Cytochalasins as Modulators of Stem Cell Differentiation

Subjects: Cell Biology

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Regenerative medicine aims to identify new research strategies for the repair and restoration of tissues damaged by pathological or accidental events. Mesenchymal stem cells (MSCs) play a key role in regenerative medicine approaches due to their specific properties, such as the high rate of proliferation, the ability to differentiate into several cell lineages, the immunomodulatory potential, and their easy isolation with minimal ethical issues. One of the main goals of regenerative medicine is to modulate, both in vitro and in vivo, the differentiation potential of MSCs to improve their use in the repair of damaged tissues. Over the years, much evidence has been collected about the ability of cytochalasins, a large family of 60 metabolites isolated mainly from fungi, to modulate multiple properties of stem cells (SCs), such as proliferation, migration, and differentiation, by altering the organization of the cyto- and the nucleo-skeleton. The ability of two different cytochalasins, cytochalasins D and B, to influence specific SC differentiation programs modulated by several agents (chemical or physical) or intra- and extra-cellular factors, is discussed herein, with particular attention to human MSCs (hMSCs).

Keywords: cytochalasins ; stem cells ; mesenchymal stem cells ; mesenchymal stromal cells ; actin microfilaments ; cytoskeleton ; cell differentiation ; osteogenesis ; adipogenesis ; chondrogenesis

1. Introduction

Regenerative medicine is an emerging field of medical research that aims to replace, repair, or regenerate damaged tissues and organs in patients suffering from severe injuries or chronic diseases, in which the body's regenerative responses are not sufficient [1][2]. Since the transplantation demands exceed the number of donated tissues and organs, research efforts in the field of regenerative medicine have focused on tissue engineering technology, i.e., the transplantation of stem cells (SCs) often combined with specific scaffolds (such as biodegradable 3D scaffolds) [3] or treatments (such as growth factors), as well as on other strategies that stimulate the regenerative potential of endogenous/resident cells in damaged tissues.

To achieve regenerative outcomes, the transplanted SCs can exert direct effects. In fact, they can survive, proliferate, and differentiate into the damaged or lost cell types, integrating themselves in a specific tissue niche and into the host's circulatory system [2]. Moreover, SCs can exert an indirect effect on the resident cells by secreting paracrine and immunomodulatory factors, thus stimulating their repairing properties [4][5]. However, the amount of SCs used during transplantation procedures affects the outcome of the regenerative process, and therefore, an ex vivo expansion of transplantable SCs is necessary to obtain an adequate cell number [2]. On the other hand, SC long-term in vitro expansion may result in the accumulation of genetic damage and intra-cellular signaling pathway alterations, ultimately leading to the senescence process, a condition in which cells perform less than early-passage SCs [6].

Therefore, the objective of this research is to identify, first in vitro and then in vivo, new strategies to modulate the biological properties of SCs and to attempt to slow down the senescence process, preserving their innate self-renewal capacity and enhancing their differentiation potential, in order to improve their efficacy in transplantation approaches.

Many factors can modulate the differentiation capacity of SCs, such as cytochalasins and cyto-permeable mycotoxins, by modifying the actin cellular organization [7][8], which can induce a specific and different cell commitment depending on the dose and type of exposed SCs [9].

Cytoskeletal networks, by influencing mechano-sensing and mechano-transduction pathways [10], may affect SC fate, as can be demonstrated by changes in mechanical properties detected during specific commitments [11]. For instance, under adipogenic and chondrogenic differentiation, mesenchymal stem cells (MSCs) lose and disorganize their cytoskeletal

framework, becoming softer and taking a round shape. In contrast, the osteogenic commitment is encouraged by a stiffer and well-organized cytoskeleton as well as the presence of focal adhesions [12].

2. Cytochalasins

Cytochalasins are a family of more than 60 different metabolites, produced by different species of fungi. Based on the size of the macrocyclic ring and the substitution of the perhydroisoindolyl-1-one residue located at the C-3 position, cytochalasins are classified into various subgroups [7][13]. The chemical diversity of each subgroup influences the biological properties of cytochalasins [13]. Cytochalasins were firstly isolated in the 1960s [7][14], and are named with the term “Phomin”, since they are isolated from the fungal species Phoma [14]; these molecules are also cytochalasins from the Greek words “cytos” (cells) and “chalasis” (relaxation), based on the observed effects of these compounds on mouse fibroblasts [15].

Cytochalasins inhibit actin polymerization and prevent microfilament assembly by binding both actin monomers and filaments [8][16]. Consequently, these mycotoxins, interfering with the cytoplasmatic actin organization, induce changes in cell morphology and affect cell division [13][17]. The chemical structure of each cytochalasin influences its specific functional properties and the possibility of having a reversible effect. For example, cytochalasin B (CB) or D (CD), widely used in research, are able to change cell morphology, and when they are removed from the culture medium, cells revert to their original shape [13][17].

CB is produced by the metabolic processes of Helminthosporium dematioideum and other fungi [18]. It inhibits actin polymerization by binding the fast-growing (barbed) end of F-actin, as well as by interacting with capping proteins (CAPZA1 and others in the F-actin capping protein α subunit family) [18][19]. CB is a well-known cytokinesis inhibitor which interferes with the contractile ring formation, as well as with the cleavage furrow development. Consequently, CB leads to the accumulation of multinucleated cells, as it blocks cell cytokinesis and does not affect the replication and division of the genetic material contained in the nucleus [13][15]. CB has other peculiar functions: (i) it can act as a glucose uptake inhibitor by competing with glucose itself in the binding of the plasma membrane transporters [20]; (ii) it can prevent the endocytosis process, leading to malabsorption of lipoproteins [21]; and (iii) it can alter cell migration by changing the cytoskeletal pattern as well as by interfering with the cytosolic Ca^{2+} storage [22].

CD is an isomeric metabolite of CB, derived from Metarrhizium anisopliae and Zygosporium mansonii [23]. Compared to CB, CD shows differences in the positioning of an ester group and the addition of a ketone group, as well as a contrast in the placement of double bonds. This different chemical structure makes CD much more potent than CB in preventing actin polymerization [24][25]. Thanks to its action on the cytoskeleton, CD can activate tumor suppressor protein 53 (p53)-dependent pathways, causing cell cycle arrest at the G1–S transition [26]. Furthermore, CD has been identified as a promising chemotherapeutic agent. Indeed, by blocking cytokinesis, CD prevents the division of neoplastic cells without interfering with DNA synthesis and karyokinesis [13].

3. Human Mesenchymal Stem Cells

The term “MSCs” is an acronym used to describe a population of multipotent stem/progenitor cells located in the stromal component of several tissues and commonly referred to “mesenchymal stem cells”, “multipotent stromal cells”, “mesenchymal stromal cells”, and mesenchymal progenitor cells. MSCs represent an important tool in regenerative medicine due to their ability to promote tissue repair and homeostasis following injury [27][28].

hMSCs have specific characteristics defined by the International Society for Cellular Therapy (ISCT). According to the ISCT criteria, hMSCs show a fibroblast-like morphology and adhere to the plastic support when grown under standard conditions in vitro. In addition, they are positive for the cluster differentiation (CD) 105, CD73, and CD90 surface markers, while they express low levels of major histocompatibility complex (MHC) class I and are negative for MHC class II, CD11b, CD34, CD14, CD45, and CD31 markers. Although the majority of the MSC population exhibits this immune phenotype, recent studies have revealed that it can change based on tissue sources and localization [29]. Moreover, hMSCs differentiate in vitro in various cell types of mesodermal origin, such as osteocytes, adipocytes, and chondrocytes [30]. To date, it is known that hMSCs can differentiate even into non-mesenchymal cell types, like skin cells, nervous cells, hepatocytes, and cardiomyocytes [27][28].

Although bone marrow represents the main source of hMSCs, MSCs can be isolated from various tissues, including adipose tissue [31], dental pulp [32], yellow ligament [33], umbilical cord blood and Wharton’s jelly [34], and the placenta and fetal membranes [35]. Compared to the hMSCs isolated from adult sources, the ones isolated from fetal and perinatal

tissues show an increased ability to proliferate and differentiate, as well as a prolonged in vitro lifespan before replicative senescence occurs [36]. Moreover, hMSCs isolated from different tissues show biological heterogeneity in their differentiation potential, being able to differentiate or not into a specific cell type [37]. Several investigations have also highlighted a reduction in stemness maintenance, proliferation rate, lifespan, and differentiation potential in hMSCs obtained from elderly donors, indicating that donor age as well as harvesting time may represent a critical factor which must be taken into consideration during MSC transplantation [38][39].

Finally, hMSCs exert their regenerative potential both directly and indirectly in organ and tissue repair [4][40] by restoring tissue homeostasis through paracrine effects that mediate cell-to-cell signaling, reducing local inflammation and increasing cell proliferation during tissue regeneration [41][42].

4. Cytochalasins and Osteogenesis

4.1. Effects of Cytochalasins on Cytoskeletal Organization and Cell Morphology during Osteogenesis

Osteogenic differentiation is a cellular process that involves specific cellular changes at both the structural and molecular level. During this process, SCs lose their typical fibroblast-like morphology and acquire an angular shape with a greater extension and size. These changes are due to a cytoskeletal structure modification, which involves the reorganization of actin microfilaments, leading to a switch from parallel-oriented distribution extending across the entire cytoplasm to a cortical location, as well as to an increase in actin polymerization and in the number of microfilaments [43][44].

Several research groups independently observed that CD inhibited hMSC osteogenic differentiation. In fact, CD-treated cells showed a reduction in calcium deposition and in the expression of alkaline phosphatase (ALP), RUNX family transcription factor 2 (RUNX2), and osteocalcin (OCN) [43][44][45][46]. Moreover, in a study by Sonowal and colleagues, it was reported that in human bone marrow MSCs (hBM-MSCs) a three-day CD treatment (100–1000 ng/mL) was sufficient to reduce osteogenic differentiation; indeed, CD affected the cytoskeletal actin polymerization, promoting a decrease in the phosphorylation levels of p38 mitogen-activated protein kinases (p38MAPKs) and in the expression of osteogenic-related genes, thus highlighting a fundamental role of actin organization in the induction of the osteogenic process [44].

Peng and colleagues used CD to study the relationship between actin filaments and MSC osteogenic differentiation induced by cyclic tensile stress. The authors demonstrated that cyclic tensile stress promoted the early stage of osteogenic differentiation in MSCs via integrin αVβ3 activation, leading to the rearrangement of the actin filaments, an increase in focal adhesion complex (FAC) formation, and Yes1-Associated Transcriptional Regulator (YAP) nuclear localization. CD treatment inhibited the effects elicited by the cyclic tensile stress, reducing the aggregation of actin filaments, nuclear YAP localization, and osteogenic marker expression [47].

To deepen the role of the cytoskeleton during the osteogenic process, Fan and colleagues demonstrated that in human adipose-derived stem cells (hASCs), CD (0.1 µg/mL) negatively affected osteogenesis by acting on the spatial organization and on the crosstalk between actin and vimentin, which are key players in the osteogenic process [48]. Actin and vimentin interact with the nucleus by binding to two nuclear proteins, Nesprin-3 and Nesprin-1/2, respectively. The expression of these proteins is modulated during osteogenic differentiation: vimentin and Nesprin-3 decrease during the osteogenic process, while the expression of actin and Nesprin-1/2 increase in the early stage of the differentiation. Consequently, the vimentin network becomes smaller and denser, localizing itself in a smaller area of the cytoplasm, while Nesprin-3, which is normally aggregated around the nucleus, spreads into the cytoplasm. On the other hand, actin filaments become thicker and multilayered on the ventral cell side, replacing the space left empty by vimentin, and as a result, cells increase their stiffness [48]. In addition, the authors demonstrated a vimentin-mediated control on actin expression, capable of modulating the osteogenic process. They showed that when vimentin expression was upregulated, there was a concomitant decrease in the expression of actin and Nesprin-1/2, and, therefore, in the cell stiffness, resulting in a reduction in *RUNX2* and *osteopontin* (*OPN*) gene levels [48]. In this context, the addition of CD to the osteogenic medium reduced the expression of actin filaments and of the associated Nesprin-1/2 while increasing the expression of vimentin and Nesprin-3, reversing their mutual cellular distribution. These changes were associated with a decrease in the expression of osteogenesis-related genes such as *RUNX2* and *OPN* and with an increase in the expression of phospho-Smad family member 2/3 (Smad2/3) and adiponectin, important markers of the adipogenic commitment, suggesting that CD can modulate SC fate by impairing the balance between actin and vimentin expression [48].

Pampanella and colleagues studied the effects of another cytochalasin, CB (0.01–5 µM), on osteogenic differentiation in human Wharton's jelly MSCs (hWJ-MSCs), a perinatal MSC model [49]. The authors observed that CB-treated cells

became more enlarged, with large focal adhesion regions connecting the stress fibers to the plastic support. Moreover, CB changed the mechanical properties of hWJ-MSCs by increasing actin bundles. In particular, at the highest CB concentration, hWJ-MSCs showed an increased stiffness in the peripheral area, probably due to the maintenance and strengthening of actin tension, which was localized at the cortical level. Moreover, vimentin supported both the cytoplasm and the nucleus and formed intense filament clusters, especially at the perinuclear level, which could maintain the structural integrity of the nucleus, counteracting cytoskeletal deformations and maintaining its biological functions. The overall reorganization of the cytoskeleton induced by CB favored osteogenic differentiation in a dose-dependent manner. In fact, CB treatment increased the expression of the osteogenic genes *RUNX2* and *OCN*, as well as the formation of calcium deposits [49]. Therefore, Pampanella and colleagues came to different conclusions than those previously discussed for CD, indicating that different cytochalasins may induce different effects on the same SC commitment, probably depending on the type of SCs treated [9][13].

CD can also modulate the cell spreading area, a parameter which influences the osteogenic process. Yao and colleagues, for example, demonstrated that in rat BM-MSCs (rBM-MSCs), osteogenesis was promoted by a high cell aspect ratio, which represents a measure of the stretching of a cell. CD treatment (0.25 mg/mL), by reducing the aspect ratio, induced alterations in cell morphology, leading to a reduction in the osteogenic potential of rBM-MSCs [50].

The ability of CD to change the cell spreading area and morphology was also investigated by Zhao's group [51]. The authors cultured mouse BM-MSCs (mBM-MSCs) in substrates of different shapes (circular or star-like micropatterned substrates) with different spreading areas (1256 or 314 mm^2) in the presence or absence of CD (1 µg/mL). The data evidenced that mBM-MSCs cultured in a 1256 mm^2 star-like pattern showed a high density in actin filaments and stress fibers at the peripheral and central regions of the cells. On the contrary, when cells were cultured in the circular-pattern substrate of 314 mm^2, the density of actin filaments decreased. The different cytoskeletal organization influenced the osteogenic differentiation, which was more pronounced in mBM-MSCs cultured in the star-like pattern of 1256 mm^2 compared to those cultured in the other substrate. Such observations were confirmed by the increase in the expression of the *ALP*, *collagen type I* (*Col I*), and *OCN* genes. Moreover, mBM-MSCs cultured in a star-like pattern and in a higher spreading area expressed a higher level of β-catenin and showed an increase in their nuclear/cytoplasm ratio. It is known that, during osteogenic differentiation, β-catenin translocates from the cytoplasm to the nucleus, where it functions as a key player in the Wnt signaling pathway. The authors also demonstrated that the CD treatment of mBM-MSCs, by altering the organization and the assembly of actin, abrogated the difference in actin density and orientation between the two investigated pattern conditions, reducing the expression of osteogenic genes in both patterns, thus affecting the osteogenic process. In addition, CD reduced β-catenin levels and nuclear translocation, indicating that the mycotoxin may also impair the osteogenic process by modulating the activity of the Wnt pathway [51].

In another study, Xu and colleagues demonstrated that the manipulation of mASCs' seeding densities resulted in large and small cells with distinguished microenvironments associated with actin cytoskeletal tension [52]. mASCs seeded at low and medium densities spread into large cells and showed robust osteogenesis, while mASCs at a high density were smaller and were differentiated into adipocytes. CD, by blocking the arrangement of actin cytoskeletal tension, induced the reorganization of cytoskeletal proteins influencing mASCs' size and tension and, consequently, cell lineage differentiation. CD completely blocked adipogenesis in the presence of an adipogenic medium, yet inverted the osteogenic effects related to cell size in an osteogenic medium. CD reduced mineralization in low-density-seeded large cells, while it increased mineralization in high-density-seeded small cells [52].

4.2. Effects of Cytochalasins on Cell–Extra-Cellular Matrix Interactions during Osteogenesis

It is known that, during the early stages of cell commitment, the microenvironment may affect the differentiation potential of SCs. Extra-cellular matrix (ECM) properties such as matrix spatial organization, composition, and stiffness have been reported to be important regulators of osteogenic commitment [44].

The ECM and the intra-cellular actin filaments are closely connected to each other through a variety of cytoskeletal linker proteins; thus, the ability of cytochalasins to change the cytoskeleton architecture clearly influences the mechanical connection between intra-cellular and extra-cellular structures.

Meka and colleagues observed that when hBM-MSCs were cultured on a 3D nanofibrous scaffold, which exhibits an architecture similar to the natural ECM, cells started to differentiate into osteocytes even in the absence of a specific osteogenic medium [53]. On this 3D substrate, hBM-MSCs showed distinct cell and nuclear morphologies, with lower areas and perimeters than cells grown on a flat two-dimensional (2D) substrate, but with higher aspect ratios. These changes were associated with a peculiar organization and localization of the actin microfilaments and microtubules that favored the osteogenic commitment. When the actin organization was impaired by using a CD treatment (1 µM), the osteogenic

differentiation of the hBM-MSCs grown on the 3D scaffold decreased, as evidenced by a reduction in bone mineralization, a redistribution of heterochromatin, and a decrease in osteogenic gene expression. CD's inhibition of osteogenesis was associated with a remodeling of cell morphology and nuclear shape that impaired the cell–ECM interactions [53].

Hu and colleagues also confirmed the ability of CD to interfere with cell–substrate interactions. Firstly, they studied the role of the surface topography in cell fate modulation using human periodontal ligament SCs (hPDLSCs) [54]. Cells cultured on a titanium surface expressed high levels of adhesion-related genes (*integrin β1* and *focal adhesion kinase—FAK*), improving their adhesion to the substrate, as well as increased stress fiber production. In this culture condition, hPDLSCs also showed an increase in the expression of osteogenic-related genes and proteins and a downregulation of adipogenic markers. Moreover, an increase in the nuclear translocation and transcriptional activity of the PDZ-binding motif (TAZ) transcription regulator was observed, revealing a contribution of the TAZ activity in the osteogenic process. In the same culture condition, CD treatment (0.2 μM), by interfering with the actin cytoskeleton and cell–substrate interactions, reduced TAZ expression and translocation into the nucleus, leading to a reduction in osteogenesis [54].

On the other hand, Keller and colleagues, studying the role of various ECM components on the modulation of the osteogenic process, showed that CD treatment (2 μM) modulated hASC osteogenic differentiation independently from the presence of specific ECM components [55]. In fact, CD treatment alone increased the expression of osteogenic markers and calcium deposition. On the contrary, ECM components alone seemed to be insufficient to guide a complete cell commitment, but they stimulated the osteogenic process by inducing changes in cell morphology. Interestingly, in the presence of both CD and fibronectin, hASCs still maintained an elongated shape, which favored the osteogenic process, despite the presence of CD, which, alone, stimulated the cells to assume a rounded shape; in these culture conditions, cells showed the highest increase in the expression of osteogenic transcription factors and markers, indicating that a strong relationship exists between the ECM, actin organization, and cellular morphology in the regulation of osteogenic differentiation [55].

Guo and colleagues tried to clarify whether cell morphology modulated cell differentiation by acting simultaneously or downstream of the matrix stiffness [56]. Firstly, the authors evaluated how cell morphology impacted cell commitment by culturing rBM-MSCs in the presence of fibronectin. They demonstrated that fibronectin (whose concentration correlates with matrix stiffness) enhanced F-actin assembly and modified cell morphology in a dose-dependent manner; such modifications enhanced the cell spreading area and promoted the osteogenic differentiation. The molecular mechanism behind the changes in F-actin organization and cell morphology involved the fibronectin-mediated translocation of YAP/TAZ proteins into the nucleus, which favored osteogenic gene expression. Later, to investigate the interplay between cell morphology and matrix stiffness, rBM-MSCs were cultivated in a soft or rigid matrix, fixing the cell spreading area to prevent changes in cell morphology. In these conditions, the authors observed that the matrix stiffness did not significantly affect YAP/TAZ activity and failed to modulate cell differentiation. All these findings indicated that cell morphology could be a downstream mediator of matrix stiffness-induced osteogenic differentiation [56].

Moreover, the authors demonstrated that CD (1 μM) alone reduced the osteogenic potential of rBM-MSCs. In fact, the mycotoxin significantly inhibited cell spreading and induced the cells' round shape, promoting a reduction in *OCN* and *RUNX2* gene levels. On the contrary, a higher concentration of fibronectin in the presence of CD supplementation promoted an osteogenic program. In fact, fibronectin rescued cell spreading, counteracting changes in cell morphology induced by the mycotoxin, as also demonstrated by Keller and colleagues [55]. Therefore, in this study, fibronectin reversed CD's osteogenic inhibition, mainly acting on cell morphology, a key player in osteogenic commitment [56].

4.3. Effects of Cytochalasins on the Cytoplasmic/Nuclear Actin Ratio in the Regulation of Osteogenesis

Different authors have speculated on the importance of actin nuclear trafficking in the modulation of osteogenic commitment. In fact, besides its mechanical functions in the cytoplasm, actin plays an important role in the nucleus, where it can exist in both monomeric and polymeric forms and can interact with multiple transcription factors, thus modulating the expression of several genes involved in osteogenic differentiation [57].

Sen and colleagues observed that, in hBM-MSCs and mBM-MSCs, CD supplementation (0.1 μg/mL) for 3 days induced a persistent actin fiber depolymerization and led to the translocation of actin monomers into the nucleus, thus increasing nuclear actin levels [57]. Such an increase promoted the cytoplasmic translocation of YAP, a transcription factor which acts in the nucleus inhibiting the expression of *RUNX2*, thus favoring an increase in the expression of osteogenesis-related genes. These molecular mechanisms were confirmed by blocking the translocation of actin towards the nucleus, which, in turn, prevents the translocation of YAP in the cytoplasm and affects CD-induced osteogenesis [57].

Wang and colleagues also observed a cytoplasmic translocation of YAP when they studied the effects of CD (0.2 µg/mL) in hPDLSCs. The translocation was associated with an increase in YAP's phosphorylated form and a decrease in the expression of its target genes (*connective tissue growth factor* (*CTCG*) and *TEA domain* (*TEAD*) family members). However, unlike what is reported by Sen and colleagues, these authors described a CD downregulation of osteogenesis by reducing the expression of ALP, RUNX2, and OSX [58]. These contradictory results could be explained by the fact that cytochalasins can behave in a different way depending on the investigated SC type [49].

In another study, Sen and colleagues observed that, in mBM-MSCs, CD (0.1 mg/mL) not only destroyed F-actin filaments in single monomers and dimers, which translocated into the nucleus, but they also found that, inside the nucleus, monomeric actin started to assemble in filaments, which acted by modulating the chromatin architecture, thus influencing mBM-MSC fate. The formation of a primary actin filament was favored by CD through the transport of diaphanous-related formin 1 (mDia1) in the nucleus, where it co-worked with diaphanous-related formin 2 (mDia2). Then, both formins recruited the actin-related protein 2/3 (Arp2/3) complex, which initiated the second filament branching, a crucial step in starting osteogenic differentiation. In support of this, the inhibition of this complex formation abrogated the CD-induced osteogenesis and stimulated the cells to differentiate into adipocytes [59].

In line with these observations, Samsonraj's group demonstrated in another cellular model, hASCs, that CD (0.1 µg/mL) stimulated osteogenesis by enhancing nuclear actin transport and the expression of genes coding for ECM proteins, cell surface receptors, cell adhesion molecules, and proteins involved in cell communication and signal transduction [60]. Some of these genes are involved in the osteogenic program, such as *HSD11B1*, which encodes the glucocorticoid-activating enzyme hydroxysteroid 11-beta dehydrogenase. A polymorphism in this gene has been associated with age-related osteoporosis and bone metabolism. Another gene upregulated by CD treatment was the *metastasis suppressor protein 1* (*MTSS1*), which is implicated in actin reorganization and in the regulation of cell motility by modulating different Arp2/3 activators. In parallel, CD decreased the expression of genes involved in nuclear processes and mitosis. One of these genes encodes an epigenetic regulator, the Polycomb Group 2 (PcG2) protein EZH2 (Enhancer of Zeste 2), a critical suppressor of osteoblastogenesis. An inverse correlation between EZH2 and RUNX2 was observed, suggesting that CD may regulate cell commitment by modulating the expression of key epigenetic regulators. Moreover, CD promoted the downregulation of TEAD4, a transcriptional factor able to interact with YAP and TAZ factors, controlling cell differentiation [60]. In another study [61], Samsonraj and colleagues compared the effect of CD (0.1 µg/mL) on gene expression in three MSC types, hASCs and h- and mBM-MSCs. Eight genes involved in cell adhesion, angiogenesis, and skeletal tissue development were commonly upregulated in all three types of MSCs. Among them, *Vestigial-like Family Member 4* (*VGLL4*), encoding for a co-regulator of the YAP/TAZ pathway, was upregulated when cells were cultured in the presence of CD. When *VGLL4* was silenced in hASCs, cells had a reduced ALP activity and mineralization process, indicating that CD enhanced the osteogenic differentiation of MSCs by modulating *VGLL4* expression [61].

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