



Zirconium phosphate nanoplatelets promote osteogenic differentiation via MAPK/AKT pathway activation in MC3T3-E1 cells

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Abstract

Zirconium-based materials have attracted considerable attention in the field of bone tissue regeneration owing to their exceptional mechanical properties and bioactivity. Zirconium phosphate (ZrP), particularly when applied as coatings or self-assembled nanonetworks on implants, enhance osseointegration and bone formation. However, the exact mechanisms by which ZrP nanoplatelets (NPs) promote osteogenesis remain unclear. This study aimed to investigate how ZrP NPs drive osteogenic differentiation in MC3T3-E1 pre-osteoblasts by analyzing both osteogenic marker expression and intracellular signaling pathways. The synthesized ZrP NPs (<30 nm in size) demonstrated an excellent biocompatibility and induced dose-dependent increases in calcium deposition and in the alkaline phosphatase (ALP) activity. Gene expression analysis revealed the time-dependent upregulation of early-stage markers, including runt-related transcription factor 2 (RUNX2) and ALP, as well as late-stage markers such as osteocalcin (OCN) and osteopontin (OPN). Western blot analysis further showed that the ZrP NPs activated critical signaling pathways, including the p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1/2 (ERK1/2), and protein kinase B (AKT) pathways. This activation led to the elevated expression of bone morphogenic protein-2 (BMP-2), RUNX2, and OPN. These findings demonstrate that ZrP NPs promote osteogenic differentiation in MC3T3-E1 cells primarily through the activation of the MAPK and AKT signaling pathways. These ZrP NPs can therefore be considered promising candidates for use in bone tissue engineering, particularly in the context of targeted bone defect repair and regeneration.

Keywords Zirconium phosphate · MC3T3-E1 cells · Osteogenic differentiation · Molecular mechanism · MAPK/AKT pathway

1 Introduction

The repair of bone defects caused by trauma, degenerative diseases, or tumor resection remains a major clinical challenge in the fields of regenerative medicine and bone tissue engineering [1, 2]. To overcome this challenge, research on the development of bioactive scaffold materials that not only

bridge bone defects, but also actively stimulate osteogenesis by interacting with osteoprogenitor cells and modulating signaling pathways is underway [3]. Although zirconia (ZrO₂; zirconium dioxide) ceramics are widely employed in dental and orthopedic applications owing to their exceptional mechanical strengths and biocompatible nature [4, 5], their clinical utility is constrained by an inherent bioinertness and poor osseointegration [6]. This has motivated research into alternative biomaterials that integrate mechanical stability with bioactive and osteoinductive properties.

More specifically, to address the limitations of traditional biomaterials, recent research has focused on developing nanostructured and composite materials that closely mimic the mechanical properties of bone, while actively promoting osteogenesis and bone integration [7]. In particular, zirconium phosphate (ZrP) presents a promising strategy by offering both structural support and bioactive effects. For example, biomimetic ZrP nanonetworks self-assembled on

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yttria-stabilized zirconia (YSZ) implants have demonstrated pro-osteogenic and anti-inflammatory properties, thereby enhancing cell adhesion, proliferation, and differentiation [8]. These nanonetworks also upregulate the expression of key osteogenic markers *in vitro*, including alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), and osteocalcin (OCN). Furthermore, scaffolds composed of biphasic calcium phosphate (BCP) doped with ZrO₂ nanoparticles have demonstrated synergistic effects, resulting in more effective bone formation, maturation, and osseointegration *in vivo* compared with undoped BCP scaffolds [9]. Similarly, hydroxyapatite (HAp) matrices containing ZrO₂ have been found to promote the osteogenic differentiation of MG-63 cells through the activation of signaling pathways, such as the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase (MAPK) pathways, thereby influencing the expression of osteogenic genes [10]. At the molecular level, zirconium ions (Zr⁴⁺) have been shown to activate the bone morphogenetic protein (BMP)/SMAD signaling pathway, which plays a central role in osteoblast differentiation [11]. Soluble Zr⁴⁺ ions upregulate BMP-2 expression and promote SMAD1/5 phosphorylation, thereby enhancing the transcription of osteogenic markers such as ALP, RUNX2, and collagen type I alpha 1 chain (COL1A1), in primary human osteoblasts.

Although previous studies have examined the osteogenic potential of various zirconium-based materials, most have primarily focused on conventional zirconium compounds or composites, emphasizing passive effects such as ion release, surface topography, or reliance on exogenous osteogenic factors. As a result, the specific cellular and molecular mechanisms by which ZrP nanoplatelets (NPs) enhance osteogenic differentiation in pre-osteoblastic cells remain poorly understood. In this study, we uniquely demonstrate that ZrP NPs exhibit intrinsic osteoinductive properties. Notably, we show for the first time that ZrP NPs directly activate key intracellular signaling pathways, specifically, MAPK and AKT, and significantly upregulate endogenous BMP-2 expression in pre-osteoblastic cells, independently of external growth factors. Furthermore, we provide comprehensive mechanistic evidence, including analysis of gene and protein expression, revealing activation of both early and late markers of osteogenic differentiation.

2 Materials and methods

2.1 Synthesis of ZrP NPs

ZrP NPs were synthesized via a modified reflux method [12]. More specifically, 0.05 M zirconyl chloride octahydrate (ZrOCl₂·8H₂O, Sigma-Aldrich) was dissolved in

deionized water (DW) and transferred to a 500 mL two-neck round flask equipped with a reflux condenser. The resulting solution was heated to 100 °C under continuous stirring, and subsequently, phosphoric acid solutions (H₃PO₄, Sigma-Aldrich) of varying concentrations (1, 6, and 9 M) were added dropwise (1 drop every 4 s) to give a 1:1 volume ratio of H₃PO₄ to the zirconyl chloride solution. The obtained mixture was subjected to reflux at 100 °C for 24 h, after which the ZrP NPs were purified through three centrifugation cycles (3,000 rpm, 10 min) using DW, and lyophilized for 48 h to yield a dry powder.

2.2 Characterization of the ZrP NPs

The morphology of ZrP NPs was characterized using field-emission scanning electron microscopy (FE-SEM, Sigma 300; Carl Zeiss) at 20 kV, in addition to field-emission transmission electron microscopy (FE-TEM, JEM-F200; JEOL) at 200 kV. Elemental composition analysis was performed using energy-dispersive X-ray spectroscopy (EDS) to confirm the presence of Zr, P, and O. The crystallographic properties of the NPs were analyzed using X-ray diffractometry (XRD; Bruker D8 Advance, Cu-Kα radiation, λ = 1.5418 Å, 40 kV, 40 mA). Fourier transform infrared (FT-IR) spectroscopy (Shimadzu 8400 S; Shimadzu) was performed in the 4,000–400 cm⁻¹ range to confirm successful synthesis of the NPs. Dynamic light scattering (DLS, SZ-100; HORIBA) was used to determine the hydrodynamic mean size, Z-average size, and polydispersity index (PDI). The stability of ZrP (1 M) NPs was evaluated by monitoring particle size using a particle size analyzer (HORIBA). ZrP (1 M) NPs (1 mg) were dispersed in 3 mL of DW or Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Welgene Inc.). To ensure homogeneous dispersion, the samples were sonicated for 3 min using a probe-type sonicator. At predetermined intervals (0, 1, 2, 3, 5, and 7 days), the particle size distribution of ZrP (1 M) NPs was measured.

For the *in vitro* degradation studies, the ZrP (1 M) NPs (1 mg, prepared using the 1 M H₃PO₄ solution) were dispersed in 0.1 M MES buffer (1 mL, pH 5.0 or 7.4; 2-(N-morpholino)ethanesulfonic acid buffer) and placed in a dialysis membrane bag (molecular weight cut-off: 1 kDa, Spectrum Laboratories). Each bag was then immersed in the corresponding MES buffer (10 mL) and gently shaken (100 oscillations/min) at 37 °C. At predetermined intervals, buffer samples were collected and replaced with fresh buffer. The released Zr⁴⁺ and phosphate ions were quantified via inductively coupled plasma-optical emission spectrometry (ICP-OES, Optima 5300 DV; PerkinElmer).

2.3 In vitro cytotoxicity assessments of the ZrP NPs

The in vitro cytotoxicity of the prepared ZrP NPs was assessed using pre-osteoblastic MC3T3-E1 cells (Korean Cell Line Bank). The cells were maintained in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Wetgene Inc.) and 1% penicillin–streptomycin (Gibco). To perform the cytotoxicity tests, cells were seeded into 96-well plates at a density of 1×10^4 cells/well, then exposed to the ZrP NPs at concentrations of 10, 50, and 100 $\mu\text{g/mL}$ for 1 and 3 days. After treatment, the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4) to remove any residual NPs, and the cell viability was quantified using the CCK-8 assay kit (Dojindo Molecular Technologies Inc.). For this purpose, the CCK-8 reagent was added to each well, followed by incubation at 37 °C for 1 h in the dark. After this time, the absorbance was measured at 450 nm using a VarioskanFlash Multimode plate reader (Thermo Scientific). All experiments were performed in quadruplicate ($n=4$).

2.4 ALP activity, calcium content, and intracellular calcium deposition imaging

To evaluate the osteogenic potential of the ZrP NPs, MC3T3-E1 cells were seeded in 24-well plates at a density of 1×10^5 cells/well, and were treated with ZrP NPs (10, 50, and 100 $\mu\text{g/mL}$). The ALP activity, which represents a key marker of early osteogenic differentiation, was assessed at days 3 and 9 post-treatment. The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, and the lysates were subjected to centrifugation (13,500 rpm, 10 min, 4 °C). The supernatants were then incubated with p-nitrophenyl phosphate (Sigma-Aldrich), a colorimetric ALP substrate, at 37 °C for 25 min. Each reaction was terminated by the addition of 3 N NaOH, and the ALP activity was quantified by measuring absorbance at 405 nm using a VarioskanFlash Multimode Reader (Thermo Scientific). Data were normalized to the total cellular protein content. The experiments presented here were performed in quadruplicate ($n=4$).

The degree of calcium deposition, which is a hallmark of late-stage osteogenic differentiation, was analyzed by treating the MC3T3-E1 cells (1×10^5 cells/well) with the prepared ZrP NPs (10–100 $\mu\text{g/mL}$) for 7, 14, and 21 days. After subsequent washing with PBS, the calcium deposits were solubilized by incubating the cell layers in a 0.5 M HCl solution at 37 °C for 24 h. The calcium content was quantified using the QuantiChrom™ Calcium Assay Kit (BioAssay Systems) according to the manufacturer's instructions. The absorbance at 612 nm was measured using a VarioskanFlash Multimode Reader (Thermo Scientific), and the calcium concentrations were calculated against a CaCl_2

standard curve. The experiments presented here were performed in quadruplicate ($n=4$).

To visualize the deposition of calcium, MC3T3-E1 cells (5×10^3 cells/well) were cultured on 4-well chamber slides and treated with the ZrP NPs (10–100 $\mu\text{g/mL}$) for 21 days. The cells were then incubated for 1 h with a 10 μM alendronate-PEG-cyanine 5.5 solution (ALN-PEG-Cy5.5), which is a calcium-specific probe developed by our group [13]. After washing with PBS (pH 7.4) and fixation with 4% paraformaldehyde at 4 °C for 20 min, the calcium deposits were imaged using confocal laser scanning microscopy (LSM 700; Carl Zeiss). The fluorescence intensity was quantified using ImageJ software (version 1.47; National Institutes of Health) by dividing the total intensity of Cy5.5 by the number of cells per field ($n=3$ independent replicates).

2.5 Evaluation of osteogenesis-related gene and protein markers

To evaluate the osteogenic potential of the prepared ZrP NPs, mRNA expression levels of key markers, including *RUNX2*, *ALP*, *OCN*, and osteopontin (*OPN*), were analyzed using the quantitative real-time polymerase chain reaction (qRT-PCR). MC3T3-E1 cells (1×10^5 cells/well) were seeded into 24-well plates and treated with the ZrP NPs (10, 50, and 100 $\mu\text{g/mL}$) for 7, 14, and 21 days. The total RNA was isolated using TRIzol® reagent (Thermo Scientific) following chloroform–isopropanol extraction. This isolated RNA (2 μg) was then reverse-transcribed into cDNA using an AccuPower RT PreMix system (Bioneer), and qRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 system (Applied Biosystems). Gene expression levels were normalized to those of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and the primer sequences are listed in Table S1.

To further confirm successful osteogenic differentiation, the secreted protein levels of OCN and OPN were quantified. Cell culture supernatants from MC3T3-E1 cells treated with ZrP NPs (10–100 $\mu\text{g/mL}$) for 7, 14, and 28 days were collected. The OCN concentrations were measured using a Human Osteocalcin ELISA Kit (Elabscience Biotechnology Co., Ltd.), while the OPN levels were determined using an Osteopontin Human ELISA Kit (Invitrogen), according to the manufacturer's instructions. The absorbance values were normalized to the untreated control groups. All experiments were performed in triplicate ($n=3$).

2.6 Western blotting analysis

To assess the activation of various signaling pathway, MC3T3-E1 cells (1×10^6 cells/well) were seeded in 100 mm culture dishes, allowed to adhere overnight, then

2.7 Statistical analysis

All data are presented as the mean $\pm$ standard deviation. Statistical analyses were performed using one-way ANOVA using Systat software (Systat Software Inc.), and differences were considered statistically significant at $p < 0.05$ or $p < 0.01$.

3 Results and discussion

3.1 Characterization of the ZrP NPs

The ZrP NPs were synthesized via a modified reflux method by reacting $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$ with varying concentrations of H_3PO_4 (1, 6, and 9 M), consistent with established protocols for α -ZrP synthesis [12, 14]. Structural characterization by TEM and SEM revealed distinct morphologies that were dependent on the H_3PO_4 concentration (Fig. 1A). More specifically, the NPs synthesized using the 1 M H_3PO_4 solution (denoted ZrP (1 M)) exhibited NP-like structures with diameters < 30 nm, whereas those prepared using the 6 and 9 M H_3PO_4 solutions formed larger hexagonal NPs (150–450 nm), aligning with the α -ZrP crystallographic phase [15, 16]. The size distribution of ZrP NPs synthesized using 1, 6, and 9 M H_3PO_4 solutions was examined by DLS. The Z-average diameters of the ZrP NPs were 154.8 nm, 519.2 nm, and 310.2 nm, respectively, with corresponding PDI values of 0.295, 0.306, and 0.214. DLS confirmed this size trend, showing increased hydrodynamic diameters with higher H_3PO_4 concentrations (Fig. S1) [14, 17]. The storage

A ZrP (1 M) ZrP (6 M) ZrP (9 M)

TEM

SEM

B

Elements	Atomic (%)
O	81.3
P	10.8
Zr	7.88

C

Intensity (cps)

(002) (110) (112) (020)

(002) (110) (112) (020)

(002) (110) (112) (020)

(002) (110) (112) (204) (020) (312)

2θ (degree)

D

Transmittance (%)

Wavenumber (cm^{-1})

3590 3510 3165 1620 1250 970

stability of ZrP (1 M) NPs was evaluated in both DW and DMEM containing 10% FBS. The Z-average diameter of ZrP (1 M) NPs in DW ranged from 188.9 to 290.5 nm, while in 10% FBS-containing medium, it ranged from 160 to 267.3 nm over 7 days (Fig. S2). These results demonstrate that the size stability of ZrP (1 M) NPs was maintained in both DW and serum-containing medium. EDS of the ZrP (1 M) NPs detected three primary elements, namely Zr (Zr K α : 2.1 keV), P (P K α : 2.01 keV), and O (O K α : 0.52 keV), thereby validating the stoichiometric formula of Zr(HPO₄)₂·2H₂O (Fig. 1B) [18].

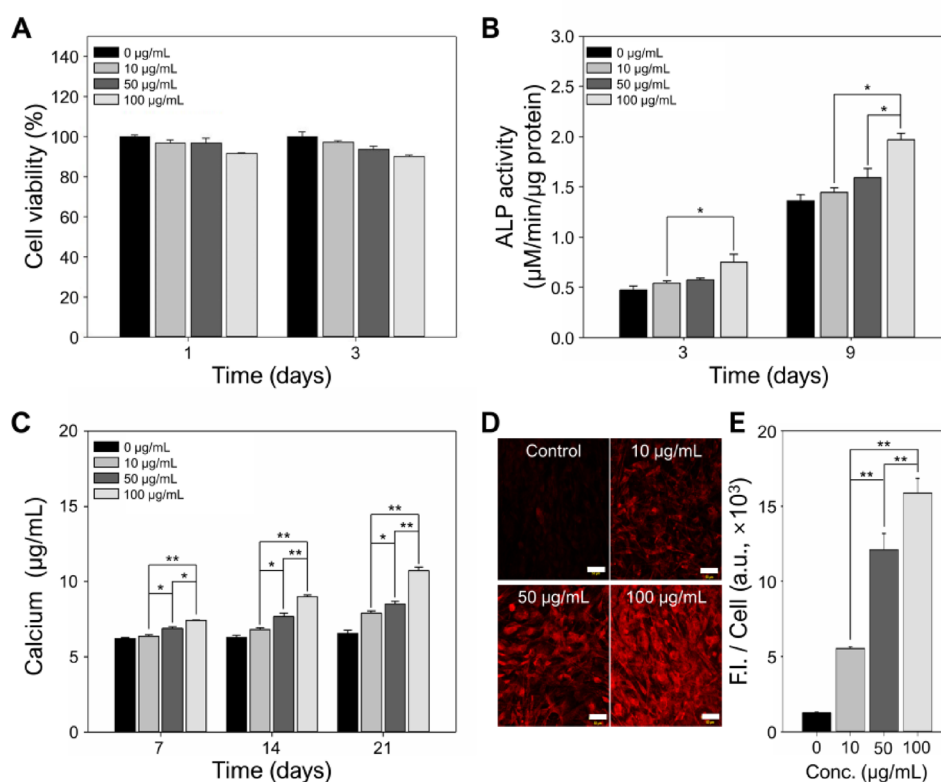
XRD analysis of the synthesized ZrP NPs revealed three characteristic α -phase peaks at $2\theta=11.6^\circ$ (002), 19.7° (110), and 24.9° (112) (Fig. 1C), consistent with the hexagonal α -Zr(HPO₄)₂·2H₂O structure (ICSD-1281) [18–21]. Compared to the ZrP (6 M) and ZrP (9 M) samples, the ZrP (1 M) NPs exhibited broader and less intense peaks, indicating a reduced crystallinity associated with their smaller sizes. FT-IR spectroscopy further confirmed the structural integrity of the ZrP (1 M) NPs, with the peaks at 3,590 and 3,510 cm⁻¹ corresponding to the asymmetric stretching vibrations of the interlayer water molecules. Furthermore, the observation of additional bands at 3,165 cm⁻¹ (symmetric -OH stretching) and 1,620 cm⁻¹ (-OH bending) confirmed the presence of hydroxyl groups within the layered structure [20]. Phosphate-related vibrations were also observed, namely at 970 cm⁻¹ (out-of-plane P–OH deformation), 1,250 cm⁻¹ (in-plane P–OH bending), and at 1,047

and 1,121 cm⁻¹ (symmetric and asymmetric P–O stretching in the PO₄ groups, respectively) [20, 22]. Collectively, these results validate the successful synthesis of α -ZrP NPs with characteristic crystallographic and chemical features.

3.2 In vitro cytotoxicity of the ZrP NPs

Previous research has highlighted the nanoparticle size as a critical determinant of cellular uptake, with smaller nanoparticles demonstrating enhanced internalization due to their higher surface area-to-volume ratio compared to those of larger counterparts [23, 24]. Additionally, nanoparticles with lower crystallinities exhibit faster degradation rates, which ultimately modulate the biological interactions and ion release kinetics [25, 26]. Based on these principles, the smaller and less crystalline ZrP (1 M) NPs were selected for subsequent cellular studies. In vitro cytotoxicity assays performed using MC3T3-E1 pre-osteoblasts revealed cell viabilities of >90% across all tested concentrations (10–100 μ g/mL; Fig. 2A), consistent with the biocompatibility profile of zirconium-based nanomaterials [27]. These results confirm the non-cytotoxic nature and high cytocompatibility of the ZrP (1 M) NPs with MC3T3-E1 cells.

Fig. 2 In vitro osteogenic differentiation effects of the ZrP (1 M) NPs. **(A)** In vitro cytotoxicity effects of the ZrP (1 M) NPs on the MC3T3-E1 cells ($n=4$ /group). **(B)** ALP activity and **(C)** calcium deposition in the MC3T3-E1 cells treated with the ZrP (1 M) NPs ($n=4$ /group). * $p<0.05$ and ** $p<0.01$. **(D)** Fluorescence images and **(E)** quantitative fluorescence intensities for evaluation of the intracellular calcium levels in MC3T3-E1 cells after treatment with the ZrP (1 M) NPs for 21 days ($n=3$ /group). ** $p<0.01$. ZrP: zirconium phosphate, NPs: nanoplatelets, ALP: alkaline phosphatase



3.3 ALP activity, calcium contents, and intracellular calcium deposition imaging

In vitro release studies demonstrated that the ZrP (1 M) NPs released significantly higher amounts of Zr^{4+} ions and phosphate ions at pH 5.0 (mimicking endosomal conditions) than at physiological pH 7.4 (Fig. S3). To evaluate osteogenic potential of these NPs, the ALP activity, which is a hallmark of early osteoblast differentiation [28], was measured in MC3T3-E1 cells treated with the prepared NPs. As shown in Fig. 2B, the ALP activity increased in a time- and concentration-dependent manner, with notably elevated levels being observed at 100 $\mu\text{g/mL}$ after 3 and 9 days of treatment. At lower concentrations (10–50 $\mu\text{g/mL}$), the ALP activity remained comparable to those of the untreated controls, suggesting insufficient osteogenic stimulation. The pronounced ALP enhancement at 100 $\mu\text{g/mL}$ aligns with prior findings that Zr^{4+} ions and phosphate ions significantly activate the osteogenic signaling pathways (e.g., BMP/SMAD) in addition to supplying mineralization substrates [11, 29], and boosting ALP expression. These results indicate that at an optimal concentration, the ZrP NPs promote early osteogenesis through the synergistic roles of Zr^{4+} and phosphate ions.

The degree of calcium deposition in the extracellular matrix, which is a hallmark of late-stage osteogenic differentiation [30], was quantitatively assessed in the MC3T3-E1 cells treated with the ZrP NPs for 7, 14, and 21 days. All treatment groups exhibited a progressive increase in mineralized matrix formation, with the highest extent of calcium deposition being observed at 100 $\mu\text{g/mL}$ (Fig. 2C). This finding is consistent with previous reports that Zr^{4+} ions facilitate extracellular matrix remodeling [11], while phosphate ions directly contribute to HAp crystal formation, as demonstrated in zirconia-hybridized, pyrophosphate-stabilized amorphous calcium phosphate systems [29]. Additionally, intracellular calcium accumulation was visualized using ALN-PEG-Cy5.5, a calcium-specific fluorescent probe [13], wherein the fluorescence intensity increased proportionally to the ZrP concentration (Fig. 2D and E), further supporting the quantitative calcium deposition results. Collectively, these results demonstrate that ZrP NPs enhance osteoblast differentiation and matrix mineralization in MC3T3-E1 cells through the release of Zr^{4+} and phosphate ions.

3.4 Effect of ZrP on the osteogenic differentiation markers in MC3T3-E1 cells

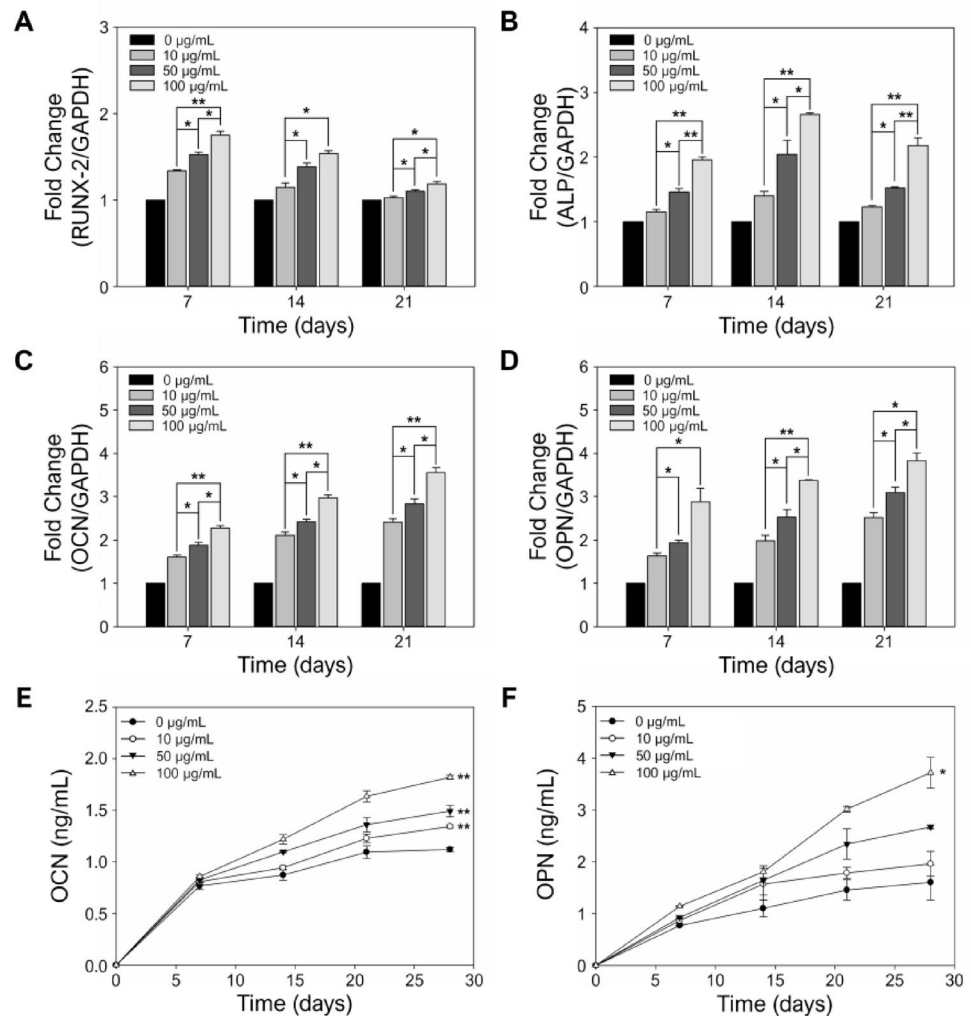
The osteogenic differentiation potential of ZrP NPs was subsequently assessed at the mRNA level by quantifying the expression of key osteogenic markers, including

RUNX2, *ALP*, *OCN*, and *OPN* [31, 32]. Early regulators of *RUNX2* and *ALP* exhibited peak expression at 7 and 14 days, respectively (Fig. 3A and B), followed by a decline at 21 days as the cellular activity shifted from osteoblast commitment to matrix mineralization. In contrast, the late-stage markers *OCN* and *OPN*, which are essential for HAp nucleation and collagen fibril organization, showed progressive increases in their expression levels, reaching maximal expression at 21 days (Fig. 3C and D). This biphasic gene expression pattern is consistent with previous findings in ZrP nanonetworks, wherein the early activation of *RUNX2* precedes matrix mineralization [8]. Furthermore, the dose-dependent upregulation of all markers, particularly at a ZrP concentration of 100 $\mu\text{g/mL}$, supports the notion that the material concentration can directly influence the osteogenic transcription factor activity, as observed in previous studies based on nano-grained tantalum surfaces [33].

ELISA findings confirmed that *OPN* exhibited the most pronounced upregulation at higher ZrP concentrations (Fig. 3E and F), consistent with its role in bridging collagen fibrils and HAp during mineral nucleation. This phenomenon is also observed in MXene-based scaffolds, where ion release enhances late-stage osteogenesis via $\text{Ca}^{2+}/\text{PO}_4^{3-}$ -mediated protein secretion [34]. Mechanistically, Zr^{4+} ions promote early osteogenic commitment by activating the BMP2/SMAD pathway, thereby upregulating the expression of *RUNX2* and *ALP* [11], while the phosphate ions released during endosomal degradation (pH 5.0) may elevate the inorganic phosphate (Pi) levels, activating Pi transporters (SLC20a1) to drive *OCN/OPN* expression and HAp deposition [35]. This synergy between Zr-mediated signaling and phosphate-driven mineralization is similar to the osteoinductive effects of strontium-substituted HAp, and it is possible that the combined action of these two ions may amplify osteogenesis through complementary mechanisms [36].

Compared to calcium phosphate ceramics, which primarily function through passive osteoconduction and ion release, ZrP NPs exhibit intrinsic osteoinductive properties through direct MAPK/AKT pathway activation and BMP-2 upregulation without requiring external growth factors. Unlike BMP-2-based scaffolds that require milligram-scale dosing and are associated with complications including ectopic bone formation and inflammation, ZrP NPs achieve comparable osteogenic effects at microgram concentrations with minimal cytotoxicity. In contrast to conventional calcium phosphate ceramics, which support bone formation via passive osteoconduction and ion release, ZrP NPs possess intrinsic osteoinductive properties by directly engaging MAPK/AKT pathways and elevating BMP-2 expression without relying on added growth factors. Furthermore, while BMP-2-based scaffolds require high-dose application

Fig. 3 In vitro expression of osteogenic-related genes and proteins in MC3T3-E1 cells treated with the ZrP (1 M) NPs. (A–D) mRNA expression for osteogenic-related genes including (A) *RUNX2*, (B) *ALP*, (C) *OCN*, and (D) *OPN*. (E, F) Expression of osteogenic-related proteins, including (E) *OCN*, and (F) *OPN*. Data are expressed as the mean $\pm$ SD ($n=3$). * $p<0.05$ and ** $p<0.01$. ZrP: zirconium phosphate, NPs: nanoplatelets, RUNX2: runt-related transcription factor 2, ALP: alkaline phosphatase, OCN: osteocalcin, OPN: osteopontin, GAPDH: glyceraldehyde-3-phosphate dehydrogenase



and carry risks of complications such as ectopic ossification and inflammation, ZrP NPs achieve robust osteogenic effects at significantly lower concentrations and with greater cytocompatibility. Collectively, these findings demonstrate that ZrP NPs enhance osteogenic differentiation in MC3T3-E1 pre-osteoblasts by regulating gene expression, protein activity, and biomineralization processes.

3.5 Osteogenic signaling pathways of the ZrP NPs in MC3T3-E1 cells

The osteogenic potential of the ZrP NPs in MC3T3-E1 cells was investigated through western blot analysis, revealing the activation of key signaling pathways and the upregulation of osteogenic markers (Fig. 4A). Treatment with 100 µg/mL ZrP NPs for 7 days significantly increased the phosphorylation levels of p38 MAPK, ERK1/2, and AKT, while elevating the expression of BMP-2, RUNX2, and OPN (Fig. 4B). These findings are consistent with previous studies on zirconium-based nanomaterials, including ZrP

nanonetworks and zirconia-doped BCP scaffolds, wherein pathway activation (e.g., MAPK/AKT) and marker induction were found to drive osteogenesis [8, 9, 11].

Upon internalization into MC3T3-E1 cells, the ZrP (1 M) NPs are degraded in an acidic endosomal compartment (pH 5.0), releasing Zr^{4+} and phosphate ions that create a synergistic microenvironment. Similar to Zn^{2+} -modified biomaterials that enhance osteoblast differentiation via ERK/p38 signaling [37], the released Zr^{4+} and phosphate ions may activate the MAPK/ERK pathways, thereby upregulating osteogenic markers such as ALP, RUNX2, OCN, and OPN (Fig. 3). While soluble Zr^{4+} ions diffuse quickly and are rapidly cleared, thereby limiting the duration of osteogenic signaling, ZrP NPs offer controlled, sustained intracellular ion release. This prolonged release facilitates continuous activation of multiple signaling pathways over an extended period, thereby enhancing osteogenic efficacy and therapeutic potential. Phosphate ions directly participate in HAP nucleation, as demonstrated in calcium phosphate systems [38], while synergizing with Zr^{4+} to upregulate BMP-2.

Fig. 4 Effects of ZrP (1 M) NPs on osteogenic-related proteins. (A) The western blotting bands and (C) the quantitative analysis of osteogenesis-specific proteins such as p38, p-p38, ERK1/2, p-ERK1/2, RUNX2, OPN, AKT, p-AKT, and BMP-2 in MC3T3-E1 after treatment with ZrP (1 M) NPs for 7 days. Data are expressed as the mean $\pm$ SD ($n=3$). * $p<0.05$, ** $p<0.01$. ZrP: zirconium phosphate, NPs: nanoplatelets, p-p38: phosphorylated p38, p-ERK1/2: phosphorylated ERK1/2, RUNX2: runt-related transcription factor 2, OPN: osteopontin, AKT: protein kinase B, p-AKT: phosphorylated AKT, BMP-2: bone morphogenic protein-2, GAPDH: glyceraldehyde-3-phosphate dehydrogenase

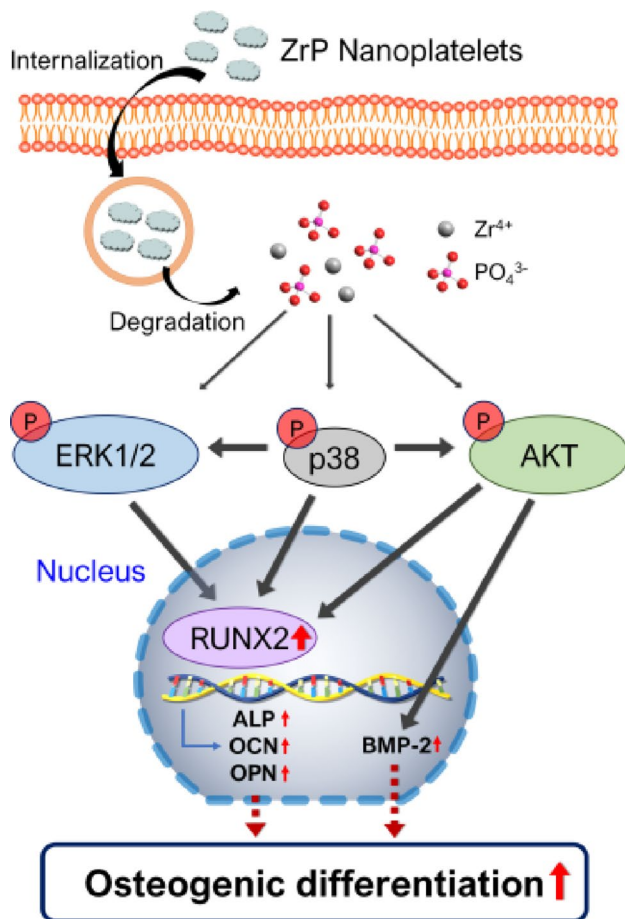
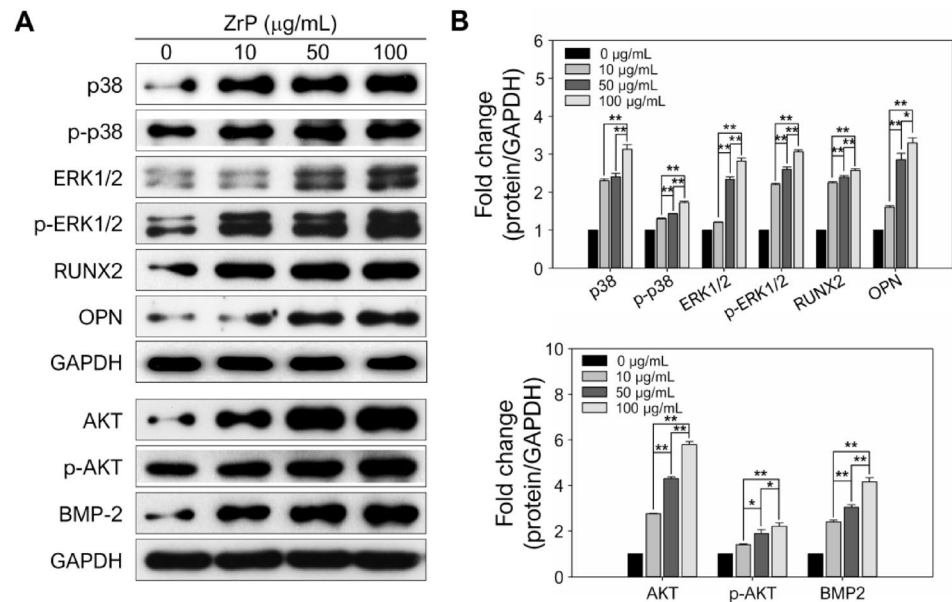


Fig. 5 Molecular mechanism of the modulation of osteogenic differentiation in MC3T3-E1 cells by ZrP NPs through the p38 MAPK and AKT signaling pathways. ZrP: zirconium phosphate, NPs: nanoplatelets, MAPK: mitogen-activated protein kinase, AKT: protein kinase B, RUNX2: runt-related transcription factor 2, ALP: alkaline phosphatase, OCN: osteocalcin, OPN: osteopontin, BMP-2: bone morphogenic protein-2

BMP-2 activates SMAD-dependent pathways, thereby further amplifying osteogenic gene expression and cross-talk with the MAPK/AKT cascades [11].

Western blot analysis revealed that the ZrP NPs induced the concentration-dependent phosphorylation of p38 MAPK, ERK1/2, and AKT (Fig. 4), which represent key kinases associated with regulating osteoblast differentiation and survival [39, 40]. p-p38 and ERK1/2 enhance the activity of RUNX2, the master regulator of osteogenesis [41], while AKT signaling promotes cell survival and matrix mineralization [42]. This pathway activation mechanism is consistent with findings in gold nanoparticles, where mechanical stimuli activate p38 MAPK to drive osteogenic commitment [43]. Additionally, the ZrP NPs were found to elevate BMP-2 expression, reinforcing SMAD and MAPK/AKT cross-talk to ultimately amplify osteogenesis. Based on these observations, we propose a dual activation mechanism by which initial non-canonical stimulation of MAPK and AKT pathways induces BMP-2 expression. This, in turn, potentiates osteogenic signaling through enhanced interaction between canonical (SMAD-dependent) and non-canonical pathways, possibly establishing a positive feedback loop that sustains osteogenic commitment [44]. While our results clearly demonstrate MAPK/AKT pathway activation and BMP-2 upregulation, further detailed investigation of SMAD pathway dynamics is warranted to fully elucidate the molecular interplay mediating ZrP NP-induced osteogenesis. This mechanism is summarized in Fig. 5, highlighting the dual role of ZrP NPs as bioactive ion reservoirs and signaling activators in bone formation.

4 Conclusion

In this study, ZrP NPs were synthesized and characterized, and their osteogenic mechanisms in MC3T3-E1 pre-osteoblasts were investigated. The ZrP NPs prepared using a 1 M H_3PO_4 solution exhibited smaller particles sizes (<30 nm) and a reduced crystallinity, in addition to demonstrating excellent biocompatibility and enhancing osteogenic differentiation in a dose-dependent manner. This was evidenced by the increased ALP activity and the extent of calcium deposition, which are hallmarks of early and late osteogenesis, respectively. Gene expression profiling revealed the time-dependent upregulation of early-stage markers (RUNX2 and ALP) and late-stage markers (OCN and OPN), reflecting the natural progression of osteoblast maturation. Mechanistically, the ZrP NPs activated key intracellular signaling pathways, including the p38 MAPK, ERK1/2, and AKT pathways. Such activation led to the upregulated expression of BMP-2, RUNX2, and OPN. Collectively, these findings demonstrate that ZrP NPs promote osteogenic differentiation in MC3T3-E1 cells, primarily through MAPK and AKT pathway activation. Furthermore, these results reveal that ZrP NPs represent promising candidates for use in bone tissue engineering, particularly in applications requiring targeted bone defect repair and regeneration. While our current investigation focused on the *in vitro* validation of ZrP NPs' osteogenic potential, future work will extend these findings to *in vivo* studies. We plan to utilize established animal models, such as rat calvarial or murine femoral defect models, to rigorously evaluate the bone-regenerative efficacy and biocompatibility of ZrP NPs.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Neither ethical approval nor informed consent was required for this study.

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