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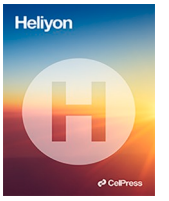

Research article

Effects of EZH2 inhibitor, trichostatin A, and 5-azacytidine combinatorial treatment on osteogenic differentiation of dental pulp stem cells

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A B S T R A C T

Objective: Epigenetic mechanisms play regulatory roles in dental pulp stem cell (DPSC) differentiation. The molecules that modulate these mechanisms can be used to enhance DPSC differentiation in experimental studies and clinical applications. We investigated the combined effects of an epigenetic modulator enhancer of zeste homologue 2 inhibitor (EZH2i), trichostatin A (TSA), and 5-azacytidine (5-AZA) on the osteogenic differentiation of DPSCs.

Results: To assess osteogenic differentiation, we measured alkaline phosphatase activity, calcium deposition, and expression of osteogenic differentiation marker genes (*RUNX2*, *BMP2*, and *ALPL*) after 7 or 21 days of combinatorial drug treatment in normal cell culture medium or osteoinductive medium (OIM). No synergistic effects were observed for any possible combination of EZH2i, TSA, or 5-AZA. However, the effects of these drugs and their combinations depend on the time and culture conditions.

Discussion: We confirmed that EZH2i and TSA have positive effects on the osteogenic differentiation of DPSCs. EZH2i activates the expression of key regulatory genes (*RUNX2*, *BMP2*, and *ALPL*) directly, whereas TSA interacts with signalling pathways induced by supplements in OIM to activate these genes.

1. Introduction

Dental pulp stem cells (DPSCs) isolated from the pulp of extracted adult wisdom teeth are a subtype of mesenchymal stem cells (MSCs) that exhibit multilineage differentiation potential [1–6]. The multipotency and easy and simple collection of DPSCs from pulp tissue make these cells promising candidates for tissue engineering in regenerative medicine [6] and an ideal *in vitro* model system to study stem cell differentiation. Among dental stem cells (DSCs), only DPSCs express all the key factors (OCT4, SOX2, and NANOG) that maintain pluripotency, suggesting that DPSCs have a better potential to differentiate into multiple cell types than other DSCs [7].

Stem cell differentiation is regulated not only at the genetic level via proteins that directly bind to different DNA motifs (e.g. transcription factors), but also via epigenetic mechanisms. These mechanisms involve DNA methylation and histone post-translational modifications, known as epigenetic markers. Components of chromatin remodelling complexes that act as writers, readers, and erasers of epigenetic marks are responsible for chromatin plasticity and dynamics [8–10]. Chromatin remodelling complexes regulate the reversible transition between the less compact, transcriptionally active euchromatin and the tightly packed, transcriptionally repressed heterochromatin, thus determining the pattern of actively transcribed genes in the whole genome [11,12].

In this study, we focused on the osteogenic differentiation of DPSCs and epigenetic mechanisms involved in this process. We and others have shown that DPSCs can differentiate into osteoblast progenitors and mature osteoblasts *in vitro* under culture conditions

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conducive to osteogenic differentiation [5,6,13–20]. Distinct epigenetic mechanisms including histone acetylation, histone methylation, and DNA methylation regulate this process by modulating the expression of osteogenic marker genes [8–10].

Several epigenetic modifying agents have been developed for clinical use in cancer therapy to inhibit cell proliferation by modifying the function of chromatin remodelling complexes [21–23]. These agents also affect stem cell fate [24] and accelerate the osteogenic differentiation and mineralisation of DPSCs [24] opening novel possibilities for the application of these drugs. Given that DPSCs are promising candidates for tissue regeneration in dentistry and general medicine, it would be beneficial to enhance their osteogenic differentiation. Epigenetic-modifying agents can be used for this purpose if DPSCs are differentiated *in vitro* [6] or if drug treatment is combined with biodegradable polymers [25–29] as delivery systems to avoid side effects. In the present study, we examined three molecules: trichostatin A (TSA), enhancer of zeste homologue 2 inhibitor (EZH2i), and 5-azacytidine (5-AZA). TSA, a histone deacetylase inhibitor, increases chromatin acetylation by inhibiting histone deacetylases (HDACs), which are responsible for removing acetyl groups from lysine residues in the N-terminal tail of histone molecules. As histone acetylation is a characteristic feature of the promoter region of transcriptionally active genes, increased chromatin acetylation leads to general gene activation [30]. EZH2i hampers the histone methyltransferase activity of EZH2, the catalytic subunit of the polycomb repressive complex 2 (PRC2), which is responsible for the methylation of the K27 residue in the H3 histone N-terminal tail. Trimethylation of K27 of histone H3 in gene promoters is a repressive epigenetic marker responsible for gene silencing [31]. Thus, similar to TSA, EZH2i treatment leads to gene activation across the entire genome. 5-AZA is a pyrimidine nucleoside analogue that can be incorporated into DNA. Because 5-AZA is incapable of accepting methyl groups from DNA methyltransferases, its incorporation leads to global hypomethylation of the genome and thus decondensation of heterochromatic regions, allowing inactive genes to be expressed [32]. Independent studies have shown that the osteogenic/odontogenic differentiation of DPSCs can be enhanced by these agents, modulating the epigenetic landscape of chromatin and thus activating key regulatory genes [33–37].

Although the effects of TSA, EZH2i, and 5-AZA on the osteogenic/odontogenic differentiation of DPSCs have been previously investigated, the combined effects of these drugs have not yet been studied. Thus, the aim of this study was to compare the effects of the three drugs in combination on the osteogenic differentiation of DPSCs and to reveal synergistic effects by measuring alkaline phosphatase (ALPL) activity, calcium deposition, and the expression of osteogenic differentiation marker genes, namely runt-related transcription factor 2 (*RUNX2*), bone morphogenetic protein 2 (*BMP2*), and *ALPL*.

2. Materials and methods

2.1. Cell culture

DPSCs were isolated from the pulp tissue of healthy human wisdom teeth based on a previously published protocol [38] (DPSCs were isolated from a 24-year-old male; patient declaration of agreement No. F0102/1ST). Isolated DPSCs were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM-F12; 11320033; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % foetal bovine serum (FBS; F9665; Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin, 100 mg/mL streptomycin (P0781; Sigma-Aldrich), and 1 % GlutaMAX (10567014; Life Technologies, Carlsbad, CA, USA) at 37 °C and 5 % CO_2 in a humidified atmosphere. In the figures, this medium is indicated as control medium (CM). Osteo-inductive medium (OIM) was prepared by supplementing CM with 10 mM β-glycerophosphate (G9891; Sigma-Aldrich), 50 μg/mL ascorbic acid (1043003; Sigma-Aldrich), 0.1 μM dexamethasone (D4902; Sigma-Aldrich), and 50 nM vitamin D3 (740292; Sigma-Aldrich). To induce epigenetic changes in the chromatin, CM or OIM was supplemented with 5 μM EZH2i (ab269816; Abcam, Cambridge, UK), 10 μM TSA (ab146598; Abcam), or 1 μM 5-AZA (ab142744; Abcam) alone or in combination.

2.2. Osteogenic differentiation

A total of 30,000 cells was seeded in each well of a 12-well cell culture plate (Z707775; Sigma-Aldrich) in 1 mL of CM. The cells were allowed to attach overnight in CM before treatment with OIM, CM, or media (CM or OIM) supplemented with EZH2i, TSA, 5-AZA, or their combination. In the case of 5-AZA, a 24-h treatment was applied on day 0; in the case of TSA and EZH2i, the treatment was continuous. The medium was changed every 2 days.

2.3. ALPL assay

After osteogenic induction, the cells were washed twice with 1 mL of 1 × phosphate-buffered saline (PBS) and lysed in lysis buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 % Triton X-100, and 1 % protease inhibitor cocktail [PIC]). Scraped lysates were transferred to sterile Eppendorf tubes. After centrifugation (10,000×*g* for 10 min at 4 °C), the supernatant was used to determine ALPL activity and protein concentration (Pierce BCA Protein Assay; 23227; Thermo Fisher Scientific). To determine ALPL activity, 0.1 % *p*-nitrophenyl phosphate (N7653; Sigma-Aldrich; in 0.1 M glycine, 1 mM $MgCl_2$, $ZnCl_2$, pH 10.4) was added to the samples, and the absorbance was measured at 405 nm with a Hidex Sense Microplate reader (Hidex, Turku, Finland). The kinetic measurements were performed every 3 min for 2 h.

2.4. Measurement of calcium deposition

After osteogenic induction, the cells were washed twice with 1 mL of 1 × PBS (P5493; Sigma-Aldrich). The samples were fixed in 1

mL of ice-cold methanol (322415; Sigma-Aldrich) for 30 min at room temperature. Afterwards, the samples were dried for 5 min at room temperature and stained with 2 % (w/v) Alizarin Red S (pH 7; A5533; Sigma-Aldrich). For staining quantification, Alizarin Red S–calcium complexes were extracted with 10 % cetylpyridinium chloride (C0732; Sigma-Aldrich) diluted in 10 mM sodium phosphate buffer adjusted to pH 7 (P5244; Sigma-Aldrich), and the absorbance was measured at 570 nm using a Hidex Sense Microplate reader. For standardisation, the protein concentration of cell lysates was determined with the Pierce BCA Protein Assay (23227; Thermo Fisher Scientific) according to the manufacturer's instructions from parallel samples that were not stained with Alizarin Red S. Calcium deposition was expressed as A570 nm/µg protein.

2.5. Total RNA extraction, reverse transcription, and qPCR

After osteogenic induction, the cells were washed twice with 2 mL of 1 × PBS. Total RNA was extracted using a Quick-RNA Miniprep Kit (R1054; Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (4368814; Thermo Fisher Scientific) was used to reverse transcribe 0.5 µg of RNA per sample into cDNA.

Gene expression levels were determined using TaqMan gene expression assays for *RUNX2* (Hs00231692_m1; Applied Biosystems, Waltham, MA, USA), *BMP2* (Hs00154192_m1; Applied Biosystems), and *ALPL* (Hs01029144; Applied Biosystems), and were normalised to the reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs02758991; Applied Biosystems) in each sample. The 5x HOT FIREPOL Probe qPCR Mix Plus (no ROX; 08-15-00001; Solis BioDyne, Tartu, Estonia) was used for qPCR.

2.6. Statistical analysis

The error bars in the figures represent the standard deviations calculated from three independent measurements. One-way analysis of variance (ANOVA) with Dunnett's post hoc test was used for statistical analysis, where multiple comparisons were performed versus the control. Statistical significance was set at $p < 0.05$. The results of the statistical analyses and the data from which bar charts were generated are available in the supplementary materials.

3. Results

ALPL activity was measured in DPSCs after 7 or 21 days of culture in CM, OIM, CM, or OIM supplemented with different combinations of EZH2i, TSA, or 5-AZA (Fig. 1). Compared to untreated control DPSCs, ALPL activity increased on day 7 in DPSCs treated with EZH2i alone or in combination with TSA or 5-AZA in CM. After day 21 of treatment in CM, ALPL activity remained somewhat, but not significantly, higher in DPSCs treated with EZH2i alone or in combination with TSA, whereas 5-AZA treatment had a negative effect on ALPL activity. Short-(7 days) or long-term (21 days) treatment with TSA alone in CM had no effect on ALPL activity (Fig. 1A). In OIM, EZH2i increased ALPL activity in DPSCs after day 7 of treatment. TSA alone or in combination with other drugs was not effective at this time point, whereas 5-AZA had a negative effect similar to that of treatment with CM. Interestingly, at day 21, only DPSCs cultured in OIM with TSA showed increased ALPL activity; in contrast, EZH2i and 5-AZA decreased ALPL activity (Fig. 1B). No synergistic effects were observed for any drug combination in CM or OIM.

Alizarin Red S staining showed mineralisation of drug-treated DPSCs in OIM, but not in CM (Fig. 2). Calcium deposition is not expected in cells cultured in CM [13] because this medium does not contain inorganic phosphate. Surprisingly, mineralisation did not increase with any drug or combined drug treatment compared to OIM alone at days 7 and 21. In the presence of EZH2i, there was a remarkable decrease in mineralisation at day 21 when EZH2i was added alone or in combination with other drugs, in line with the reduction in ALPL activity.

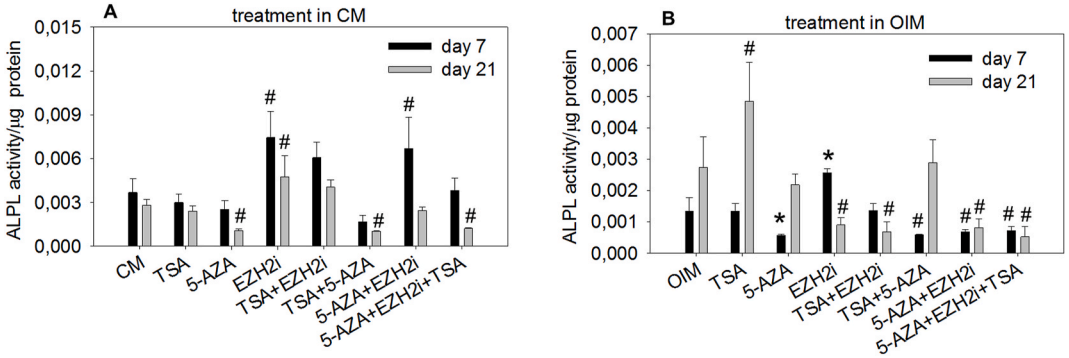

Fig. 1. Measurement of ALPL activity (A) ALPL activity of DPSCs cultured in CM supplemented with TSA, 5-AZA, or EZH2i alone or in any possible combination, measured after days 7 and 21. (B) ALPL activity of DPSCs cultured in OIM supplemented with TSA, 5-AZA, or EZH2i alone or in any possible combination, measured after days 7 and 21. Height of bars represents mean, and error bars represent standard deviation of three independent experiments. One way ANOVA with Dunnett's post hoc test was used for statistical analysis ($\alpha = 0.05$; #$p \leq 0.036$; *$p \leq 0.001$), where drug treated samples were compared to untreated control.

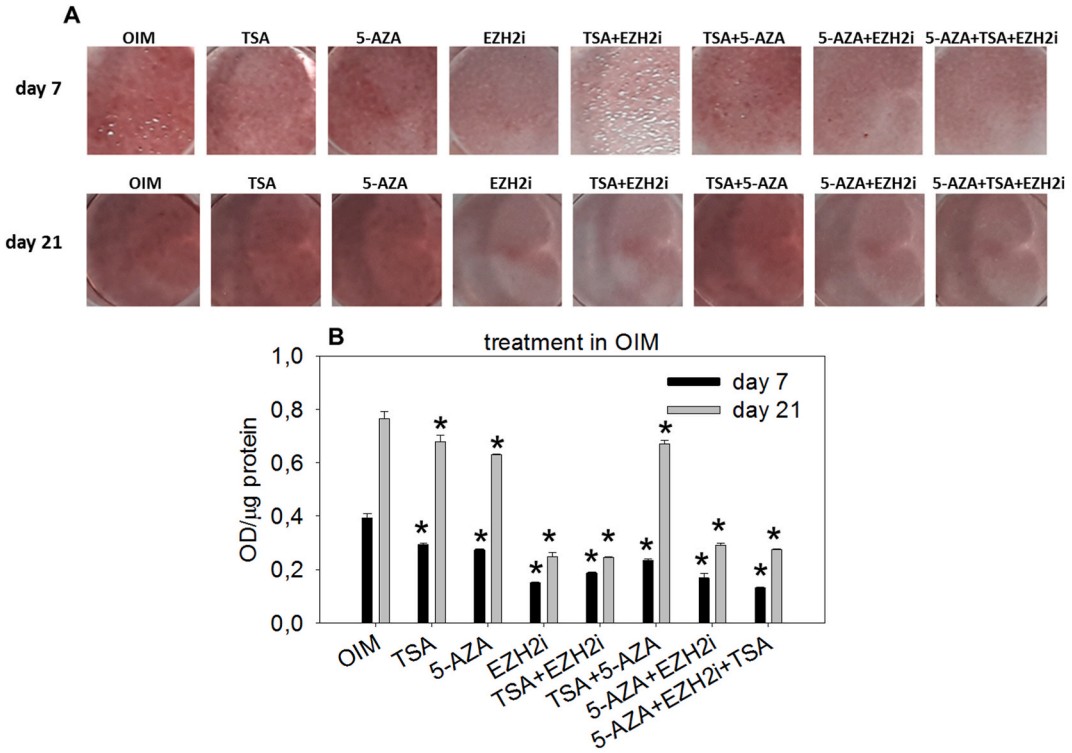

Fig. 2. Measurement of mineralisation (A) Visualisation of calcium deposits of DPSCs cultured in OIM supplemented with TSA, 5-AZA, or EZH2i alone or in any possible combination, stained with Alizarin Red S, and measured after days 7 or 21. (B) Quantitative analysis of calcium-bound dye based on spectrophotometry. Height of bars represents mean, and error bars represent standard deviation of three independent experiments. One way ANOVA with Dunnett's post hoc test was used for statistical analysis ($\alpha = 0.05$; *$p < 0.001$), where drug treated samples were compared to untreated control.

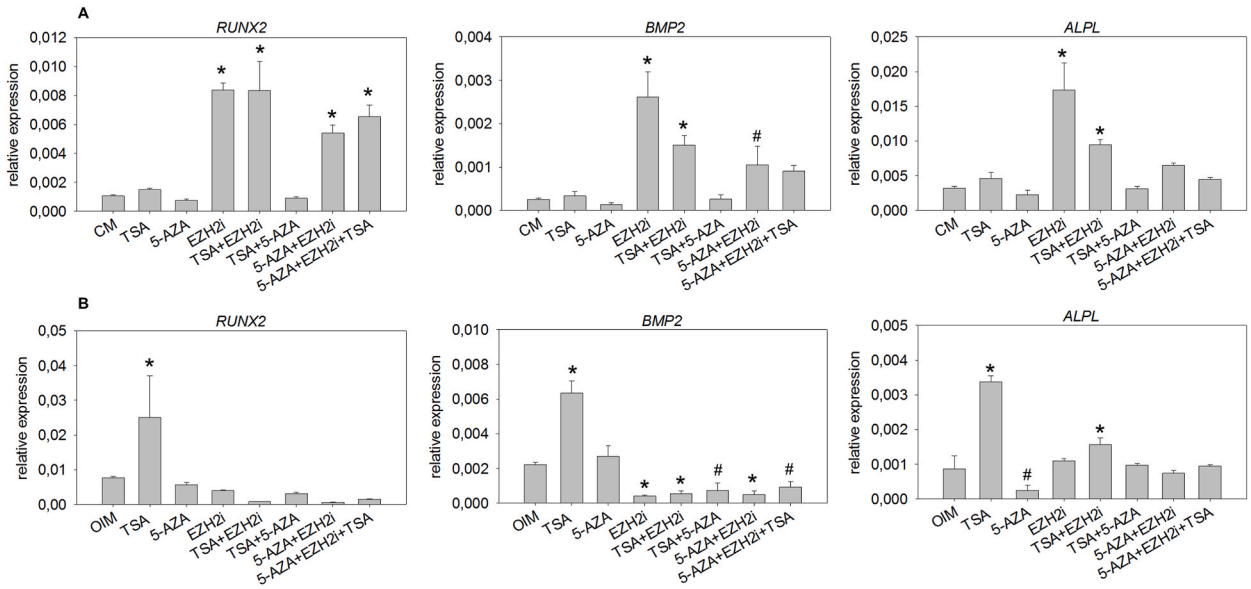

Fig. 3. qPCR analysis of osteogenic marker genes (A) *RUNX2*, *BMP2*, and *ALPL* expression in DPSCs cultured in CM supplemented with TSA, 5-AZA, or EZH2i alone or in any possible combination, measured after day 21. (B) *RUNX2*, *BMP2*, and *ALPL* expression in DPSCs cultured in OIM supplemented with TSA, 5-AZA, or EZH2i alone or in any possible combination, measured after day 21. Height of bars represents mean, and error bars represent standard deviation of three independent experiments. One way ANOVA with Dunnett's post hoc test was used for statistical analysis ($\alpha = 0.05$; #$p \leq 0.015$; *$p \leq 0.001$), where drug treated samples were compared to untreated control.

Finally, we measured the expression of the *RUNX2*, *BMP2*, and *ALPL* osteogenic marker genes. After day 21, treatment with EZH2i alone or in combination with other drugs in CM had a positive effect on the expression of these genes (Fig. 3A). However, in OIMs, only TSA alone elevated these expression levels (Fig. 3B). EZH2i, alone or in combination with OIM, inhibited *RUNX2* and *BMP2* expression (Fig. 3B). These results are consistent and correlated with ALPL activity measurements. No synergistic effects were observed in any of the tested drug combinations.

4. Discussion

Based on our data, TSA and EZH2i activated *RUNX2*, *BMP2*, and *ALPL* (Fig. 3), which are involved in the early stage of osteogenic differentiation, in agreement with published data [39,40]. Interestingly, EZH2i only affected these genes in DPSCs cultured in CM when the osteogenic differentiation signalling pathways were not activated by the components of OIM. This suggests that EZH2i can directly induce *RUNX2*, *BMP2*, and *ALPL* expression, which is in line with the chromatin immunoprecipitation sequencing (ChIP-seq) experiments demonstrating that these genes are specific targets of EZH2 [41]. The OIM constituents β-glycerophosphate, ascorbic acid, vitamin D3, and dexamethasone interfere with the action of EZH2i. Among these agents, dexamethasone [42] and vitamin D3 [43,44] can activate the Wnt/β-catenin signalling pathway, which is involved in the regulation of osteogenic differentiation, either enhancing [42–44] or inhibiting this process. WNT1 inhibits the odontogenic differentiation of DPSCs [45], and WNT1 and WNT3A inhibit the osteogenic differentiation of human MSCs [46–48]. Given that *WNT1* and *WNT3A* are EZH2 targets [41], EZH2i treatment may release the repressed state of these genes, leading to decreased ALPL activity and lack of mineralisation (Figs. 1 and 2), similar to WNT1 treatment [45]. The BMP antagonists noggin [49] and chordin [50] are also regulated by EZH2-dependent H3K27 methylation [41, 51]. Noggin and chordin can bind to BMPs that are expressed and secreted as autocrine signals during osteogenic differentiation, inhibiting their binding to BMP receptors and activating BMP-induced signalling pathways. Among the EZH2 targets [41], HDACs also inhibit osteogenic differentiation by deacetylating histones at the promoter region of the key regulator genes *RUNX2*, *BMP2*, and *ALPL* [41], which encode signalling pathway-related proteins [41], or by directly binding to RUNX2 [52].

In contrast to EZH2i, TSA was effective in OIM culture conditions permissive for osteogenic differentiation, but not in CM, suggesting that TSA has no direct effect on *RUNX2*, *BMP2*, and *ALPL* expression, although these genes are specific targets of HDAC1, HDAC2, and HDAC6 [41]. Rather, TSA acts on these genes through other genes activated by or involved in signalling pathways induced by OIM supplements, such as WNT, BMP, BMP receptor, SMAD, and MAPK, which are also HDAC targets [41]. Treatment with dexamethasone leads to the expression of *RUNX2* and its co-activator, the transcriptional co-activator with PDZ-binding motif (*TAZ*) [42], another target of HDACs [41]. Dexamethasone treatment also induces the expression of mitogen-activated protein kinase phosphatase 1 (*MKP1*) and collagen type 1 (*COL1*) [42], an HDAC target [41] that encodes an extracellular matrix protein important for mineralisation. MKP1 dephosphorylates RUNX2, which is required for TAZ binding [42]. Ascorbic acid plays a role in COL1 secretion, and integrins can activate the MAPK signalling pathway, leading to the accumulation of the phosphorylated form of extracellular signal-regulated kinase (pERK) in the nucleus. Together with RUNX2, pERK binds to osteocalcin (*OCN*) and bone sialoprotein (*BSP*), which are responsible for mineralisation in the late stage of osteogenic differentiation [42]. B-Glycerophosphate serves as a phosphate source needed for hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) production during mineralisation. Furthermore, phosphate can enter cells where it regulates the expression of osteopontin (*OPN*) and *BMP2*, acting as an intracellular signalling molecule [42].

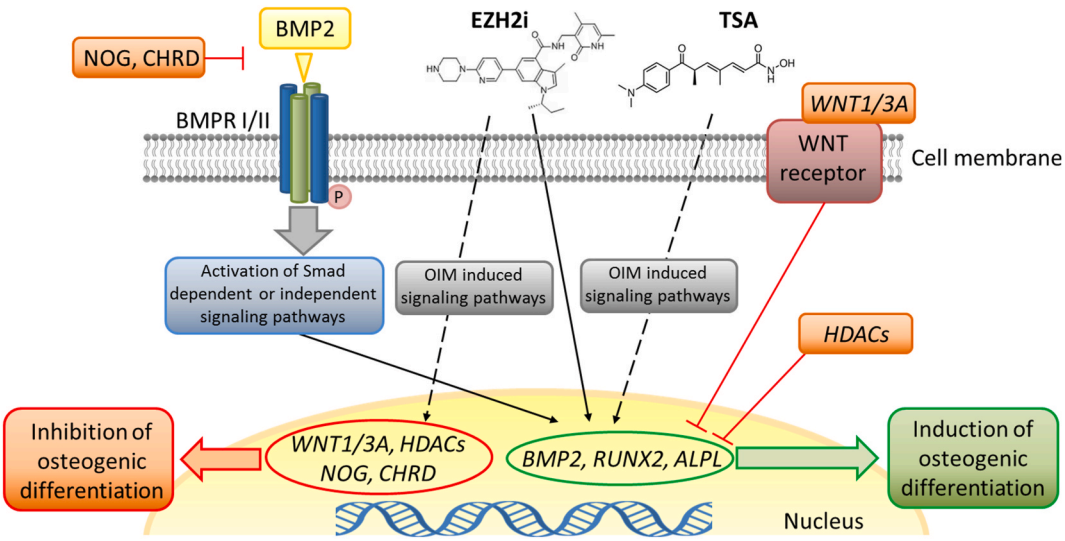

Fig. 4. Possible mechanisms of action of EZH2i and TSA on osteogenic differentiation of DPSCs EZH2i and TSA act via distinct mechanisms involving direct (arrow with a solid line) or indirect effects (arrow with a dashed line) on expression of key regulatory genes *RUNX2*, *BMP2*, and *ALPL*. EZH2i may also activate genes that counteract osteogenic differentiation by inhibiting BMP signalling (*NOG* and *CHRD*), reducing *ALPL* expression (*WNT1* and *WNT3A*), or deacetylating key regulatory and signalling pathway genes (HDACs).

Because mineralisation was not affected, TSA had no effect on genes responsible for mineralisation activated in the late stage of differentiation, a finding consistent with the ChIP-seq data showing that the *OCN*, *OPN*, and *BSP* genes are not specific targets of HDACs [41].

5-AZA had no positive effect on DPSC differentiation when administered alone or in combination with other drugs; these results differ from published data [37]. This contradiction could be the result of the suboptimal dosage of 5-AZA, or that this drug may be incorporated into DNA as well as RNA, in contrast to the 5-aza-2-deoxycytidine used by Zhang et al. [37]. The heterogeneity of DPSCs isolated from the same [2] or different [53] donors may have also led to inconsistent conclusions from independent studies. Kobayashi et al. [2] showed striking variability in the proliferation, cell surface marker expression patterns, and odontogenic, adipogenic, and chondrogenic differentiation potentials of single cell-derived clones of DPSCs obtained from the same donor. Macrin et al. [53] showed that the osteogenic potential of slow and rapid aging DPSCs vary depending on the upregulation of the TGF-β pathway and cytoskeletal proteins as well as on the metabolic state of the cells, which can differ among DPSCs originating from different donors. The effects of epigenetic modulators may also differ in DPSCs isolated from different donors, which is a limitation of this study because DPSCs were isolated from only one patient.

5. Conclusions

There were no synergistic effects when TSA, EZH2i, and 5-AZA drug combinations were applied, and only TSA had positive effects on the expression of early osteogenic marker genes and alkaline phosphatase activity, but had no effect on mineralisation under culture conditions permissive for osteogenic differentiation. Based on the present study, we hypothesise that these drugs act via distinct mechanisms involving a direct or indirect effect by EZH2i or TSA, respectively, on the expression of the key regulatory genes *RUNX2*, *BMP2*, and *ALPL*, and thus on osteogenic differentiation (Fig. 4) which requires further investigation. Because EZH2i showed inhibitory effects, TSA would be a better choice for medical applications aimed at bone regeneration; however, the effects of epigenetic modulators may vary among DPSCs originating from different patients. Thus, comparative studies with an increased number of donors are needed.

Data availability statement

No data has been deposited into a publicly available repository.

CRediT authorship contribution statement

Edit Hrubi: Writing – original draft, Visualization, Investigation. **László Imre:** Writing – review & editing. **Csaba Hegedüs:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:mmcdoino

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