encapsulation of fisetin, and FITC has no noticeable impact on the size of the nanoparticles.

We also examined the fisetin release rate from PLGA nanoparticles at different temperatures (4 and 37°C) and in citric acid (pH 5) in a final volume of 1 ml. As can be seen from the Figure 25A, after an initial burst loss, the release kinetics rapidly decreased. The temperature rise, as expected, causes fast kinetics, whereas an acidic pH appears to slow the loss.

Then, an *in vitro* release study was carried out (Figure 25B). PLGA-(Fis) exerted a sustained release trend that fits with a second-degree polynomial function.

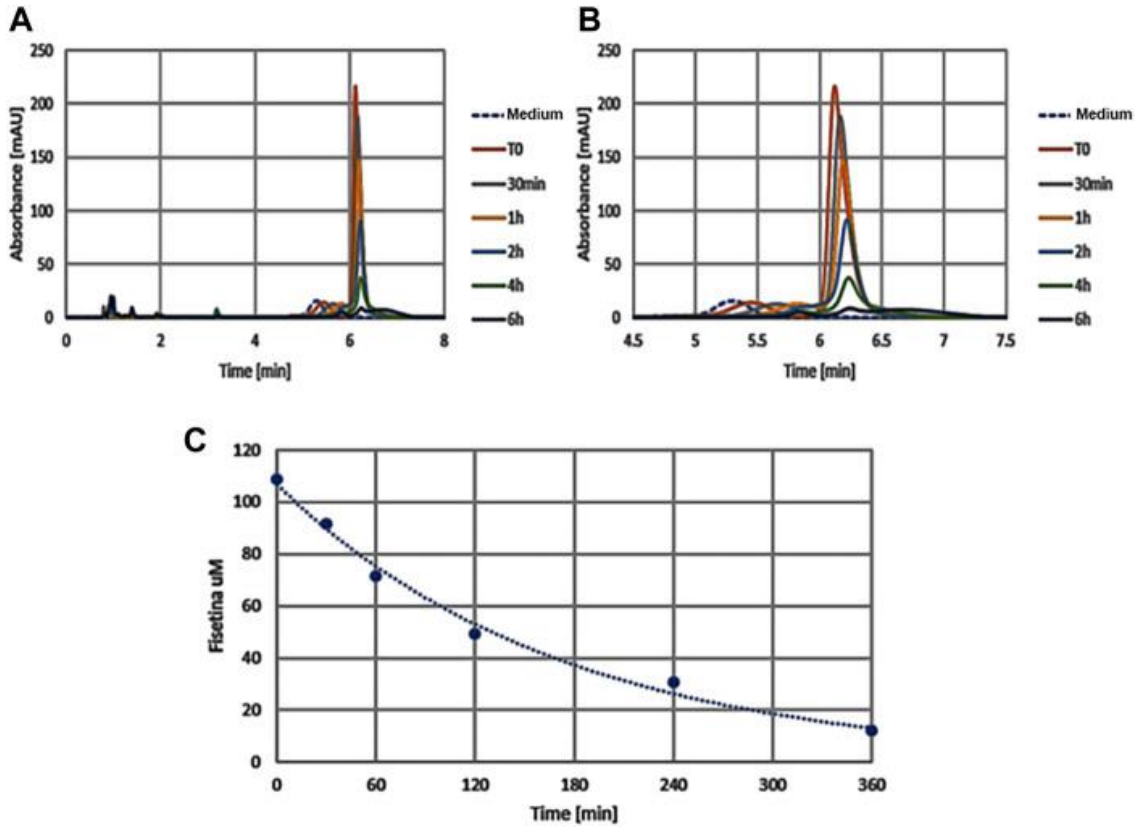

Figure 24. Evaluation of fisetin stability in cell culture medium upon time lapse observation. (A, B) The graphs illustrate absorption peaks of fisetin in culture medium at different times (in *x* axis). **(A)** The image shows the entire duration of the run (from 0 to 8 h time lapse in culture medium), while the one on the right **(B),** shows better the peaks around the retention time (from 4.5 to 7.5 h). **(C)** The graph shows the degradation kinetics of fisetin. As time passes, the molecule degrades and the concentration lowers, until it almost disappears after 6 h. Culture medium without the addition of fisetin was used as control.

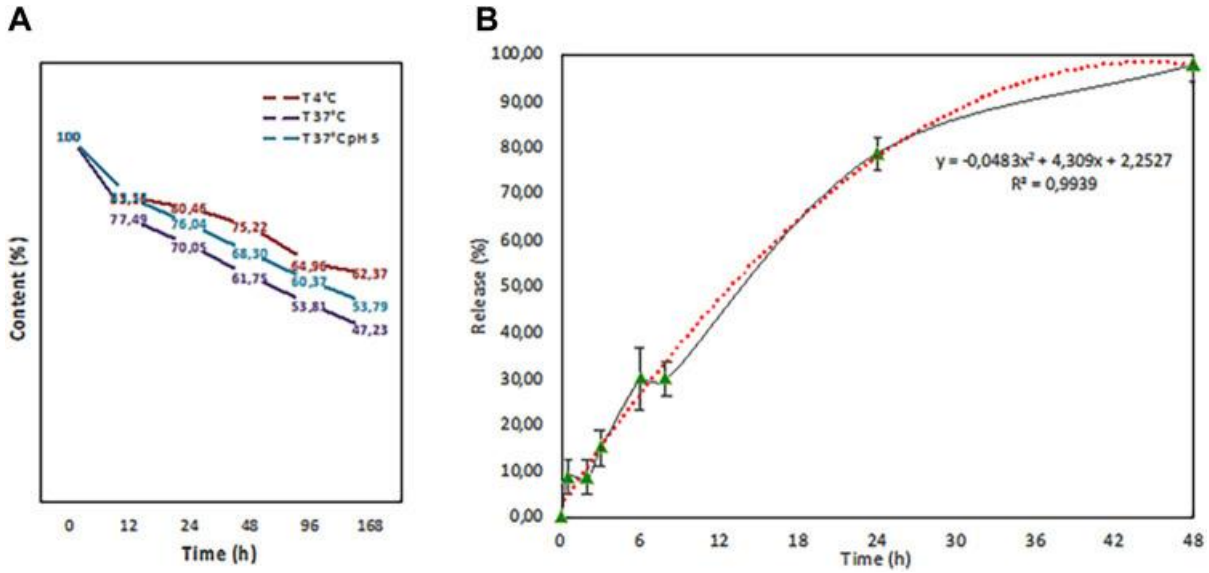

Figure 25. Evaluation of fisetin release rate from PLGA nanoparticles. (A) Release tests were conducted at different temperatures (4°C (red line) and 37°C (purple line) in PBS and 37°C (blue line) in citric acid pH 5 in a final volume of 1 ml. **(B)** *In vitro* release study in 100 ml of physiological solution that fits with a second-grade polynomial function curve (red dots).

To evaluate the ability of PLGA-(Fis) to cross the intestine, a microfluidic system containing human intestinal epithelial tissue was developed (Figure 25). As expected, PLGA-(Fis) could cross the intestinal epithelium. This is explained by the fact that nanoparticles with negative zeta potential are taken up by Peyer's patches and are transported into the blood circulation. [101] The Table 1 displays our data: after 5 hours of incubation, less than 5% of the initial concentration crossed the epithelium, while a value of 30% was reached within 16 hours. A fluorescence calibration curve was used to calculate amount of fisetin.

Furthermore, PLGA-(Fis) recovered in the volume filtered by the epithelium after the experiment, showed a slight increase in size, with values around 190 nm. This increase in PLGA-(Fis) size could be attributed to the well-known protein corona phenomenon or to some interactions occurring during the epithelial tissue crossing.

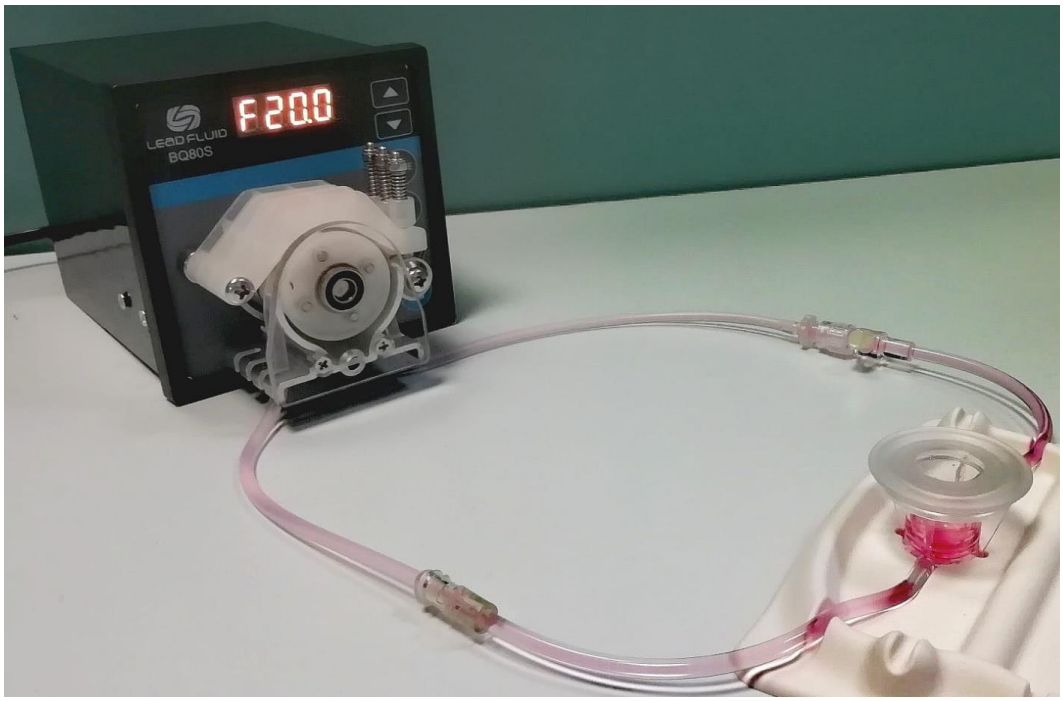

Figure 26. MIVO® organ-on-chip platform experiment. With a peristaltic pump we generated a microfluidic system containing human intestinal epithelial tissue inside the MIVO® chamber to assess the ability of PLGA-(Fis) nanoparticles to cross the intestinal epithelium. MIVO® platform was purchased from React4Life (S.r.l., IT).

Concentration of PLGA-(Fis) added to the intestinal epithelial tissue (µM)	Concentration of PLGA-(Fis) crossing the epithelium after 5 h (µM)	Concentration of PLGA-(Fis) crossing the epithelium after 16 h (µM)
1550 ± 2828	7344 ± 714	43617 ± 795

Table 1. Quantification of fisetin concentration in the medium volume filtered by the epithelium after 5 and 16 hours of incubation.

Then, we decided to culture MSCs with FITC-PLGA-(Fis) to investigate the timing of cellular internalization. After 4 hours following the addition of the complete medium, FITC-PLGA-(Fis) (green dots) were detectable in intercellular spaces (Figure 27A). FITC-PLGA-(Fis) were entirely absorbed into the cells after 6 h of treatment (Figure 27B).

We also cultured MSCs for 7 days in osteogenic differentiation medium with or without PLGA-(Fis) nanoparticles. Fresh PLGA-(Fis) were added to every medium change (every 2 or 3 days). After 7 days of PLGA-(Fis) in osteogenic differentiation medium, cells were collected to perform gene expression analyses. As shown in Figure 27C, cells treated with PLGA-(Fis)

had higher expression levels of the osteogenic transcription factors RUNX2 and SP7 than cells supplemented with free fisetin. Accordingly, the protein expression of COL1A1, a marker of osteogenic maturation, was higher in cells treated with PLGA-(Fis) than in control cells (Figure 27D).

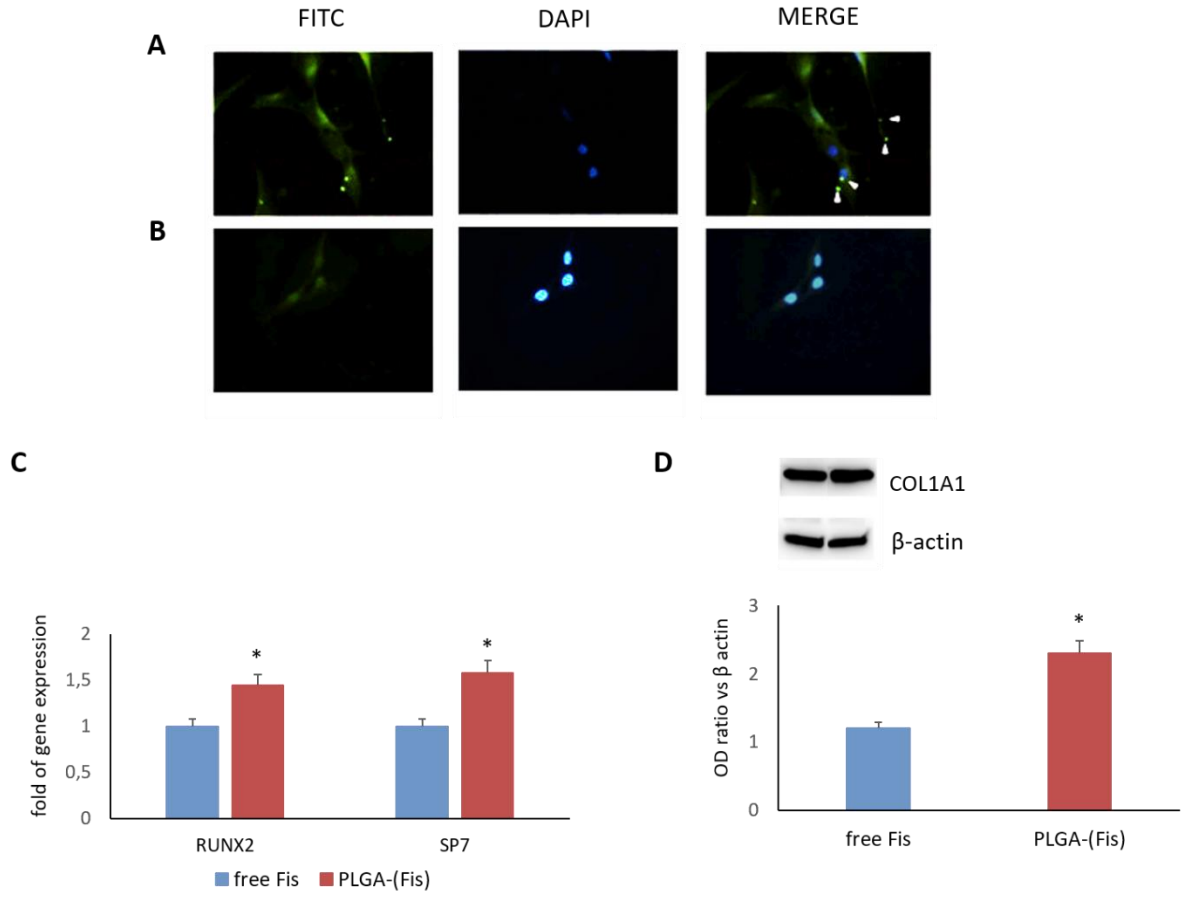

Figure 27. PLGA-(Fis) nanoparticles cell internalization and their effects on MSCs during osteogenic differentiation. (A) FITC-PLGA-(Fis) nanoparticles (green dots) were visible in culture after 4 h of treatment, showing that fisetin diffused in intercellular spaces. **(B)** After 6 h, fisetin was completely absorbed, and only cellular nuclei (blue, DAPI staining) were visible. The images were taken at 40X magnification. **(C)** After 7 days of MSCs osteogenic differentiation, PLGA-(Fis) treatment increased the expression of RUNX2 and SP7 compared to control. **(D)** COL1A1 protein levels increased in osteogenic differentiated cells treated with PLGA-(Fis) compared to control. Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$ vs. free fisetin.

4.13 Effects of training on mesenchymal stem cells commitment

In order to determine the effects of training and the ultra-endurance unsupported cycling adventure known as NorthCape4000 (NC4000) on MSCs commitment, we analyzed circulating MSCs for the expression of the transcription factors RUNX2 and PPARG, which are involved in osteogenesis and adipogenesis, respectively. As shown in Fig. 28, the osteogenic and adipogenic transcription factors, RUNX2 and PPARG were upregulated in MSCs collected before the preparation period (BN) and in MSCs collected 1 week before NC4000 (AN). Both training and ultra-cycling, increased the expression of Sestrin 1 (SESN1) expression in circulating MSCs.

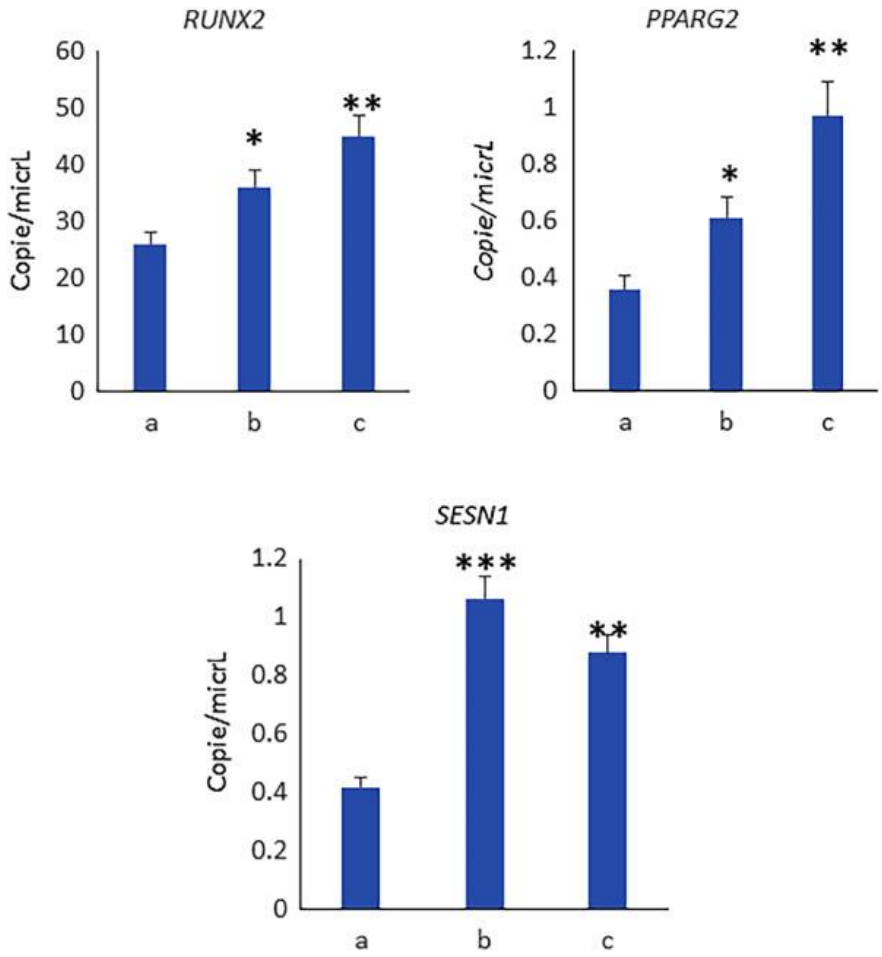

Figure 28. Training and ultra-cycling influence MSCs commitment. The graphs show the expression levels of RUNX2, PPARG, and SESN1 in circulating MSCs collected before and during the training, and after NC4000 cycling adventure. RUNX2 and PPARG2 were upregulated in BN **(b)** and AN **(c)** compared to BPP **(a)**. Moreover, BN and AN performance upregulated SESN1 expression in circulating MSCs. BPP = before the preparation period; BN = the week before NC4000; AN = 10 days after NC4000. Data are expressed as mean value (± SD) of three independent experiments. Statistical significance calculated with Student's t-test, was set at *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ vs. BPP.

5. DISCUSSION

In recent decades, human lifespan has been increasing. Consequently, aging-related diseases such as osteoporosis and osteoarthritis have been increasing in turn. In diseases associated with the aging process, cartilage and bone are the tissues most commonly damaged. Therefore, the characterization of the molecular mechanisms that regulate tissue homeostasis and regeneration, as well as testing compounds capable of reducing the degenerative effects of aging-related disorders, are important challenges. Since mesenchymal stem cells (MSCs) are the precursors of chondrocytes and osteocytes, the cells responsible for cartilage and bone formation respectively, it would be interesting to investigate the role of signaling molecules and the effects of bioactive compounds in these cells during their differentiation process. Moreover, since physical activity has been demonstrated to promote the commitment and differentiation of MSCs, it would be interesting to evaluate the impact of physical exercise on circulating MSCs commitment.

Methylsulfonylmethane (MSM) is a nutraceutical compound that has been shown to be effective in counteracting age-related diseases associated with inflammation, such as osteoarthritis. Few studies have investigated MSM's role in the differentiation of MSCs, and none have assessed the MSM's overall impact on both osteogenic and chondrogenic differentiation. These two mutually regulated processes share the same progenitor cells, the MSCs.

Flavonoids are phenolic compounds typically present in fruits and vegetables. Various flavonoids have been described, and some clinical studies have shown that flavonoid intake increases bone mineral density. [104], [105] In addition, studies conducted *in vitro* have demonstrated that flavonoids can modulate osteogenesis by altering the physiology of osteoblasts, the cells that form the bone. [106]

It is well known that different molecules induce the commitment and differentiation of MSCs to osteogenic or chondrogenic lineages by regulating the expression of transcription factors such as Runt-related transcription factor 2 (RUNX2), SRY-box 9 (SOX9) and SP7/Osterix (OSX). [107]

Epigenetic factors, such as microRNAs, play a crucial role in regulating stem cell differentiation fate, and consequently, the formation of bone and cartilage. [102] MicroRNAs are small non-coding RNAs that have the potential to suppress the expression of specific

mRNAs. [60] A dysregulated miRNA expression has been associated to skeletal disorders. [37], [103]

Numerous studies have revealed that miR-204 plays a role in MSCs fate and differentiation. According to the majority of these studies, miR-204 inhibits osteogenic differentiation by targeting RUNX2. [74], [108] Instead, contradictory data has been published on its role in chondrogenic differentiation, as well as in promoting or counteracting osteoarthritic pathology. [78], [109]

Therefore, our aim was to shed light on the function of miR-204 in chondrogenesis by investigating its modulation both during MSC differentiation and during the development in animal model. Additionally, we sought to determine the interaction between miR-204 and downstream proteins and transcription factors involved in MSC chondrogenic commitment and differentiation.

Our data demonstrated that miR-204-5p can either promote or reduce chondrogenesis by affecting specific phases of differentiation. During chondrogenesis (3,7, and 14 days of differentiation), we discovered an increased expression of miR-204-5p which correlated with the increased expression of the chondrogenic transcription factor SOX9, as well as an increased expression of genes associated to chondrogenic maturation such as COMP and COL2A1. RUNX2 mRNA expression was not altered during differentiation, although RUNX2 protein levels decreased during differentiation, confirming that RUNX2 is regulated post-transcriptionally by miR-204. [74]

Additionally, during chondrogenesis, we observed a decrease in β catenin levels, which degradation is promoted by SOX9 [110], as well as reduced p-ERK levels, which activation has been shown to repress chondrogenic commitment. [111]

Recently, it has been demonstrated that miR-204 inhibits ERK pathway activation and that the miR-204/FOXC1/GDF7 axis regulates osteogenic differentiation. [112], [113] As a result, these findings, together with the inverse modulation of miR-204 and pERK observed in our study, imply that miR-204 can influence differentiation by modulating important cellular signaling pathways (RUNX2, ERK).

We also evaluated the modulation of miR-204-5p expression during development and aging, in zebrafish models (larvae and adult). Our data showed that miR-204-5p expression increased in the larvae during embryogenesis and up to six months of growth. However, we observed that miR-204-5p expression decreased with aging, that ERK phosphorylation increased, and that aggrecan (a chondrocyte maturation marker) levels decreased. It has been proven that a

decreased ERK activity promotes chondrogenesis. [114] As a result, our findings showing an increased in pERK levels in aged zebrafish (>2 years old) suggest a reduction in chondrogenesis during the aging process.

To better understand the role of miR-204 in MSCs differentiation, we performed *in vitro* transfection experiments to inhibit miR-204 activity in MSCs, during chondrogenic and also osteogenic differentiation. By silencing miR-204, during the early chondrogenic differentiation phase, we discovered that SOX9 and chondrogenic related genes (COL2A1, COMP) were upregulated compared to controls. In particular, we observed that SOX9 and COL2A1 expression were 1.5- and 2.2-fold higher, respectively, when compared to controls. Furthermore, miR-204-5p downregulation had a significant impact on chondrocyte maturation. Indeed, we found reduced SOX9 and COL2A1 expression in miR-204 silenced cells during the late differentiation phase.

During the early osteogenic differentiation phase, we observed an upregulation of COL1A1 (a RUNX2 downstream gene) in miR-204-5p silenced cells, whereas RUNX2 expression was slightly affected and RUNX2 protein levels were higher in miR-204-5p silenced cells than control cells. Moreover, during the late osteogenic differentiation phase, the silencing of miR-204-5p had a positive effect on osteogenic differentiation. However, after 10 days of osteogenic differentiation, miR-204-5p silenced cells had lower expression of osteogenic maturation-associated genes than control cells.

Interestingly, our results suggest that miR-204 negatively regulates the osteochondrogenic commitment of MSCs, while it positively regulates chondroblasts and osteoblasts maturation by targeting the RUNX2 gene. Indeed, it has been previously discovered that RUNX2 is expressed in osteo-chondro precursors [115] and that its expression must be reduced to promote the maturation of osteoblasts [29] as well as the chondrogenic differentiation. [116]

Finally, in order to test if miR-204 plays a role in promoting or counteracting inflammatory diseases, like osteoarthritis, we treated chondrocytes and chondro-differentiating MSCs with IL-1β. We observed a decrease in the expression of both miR-204-5p and SOX9 in IL-1β treated cells. Moreover, we observed that the addition of the antioxidant methylsulphonylmethane (MSM) compound could reverse the effect of IL-1β on miR-204-5p and SOX9. In addition, clodronate, a non-aminobisphosphonate used to treat osteoarthritis, had no effect on miR-204-5p modulation, but it was able to upregulate SOX9 expression and prevent miR-204-5p downregulation induced by IL-1β treatment. Clodronate was also able to

prevent cyclooxygenase2 upregulation in IL-1β treated cells. These last findings suggest that clodronate may indirectly upregulate miR-204-5p by reducing inflammatory factors.

Some studies have indicated that MSM may be beneficial in counteracting inflammation-associated diseases related to the aging process.
Cartilage and bone are the tissues mainly damaged by the aging process. There are currently only a few studies that have investigated the effects of MSM on osteogenic differentiation or on prevention of cartilage degradation. These studies demonstrated that MSM can enhance osteogenic differentiation as well as prevent chondrocytes degradation. However, no study has been conducted to evaluate MSM's overall effects on progenitor cells and to understand how MSM may promote both osteogenic and chondrogenic differentiation. This is especially important because osteogenesis and chondrogenesis are both highly regulated processes that originate from the same progenitor cells, the MSCs [36]
Our aim was therefore to evaluate the MSM's effects on MSCs, since they are the precursors of chondrocytes and osteoblasts.

In this thesis project, we used MSM to treat MSCs during chondrogenic and osteogenic differentiation. We observed an increased chondrogenic differentiation in MSM-treated MSCs, as evidenced by the upregulation of the chondrogenic transcription factor SOX9 and the cartilage ECM marker protein COL2A1. SOX9 was also found to be upregulated in chondrocytes treated with MSM. In addition, we observed an increased expression of SOX9, Sestrin 1, and COMP, the cartilage oligomeric matrix protein, in chondrocytes treated with MSM and IL-1β, confirming MSM's protective role against chondrocyte degradation.

It has been demonstrated that Sestrins (Sesns) expression is suppressed in osteoarthritic cartilage chondrocytes. Sestrins can enhance chondrocyte survival under stress conditions: SENS1 and SENS2 counteract oxidative stress damage by activating the Nrf2 (nuclear factor erythroid 2-related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) signaling pathway [117], which mainly regulates anti-inflammatory gene expression and inhibits the progression of inflammation.
Accordingly, in our study, we found that MSM increased the expression levels of ACAN (aggrecan) in chondrocytes even in the presence of IL-β1, compared to controls. This finding is noteworthy since ACAN depletion predisposes to cartilage erosion. [118]

In order to investigate whether MSM induces ACAN expression also in vivo, we evaluated the effects of MSM treatment in adult middle-aged zebrafish. Surprisingly, we found increased

aggrecan levels in the fins of treated zebrafish, but aggrecan expression was not affected in the scales.

As a result, our findings confirmed that MSM induces chondrogenic differentiation in MSCs and that it mitigates the negative effects of IL-1β on chondrocytes. In addition, increased aggrecan levels in MSM-treated adult zebrafish fins imply that MSM promotes cartilage matrix synthesis. The more pronounced aggrecan synthesis in fins is most likely due to a higher number of progenitor cells in fins compared to scales. It is well known that fin, which is composed of multiple bony rays, contains nerves, blood vessels, and mesenchymal cells, whereas scale contains bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). [119], [120]

According to our results, MSM treatment has an impact also on osteogenic differentiation, both *in vitro* and *in vivo*. In MSM-treated cells, we noticed that RUNX2-induced genes were upregulated, whereas RUNX2 itself expression was reduced. SP7 expression increased during the osteogenic differentiation in MSM-treated samples. Since SP7 is mandatory for the final commitment of progenitors to pre-osteoblasts [121], our data indicate a role of MSM in promoting pre-osteoblast formation. In addition, the increased percentage of osteocalcin-positive cells and calcium deposition (evaluated by AR staining) in MSM-treated cells, suggest that MSM has a role in promoting osteoblasts maturation rather than osteogenic commitment.

The induction of osteogenic maturation was also observed in zebrafish model by an increased calcein staining after 7 and 14 days of treatment. According to some studies, the majority of zebrafish bones exhibit the sequential gene expression necessary for osteogenic differentiation in mammals. [122] As in mammals, three stages can be identified during the osteogenic differentiation: an early stage characterized by runx2a and runx2b expression; an intermediate stage characterized by SP7 (Osterix) gene expression; and a maturation stage characterized by the expression of osteocalcin, osteonectin, collagens, and other bone matrix genes. [122]

Interestingly, after 14 days of MSM supplementation, we observed increased gene expression of runx2b but not runx2a. Flores et al. demonstrated that runx2b is expressed in the vertebral column and in the fin in a juvenile fish, while runx2a is expressed in mesenchymal cells during caudal fin regeneration. [123] Therefore, the higher modulation of runx2b versus runx2a observed in MSM-treated zebrafish suggests that MSM influences osteoblasts maturation rather than MSC commitment, and this is consistent with the RUNX2 downmodulation observed in *in vitro* experiments with mesenchymal stem cells.

Runx2b has been shown to regulate SP7/osterix and osteocalcin expression, indicating its central role in skeletal development. [124] In adult zebrafish treated with MSM, we observed an increased osteogenic maturation: fins and scales showed a distinct gene expression pattern. The increased runx2b and decreased tnfrsf1b expression we observed in scales but not in fins reflect the different cellular composition of these two structures (i.e., the prevalence of osteoblasts and in scales compared to fins). However, a further more prolonged treatment, have demonstrated an increased runx2b expression even in fins.

The decreased pERK and pERK/ERK ratio, as well as the increased ERK levels, suggest the inhibition of Mitogen-activated protein kinases (MAPKs), the proteins involved in ERK phosphorylation. [125] Since reactive oxygen species (ROS) activate MAPK pathway [126], we hypothesize that the reduced ERK phosphorylation is due to the reduction of ROS promoted by MSM treatment. Indeed, MSM has been demonstrated to reduce mitochondrial ROS generation. [127]
Interestingly, we discovered a decrease in miR-146b expression in MSM-treated fins and scales. The miR-146 family is highly conserved between fish and human, and it shares the same seed sequence. [128] In humans, miR-146b has been shown to be downregulated in bone marrow stem cells (BMSCs) during chondrogenesis and osteogenesis. [129], [130] Moreover, it has been shown that miR-146b promotes adipogenesis. [131] Our findings therefore suggest an epigenetic effect of MSM.

Fisetin, a polyphenol, has been discovered to have an influence on a wide number of cellular processes *in vitro*. Indeed, fisetin exerts a proapoptotic activity on cancer cells [132], it acts as anti-oxidant agent in neuronal cells [133] and as anti-inflammatory agent in macrophages and fibroblasts. [134] *In vivo*, it has been demonstrated that fisetin prevent both rheumatoid arthritis and inflammation-induced bone loss. [89] Additionally, fisetin has been revealed to stimulate osteoblast differentiation via phosphorylating GSK-3β and subsequently activating β-catenin. [91] Therefore, these prior works highlight its potential to treat skeletal diseases.

Our aim was therefore to better understand the effects of fisetin during osteogenic differentiation and to investigate a method to improve fisetin administration *in vitro* that could be used in clinical.

Our data confirmed that fisetin is able to promote the osteogenic maturation of MSCs. We discovered that the addition of fisetin to MSCs during osteogenic differentiation increased the expression of osteogenesis-related genes as well as two other molecules (ascorbic acid (AsA) and pigment epithelium-derived factor (PEDF) that have already been investigated and are

known to improve bone tissue quality. In some cases, for example, for COL1A1 and SPARC expression at 14 days of differentiation, we observed higher gene expression levels in fisetin-treated MSCs than in AsA and PEDF-treated cells.

Zebrafish are considered as an excellent *in vivo* model for studying cell signaling in bone and evaluating the effects of bone treatments, as was already demonstrated in our Lab. [135], [136] Zebrafish are also commonly used as an experimental model in developmental biology. However, the effects of osteoanabolic supplementation on mature zebrafish skeleton have received little attention. Experiments focusing on skeletal changes in adult zebrafish following stimulation could allow the evaluation of molecular treatments for aging-induced bone loss.
In our study, we discovered that fisetin supplementation promoted bone formation in zebrafish larvae and adults by increasing the expression of bone-specific genes. Calcein and AR staining confirmed our findings, as fisetin-treated zebrafish had more intense calcein and AR staining than untreated zebrafish.

It is well known that different skeletal diseases or conditions show deregulated bone formation, for example, osteoporosis and systemic diseases like diabetes, aging-associated bone loss diseases, drug-induced bone disorders, osteogenesis imperfecta, and cleidocranial dysplasia (CCD). In particular, L. Dalle Carbonare et al. have reported that in patients affected by CCD, an hereditary autosomal dominant skeletal disease, RUNX2 mutations disrupt osteogenic differentiation. [98]

In this thesis work, we discovered that fisetin promotes osteoblast maturation in samples from pediatric patients with CCD. Indeed, during MSC osteogenic differentiation, we observed that fisetin stimulated the expression of SPARC, a downstream osteogenic gene associated with the osteoblast maturation phase. However, evaluating the effect of fisetin in RUNX2 KO animal models would be interesting in order to understand fisetin's role and action in CCD pathology.

Mineralization has been shown to coincide with a reduction in intracellular ROS levels [137] and oxidative stress impairs MSCs' ability to differentiate into osteogenic lineage. [138] Therefore, taking into consideration the antioxidant properties of fisetin, we have considered its activity beneficial for reducing oxidative stress levels and for promoting MSCs osteogenic maturation. Indeed, we found that, in presence of fisetin, the ROS levels in MSCs decreased specifically during the late phase of osteogenic differentiation (14 days of differentiation), while they did not change during the early and middle phases. We did not detect a reduction in ROS levels during the early phase of differentiation probably as ROS accumulation occurs during differentiation. [139]

All of these findings strongly support the idea that fisetin is an excellent molecule for promoting bone formation, suggesting that it could be employed to treat bone diseases caused by osteogenic differentiation deregulation.

However, the disadvantages of fisetin are its low water solubility and its chemical instability. [100] Previously, Sechi et al. and Kadari et al. used nanoprecipitation and double emulsion methods to successfully encapsulate fisetin into PLGA nanoparticles. Alternatively, in this thesis work, we report that the single emulsion displacement method produced comparable results in terms of size and encapsulation efficiency (EE) than those previously reported, and the method we performed was simpler. In addition, the nanoformulation performed by us has a higher negative ζ-potential than that reported by Kadari et al., conferring it a good colloidal stability. Moreover, we succeeded in achieving a higher drug loading (DL) value, corresponding to 23.51%, which was 6 to 8 times higher than the result reported by Sechi et al. In the Sechi et al. study, fisetin was complexed with hydroxyl propyl beta-cyclodextrin to increase its solubility in water, and then emulsified to produce PLGA nanoparticles. Instead, Kadari et al., used nanoprecipitation and two different polymers (PLGA-peg and polycapryl lactone) in their study. Interestingly, in our study, fisetin did not complex with other molecules, and it interacted directly with PLGA, resulting in the release of “pure” fisetin rather than a fisetin-excipient complex. Furthermore, the effects of nanoencapsulated fisetin on osteogenic differentiation and the percentage of PLGA-encapsulated fisetin intestinal filtration have not previously been investigated.

In our study, we found that at equal fisetin concentrations, MSCs supplemented with PLGA-encapsulated fisetin had higher expression of osteogenesis genes than fisetin alone. This last finding is significant to us because fisetin is easily degraded if not protected by nanoparticles.

Since progenitor cell homeostasis is a critical factor in the pathogenesis of degenerative diseases, all factors influencing and sustaining this homeostasis are potential targets in disease prevention. [140] Physical activity has been reported to promote the differentiation of mesenchymal stem cells. [141] As was previously observed in our Lab [142], we found increased expression of RUNX2 in circulating MSCs (cMSCs) after training, indicating osteogenic commitment. The increased RUNX2 expression in cMSCs persisted even after the ultra-cycling performance (NC4000). We also observed an increase in PPARG2 levels after the training as well after NC4000. These findings could suggest an increase in adipogenic commitment too. On the other hand, the increased PPARG2 expression is consistent with its role in regulating insulin sensitivity and glucose utilization to maintain energy homeostasis. [143]

Accordingly, we also evaluated in cMSCs the expression of SESN1 gene, coding for a small stress-inducible protein which has been demonstrated to enhance insulin sensitivity. [144] We investigated the expression of Sestrin in circulating progenitor cells. Given the protective function of Sestrins, the increased expression of SESN1 in progenitors highlights the beneficial role of physical exercise in degenerative diseases. [145]

6. FINAL CONCLUSIONS

In conclusion, in this thesis work, we have shed light on the role of miR-204 in chondrogenic and osteogenic differentiation.

Previous studies had reported that miR-204 had a negative role in osteogenesis and either a positive or a negative role in chondrogenesis. Our study suggests that the negative or positive role of miR-204 depends on the differentiation phase in which its expression increases. Moreover, based on our data, we believe that miR-204 role in differentiation is most likely dependent on its ability to turn down RUNX2 levels.

We demonstrated that miR-204-5p is highly modulated during chondrogenesis and the aging process, and that inflammatory stimuli can affect its expression directly or indirectly. Therefore, it would be interesting to investigate miR-204 role in other pathways, such as the ERK/c-Fos pathway or SMAD signaling, during the differentiation process.

Regarding our study on MSM, we demonstrated that MSM modulates the expression of genes involved in chondrogenic and osteogenic differentiation, most likely by acting on pathways involved in oxidation processes. These findings shed new light on the mechanism of action of MSM supplementation on bone and cartilage differentiation, and they support the use of MSM in patients with osteoarticular disorders. In addition, our study highlights miR-146b involvement in zebrafish mesenchymal stem cells differentiation. Further studies may be performed to evaluate MSM effects on adipogenesis, a process closely related to osteogenesis.

Regarding the study on fisetin, our results corroborate the knowledge about its properties and demonstrate that fisetin stimulates osteogenic genes expression at levels comparable to or higher than those of other pro-osteogenic molecules such as ascorbic acid (AsA) and pigment epithelium-derived factor (PEDF). Moreover, we demonstrated that fisetin promotes mineralization in mature zebrafish, suggesting that fisetin may be useful in preventing aging-induced bone loss. In addition, the increased expression of the osteogenic maturation SPARC gene observed in fisetin-treated CCD “ex vivo” cells, suggests that this flavonoid could be used to treat bone diseases caused by dysregulated osteogenesis. Notably, we were able to generate PLGA-encapsulated fisetin nanoparticles with a high drug loading value and the ability to cross the human intestinal epithelial tissue.

Ultimately, we demonstrated that physical activity derived from ultra-endurance cycling training promotes the osteogenic and adipogenic differentiation of circulating MSCs (cMSCs). In addition, we discovered that following training, SESN1 expression increased in cMSCs, supporting the beneficial role of physical exercise on degenerative diseases.

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