

ORIGINAL RESEARCH ARTICLE

Jawbone periosteal cell spheroids exhibit early osteogenic responses through F-actin/NF- κ B-mediated mechanotransduction

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Abstract

Three-dimensional (3D) spheroids have emerged as a powerful approach for studying ossification and bone regeneration. However, it remains unclear whether a 3D self-assembly process can initiate early osteogenic responses under physiologically relevant, induction-free conditions. In this study, we established a scaffold-free, self-assembling spheroid model using mouse jawbone periosteum-derived cells (jb-PDCs) without exogenous osteogenic inducers. Compared with monolayer cultures, spheroid formation induced the sequential upregulation of osteogenic markers, including *Col1a2* at 12 h, *Sp7* at 24 h, *Spp1* at 48 h, and *Runx2* after seven days. Transcriptomic profiling revealed significant enrichment of nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) signaling. Increased phosphorylation of the p65 subunit further confirmed NF- κ B pathway activation. Pharmacological inhibition of NF- κ B abolished the spheroid-associated upregulation of *Col1a2*, *Sp7*, and *Spp1*, whereas direct NF- κ B activation in conventional monolayer cultures recapitulated this osteogenic gene expression program. Furthermore, spheroid formation was accompanied by marked F-actin disassembly. Pharmacological stabilization of actin filaments reduced both p65 phosphorylation and osteogenic gene expression. Together, these findings identify an F-actin/NF- κ B mechanotransduction axis through which 3D self-assembly promotes early osteogenic gene expression in jb-PDCs. Our results suggest that cellular aggregation can intrinsically couple cytoskeletal remodeling to initiate early osteogenic responses. The inducer-free jb-PDC spheroid system also provides a useful platform for investigating the mechanobiology of early osteogenic responses and may inform the development of biomimetic strategies for craniofacial bone regeneration.

Keywords: Periosteal progenitor cells; Spheroids; Osteogenic Differentiation; Nuclear factor κ -light-chain-enhancer of activated B cells signaling; Filamentous actin; Mechanotransduction

1. Introduction

Jawbone defects caused by trauma and tumor resection possess high morbidity and thus remain a formidable

clinical challenge.¹ Current therapeutic strategies, including autologous bone grafting and distraction osteogenesis, are often limited by donor site morbidity, unpredictable graft resorption, and insufficient regeneration of critical-sized

defects.^{2,3} These persistent challenges underscore the need for a profound understanding of the biological mechanisms underlying jawbone repair.

In nature, the periosteum serves as an indispensable source of skeletal stem/progenitor cells.⁴ Our previous studies comprehensively characterized the biological properties of jawbone periosteum-derived cells (jb-PDCs) and demonstrated that jb-PDCs possess a distinct neural crest origin, which is associated with enhanced mineralization potential and superior *in vivo* bone-regenerative capacity.^{5,6} These cells actively respond to local microenvironmental signals and undergo osteogenic differentiation, thereby orchestrating intramembranous ossification, a predominant mechanism of jawbone healing.⁴ However, the early molecular events that prompt jb-PDCs to initiate osteogenic responses remain largely unexplored.

The osteogenic differentiation cascade has been extensively studied using conventional two-dimensional (2D) monolayer cultures supplemented with osteogenic induction cocktails containing dexamethasone, β -glycerophosphate, and ascorbic acid.⁷ However, this approach has several limitations that may hinder the identification of endogenous osteogenic triggers. First, monolayer culture does not recapitulate the three-dimensional (3D) microenvironment experienced by cells *in vivo*.⁸ Second, the use of supraphysiological glucocorticoid concentrations may bypass or obscure endogenous mechanosensitive pathways activated under physiological conditions. Third, substantial upregulation of osteogenic markers in conventional 2D cultures typically requires 7–21 days,^{9,10} potentially overlooking early instructive signaling events. Consequently, how jb-PDCs initiate early osteogenic responses in a physiologically relevant, induction-free context remains unclear.

Three-dimensional spheroid technology has emerged as a useful approach for studying tissue morphogenesis by preserving spatial architecture, cell–matrix interactions, and biomechanical signaling that are not fully recapitulated in monolayer systems.^{11–13} In the skeletal field, spheroid-based approaches have rapidly progressed in recent years. Park *et al.*¹⁴ established a mineralized spheroid model by combining osteoblasts with demineralized bone matrix, thereby recapitulating features of trabecular bone architecture. In our previous study, we employed a scaffold-free self-aggregation protocol to generate jb-PDC spheroids that underwent endochondral ossification and accelerated jawbone repair *in vivo*.¹⁵ Similarly, Zhang *et al.*¹⁶ generated spheroids from long bone periosteal cells that developed ossification centers and were subsequently incorporated into 3D-printed constructs for *in vivo* bone repair. Despite these advances, current osteogenic spheroid models mostly rely on exogenous induction factors and primarily

emphasize long-term tissue formation or regenerative outcomes. The early endogenous signaling events that initiate osteogenesis during self-assembly therefore remain insufficiently understood.

In this study, we developed a scaffold-free, self-assembling spheroid system using mouse jb-PDCs that exhibited early osteogenic features in the absence of exogenous osteogenic inducers. In contrast to conventional 2D monolayer cultures, in which substantial upregulation of osteogenic markers typically requires 7–21 days,¹⁷ our spheroid system revealed early transcriptional responses within 48 h of self-assembly. This model provides a controlled 3D platform for investigating early mechanotransduction associated with morphological changes and cytoskeletal remodeling during cell condensation. Using this system, we examined the relationship between 3D morphology and initiation of osteogenic signaling in an inducer-free microenvironment, with particular emphasis on the F-actin/nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) mechanotransduction axis. Our findings provide insight into how periosteal cells may naturally initiate osteogenic responses during early cell condensation.

2. Materials and methods

2.1. Isolation and expansion of mouse jawbone periosteum-derived cells

Mouse jb-PDCs were harvested from C57BL/6 mice (6- to 8-week-old) according to our previous studies.^{5,15} To obtain sufficient primary cells, mandibles from at least six mice were pooled for each independent preparation, and cells were expanded under 5% CO₂ at 37 °C with growth medium (α -Minimum Essential Medium, #SH30265.01, Cytiva, USA) supplemented with 10% fetal bovine serum (FBS; #SV30208.02, Cytiva, USA) and 1% penicillin/streptomycin (#G6251, Gibco, USA). The cells were expanded to passage 3 before use in subsequent experiments.

2.2. High-throughput formation of jawbone periosteum-derived cell spheroids

For the 3D group, the mouse jb-PDCs (passage 3) were seeded in our previously reported agarose 24-well inserts containing approximately 2,000 microwells,^{15,18} at a concentration of 400,000 or 100,000 cells per well. For 2D controls, cells were seeded in 24-well plates at a density of 100,000 cells per well. Cells were cultured in growth medium at 37 °C and 5% CO₂ for up to seven days. The growth medium was half-refreshed daily. The obtained spheroids were observed and imaged at predetermined time points (12, 24, and 48 h) using an inverted microscope (Olympus IXplore Pro, Olympus Corporation, Japan). The obtained images were further analyzed using Fiji software (version 2.14.0, National Institutes of Health, USA) to assess spheroid size (<https://fiji.sc>, RRID: SCR_002285).¹⁹

At each time point, three random images were captured from each of three independent wells, and the diameters of approximately 20 spheroids were measured per image.

2.3. Flow cytometry analysis of cellular size and population

Flow cytometry was performed on 3D spheroids and 2D monolayer controls to assess cell size and the skeletal stem cell populations according to our previous method.¹⁵ Specifically, the monolayer-cultured cells were detached using 0.25% trypsin-EDTA (Gibco, USA). Spheroids were harvested after 24 and 48 h by pipetting, centrifuged at 1,000× *g* for 5 min, and subsequently digested for 10 min using 0.25% trypsin and 3 mg/mL collagenase I (Biofrox, Germany). The obtained cell suspensions were blocked for 10 min in phosphate-buffered saline (PBS; pH 7.4; Servicebio, China) containing 10% FBS, followed by staining with the following antibodies: PE-conjugated anti-mouse CD105 (1:150, #120407, Biolegend, USA), APC-conjugated anti-mouse CD140a (1:50, #135908, Biolegend, USA), FITC-conjugated anti-mouse CD31 (1:75, #102406, Biolegend, USA), and FITC-conjugated anti-mouse CD45 (1:150, #103108, Biolegend, USA). After washing to remove unbound antibodies, the samples were analyzed using CytoFLEX (Beckman, USA).

Single-color controls were used for compensation adjustment, and fluorescence-minus-one controls were employed to account for fluorescence spillover in other channels.^{20,21} Cell size distribution was determined by forward scatter, and the skeletal stem cell populations were defined by the phenotype CD105⁺CD140a⁺CD31⁺CD45⁺.

2.4. Live/dead staining

After 12, 24, and 48 h of culture, the culture medium containing the 2D monolayer-cultured cells and 3D spheroids was removed, and the cultures were rinsed three times with PBS. Live/dead staining was performed using Calcein-AM/EthD-I (#L6023L, Uelandy, China) according to the manufacturer's instructions. Fluorescent images of live and dead cells were captured using a fluorescent microscope (Olympus FV1200, Olympus Corporation, Japan) with excitation wavelengths of 488 nm and 594 nm.

2.5. Immunofluorescent staining

Cell spheroids were collected from the culture plates and fixed with 4% paraformaldehyde (Servicebio, China) for 15 min. Thereafter, cells were permeabilized with 0.1% Triton X-100 (Biofrox, Germany) for 20 min, and then cells were blocked with blocking buffer (PBS containing 5% FBS and 0.1% Triton X-100) at room temperature for 1 h.

Next, cell spheroids were incubated with the following primary antibodies: COL1 (1:200, #72026, Cell Signaling Technology, USA) and SP7 (1:200, #ab209484, Abcam,

United Kingdom), respectively, at 4 °C overnight. After three washes, spheroids were incubated in the dark with the secondary antibody Alexa Fluor 488-labeled Goat anti-rabbit (1:500, #ANT024, Antgene, China) and YF594-Phalloidin (1:200, #YP0052L, Uelandy, China) in the blocking buffer at room temperature for 1 h. Finally, spheroids were washed three times and then counterstained and mounted with Antifade Mounting Medium containing 4',6-diamidino-2-phenylindole (#P0131, Beyotime, China). Monolayer cells were grown on coverslips and stained using the same protocol as described above. Fluorescent images were captured using a confocal laser scanning microscope (Olympus IXplore SpinSR, Japan) with excitation wavelengths of 488 nm and 594 nm, respectively. Images were analyzed using Fiji software.¹⁹

2.6. RNA sequencing and data analysis

After 48 h of culture, the 3D spheroids and 2D monolayer cultures were harvested, and total RNA was extracted using the EZNA HP Total RNA Kit (#R6812, Omega Bio-Tek, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA sequencing and data analysis were performed as described in our previous studies.^{5,15,18} The raw and processed RNA sequencing data are available in the Gene Expression Omnibus (GEO) under accession number GSE343220.

In brief, library preparation and sequencing were performed by the Beijing Genomics Institute (BGI, China), where all samples were sequenced in a single batch on the BGISEQ platform (SE50, stranded library). Raw reads were processed with SOAPnuke software (v1.5.2, Beijing Genomics Institute, China) to obtain clean reads, which were then aligned to the NCBI RefSeq GRCm38.p6 build using HISAT and the org.Mm.eg.db package (v3.21.0, Bioconductor, USA). Subsequently, Bowtie 2.28 was used to map the reads to reference gene sequences.²² Gene expression levels were calculated with RSEM.²³ A quality control summary (read depth, mapping rate, Q30, and RIN) is provided in **Table S1**.

Gene annotation was based on the NCBI RefSeq GRCm38.p6 build using the org.Mm.eg.db package (v3.21.0). For differential expression, we used DESeq2 (v1.42.0) with the Benjamini-Hochberg procedure for multiple testing correction, with a *Q*-value (adjusted *p*-value) ≤ 0.05 as the significance threshold.²⁴ The complete DEG table (containing log2FC, *p*-values, and adjusted *p*-values for all genes) is provided in the GEO database under accession number GSE343220.

Visualization of the results, including heatmaps, volcano plots, and principal component analysis, was carried out using R software (version 4.5.0, R Core Team, New Zealand). Functional enrichment analyses for Gene Ontology terms

and Gene Set Enrichment Analysis (GSEA) were conducted using online databases.²⁵⁻²⁷ GSEA was performed using the MSigDB Hallmark gene sets (v2026.1).

2.7. Quantitative reverse transcription polymerase chain reaction

Total RNA isolation was performed using the EZNA HP Total RNA Kit (#R6812, Omega Bio-Tek, USA) and reverse transcribed using HiScript III All-in-one RT SuperMix (#R222, Vazyme, China). Quantitative reverse transcription polymerase chain reaction was conducted using the HiScript II One Step qRT-PCR SYBR Green Kit (#Q711, Vazyme, China) on the CFX Connect Real-Time System (Bio-Rad, USA). Primers were synthesized by Sangon Biotech (China), and the primer sequences are listed in Table 1. The expression of genes was quantified using *Gapdh* as a housekeeping gene, and the relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers used in this study

Primer	Primer sequence (5'-3')
<i>Col1a2</i>	Forward GTAACTTCGTGCTAGCAACA
	Reverse CCTTTGTCAGAATACTGAGCAGC
<i>Sp7</i>	Forward ATGGCGTCCTCTCTGCTTG
	Reverse TGAAAGGTCAGCGTATGGCTT
<i>Spp1</i>	Forward CTGTTGCCAGCTTCTGAGCA
	Reverse TGTGGCTCTGATGTTCCAGGCT
<i>Runx2</i>	Forward CACTGGCGGTGCAACAAGA
	Reverse ATGACGGTAACACAGTCCCATC
<i>Rela</i>	Forward GCAGAAAGAAGACATTGAGGT
	Reverse CGATGCACATCAGCTTGAG
<i>Nfkb1</i>	Forward CTTCTCAAAGCAGCAGGAG
	Reverse GTCAAATACCTGCCAGTTGG
<i>Nfkb1a</i>	Forward CACCAACTACAATGGCCAC
	Reverse AAAGTCACCAAGTGCTCCA
<i>Tnfaip3</i>	Forward TTAAATTCCGCTGGCAGC
	Reverse ATTCCAGTTCCGAGTGTCG
<i>Gapdh</i>	Forward TGTGTCCGTCGTGGATCTGA
	Reverse TTGCTGTTGAAGTCGCAGGAG

2.8. Western blotting

Since spheroid formation is typically completed by 3 h,¹⁵ spheroids were collected at 3 h and 6 h after self-aggregation. Cells were then lysed on ice in lysis buffer (Merck Millipore, USA) supplemented with protease and phosphatase inhibitors (Roche, Switzerland). Protein concentrations were measured using a BCA Protein Assay Kit (#PA101, Genesand, China) according to the

manufacturer's instructions.

For each sample, 12 µg of denatured protein was loaded onto 10% polyacrylamide gels, separated by electrophoresis, and then transferred onto nitrocellulose membranes (Merck Millipore, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated overnight at 4 °C with the following primary antibodies: anti-p65 phosphorylated at Ser536 (1:1,000, #3033, Cell Signaling Technology, USA), p65 (1:1,000, #8242, Cell Signaling Technology, USA) and glyceraldehyde-3-phosphate dehydrogenase (1:5,000, #81640-5-RR, Proteintech, China). The next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2,000, #ANT020, Antgene, China) diluted in 5% non-fat milk for 1 h at room temperature. Signals were detected using WesternBright ECL solution (#K-12045-D50, Advansta, USA), and densitometric analysis normalized to glyceraldehyde-3-phosphate dehydrogenase was performed with Fiji software.¹⁹

2.9. Pharmaceutical intervention experiments

The NF-κB inhibitor BAY11-7082 (MedChemExpress, USA) or cytoskeleton stabilizer jasplakinolide (MedChemExpress, USA) was dissolved in dimethyl sulfoxide (DMSO; MedChemExpress, USA) and added to 3D spheroids during self-aggregation in the microwell platform. The NF-κB agonist diprovocim (MedChemExpress, USA) was dissolved in DMSO and added to 2D controls when the cells were seeded for monolayer culture. The final working concentrations are as follows: BAY11-7082 (1 µM), jasplakinolide (50 nM), and Diprovocim (500 nM). The final DMSO concentration was below 0.1% in all groups. All treatments were continued for 48 h in inducer-free culture medium. Protein samples were collected at 6 h for Western blotting. RNA samples were collected at 24 h and 48 h for further quantitative reverse transcription polymerase chain reaction analysis.

2.10. Statistical analysis

All data are presented as mean ± standard deviation. Individual data points were displayed in the plots, with bars indicating the mean values. Statistical analyses were performed using GraphPad Prism software (version 10.1.2, GraphPad Software, USA). Normality of the data distribution was assessed using the Shapiro–Wilk test. For comparisons involving more than two groups, either one-way ANOVA followed by Tukey's post hoc test or the Kruskal–Wallis test with Dunn's post hoc test was applied, depending on the data distribution. For two-group comparisons, an unpaired two-sample Student's *t*-test assuming unequal variances or the Mann–Whitney *U* test was used. Unless otherwise stated, the sample size is 3 for

each condition ($n = 3$). The independent culture wells were used as the statistical unit.

3. Results

3.1. Self-assembly of mouse jb-PDCs into spheroids induces morphological changes

Mouse jb-PDCs (passage 3) were seeded in our previously established microwell-array system at a concentration of 200 cells per microwell. Within 12 h, each microwell contained a spheroid with an average diameter of $80.6 \pm 11.1 \mu\text{m}$ and maintained its spheroid structure throughout the 48-h *in vitro* culture period. However, measurements indicated a reduction in diameter to $61.4 \pm 11.1 \mu\text{m}$ and $49.3 \pm 14.7 \mu\text{m}$ at 24 and 48 h, respectively (Figure 1A and 1B). Flow cytometry analyses elucidated the effects of the spheroid culture on cellular morphology. Compared with cells grown in 2D monolayers, the spheroid cultures exhibited a notable decrease in cell size over time, evidenced by significantly altered forward scatter profiles at the 24-h and 48-h time points (Figure 1C and 1D).

Throughout the 48-h culture period, we observed remarkable remodeling of the cytoskeletal architecture in jb-PDC spheroids (3D) compared with the 2D monolayer cells (Figure 1E). Live/dead staining further confirmed that the majority of cells remained viable within the spheroids during 48 h of culture (Figure 1F and S1).

3.2. Mouse jb-PDC spheroids exhibit early osteogenic gene expression under induction-free conditions

We performed flow cytometry to assess changes in the expression of stemness-related markers in jb-PDCs following 3D spheroid culture under induction-free conditions. Results indicated that, compared with 2D monolayer culture, the proportion of the skeletal stem cell population ($\text{CD140a}^+\text{CD105}^+\text{CD45}^-\text{CD31}^-$) in the 3D condition decreased from 63.1% to 43.1% after 24 h (Figure S2), and further decreased to 33.1% after 48 h (Figure 2A and 2B). In addition, we observed increased mRNA expression of *Runx2*, a core transcription factor regulating osteogenic differentiation, after seven days of culture without exogenous osteogenic inducers (Figure 2C).

Furthermore, we explored the early events of self-assembly within 48 h. We found a significant upregulation of osteogenic marker genes in jb-PDC spheroids during the 48-h culture. Specifically, the expression of *Col1a2* in 3D spheroids peaked at 12 h, subsequently declining but remaining elevated at both 24 and 48 h. In contrast, *Sp7* and *Spp1* demonstrated substantial upregulation at both 24

and 48 h (Figure 2D). However, the expression of *Runx2* showed no significant change during the 48-h observation period (Figure S3).

We further performed immunofluorescence staining after 48 h of culture to validate the upregulation of type I collagen (COL1) and SP7 at the protein level. Results showed that the mean fluorescence intensities of COL1 and SP7 in the spheroids were significantly higher than those in the monolayer culture, with increases of 1.58-fold and 1.46-fold, respectively (Figure 2E–2H).

Additionally, we established two density-matched groups: (i) a 2D monolayer group (100,000 cells per well) and (ii) a 3D spheroid group with an equivalent total cell number, corresponding to 50 cells/spheroid per microwell. Under matched cell density, the 3D spheroids still exhibited significantly higher expression levels of *Col1a2*, *Sp7*, and *Spp1* compared with the 2D group (Figure S4).

3.3. Mouse jb-PDC spheroids exhibit enhanced canonical NF- κ B signaling activation

We conducted RNA sequencing analysis of 2D monolayer and 3D spheroid cultures of mouse jb-PDCs to explore the mechanism underlying the osteogenic responses within mouse jb-PDC spheroids. The complete counts table is available in the GEO database (GSE343220). Principal component analysis also indicated that the transcriptome profiles between the 2D and 3D groups were significantly different and that no outlier samples were observed (Figure 3A). Compared with the 2D monolayer cultures, jb-PDC spheroids displayed a total of 1,879 upregulated genes ($\log_2\text{FC}[3\text{D}/2\text{D}] > 1, q < 0.05$) and 956 downregulated genes ($\log_2\text{FC}[3\text{D}/2\text{D}] < -1, q < 0.05$) after 48 h of culture (Figure 3B).

Gene Ontology enrichment analysis of the upregulated genes revealed significant enrichment in cell differentiation, bone mineralization, and canonical NF- κ B signal transduction (Figure 3C). GSEA suggested an upregulation of the NF- κ B signaling pathway in mouse jb-PDC spheroids (Figure 3D and 3E).

Quantitative polymerase chain reaction data confirmed that jb-PDC spheroids showed significantly upregulated NF- κ B target genes *Rela*, *Nfkb1*, *Nfkbia*, and *Tnfaip3* (Figure 3F). To determine when NF- κ B was activated, we examined p65 phosphorylation by Western blotting at 3 h and 6 h after spheroid formation. No significant increase was detected at 3 h (Figure S5), whereas phosphorylated p65 was significantly elevated at 6 h (Figure 3G and 3H), indicating that NF- κ B signaling was activated between 3 and 6 h after 3D self-assembly.

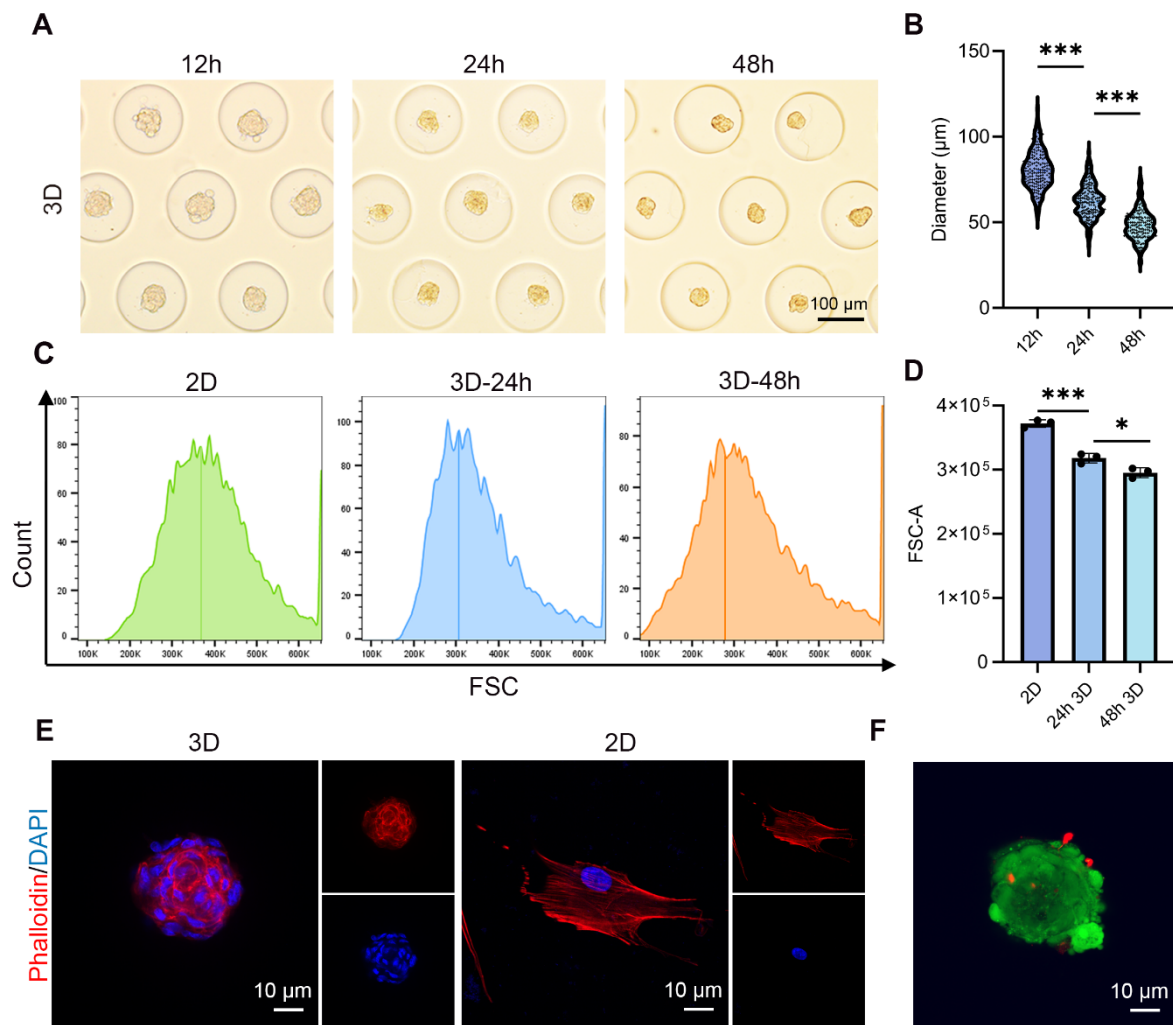


Figure 1. Self-assembly of jb-PDCs into spheroids is accompanied by cellular size reduction and F-actin remodeling. (A) Representative bright-field images showing the morphology and self-assembly of jb-PDCs into spheroids during 48 h of *in vitro* culture in agarose microwell plates (scale bar = 100 μm, magnification = 10×). (B) Quantification of spheroid diameter at 12, 24, and 48 h. Three random images were acquired from each of three independent wells at each time point, and the diameters of all visible spheroids were measured. (C) Representative flow cytometry plots showing changes in individual cell size in jbPDC spheroids, as indicated by FSC. (D) Quantification of individual cell size based on median FSC ($n = 3$). (E) Representative confocal microscopy images of 3D jbPDC spheroids and 2D jbPDC monolayers after 48 h of culture, showing F-actin organization by phalloidin staining (red) and nuclei by DAPI staining (blue) (scale bar = 10 μm, magnification = 40×). (F) Representative live/dead staining images of jbPDC spheroids after 48 h of culture. Live cells were stained with Calcein AM (green), and dead cells were stained with ethidium homodimer (red) (scale bar = 10 μm, magnification = 40×). * $p < 0.05$ and *** $p < 0.001$ indicate statistical significance.

Abbreviations: 2D: Two-dimensional; 3D: Three-dimensional; DAPI: 4',6-diamidino-2-phenylindole; FSC: Forward scatter; jb-PDC: Jawbone periosteum-derived cell.

3.4. NF-κB signaling mediates spontaneous osteogenic differentiation in mouse jb-PDC spheroids

We conducted pharmacological intervention experiments to determine whether the NF-κB pathway is the key signaling cascade driving the upregulation of osteogenic marker genes in mouse jb-PDC spheroids. We cultured 3D jb-PDC spheroids with or without the NF-κB inhibitor

BAY11-7082 (1 μM) (Figure 4A and 4B). Bright-field images and live/dead staining images confirmed that the addition of BAY11-7082 did not significantly affect spheroid formation and cell viability within spheroids (Figure S6). After 6 h of treatment, we observed a significant reduction in phosphorylated p65 in the inhibitor-treated (BAY) spheroids compared with the DMSO controls (Figure 4C and 4D). Consistently, the expression levels of the osteogenic marker genes *Col1a2*, *Sp7*, and *Spp1* were

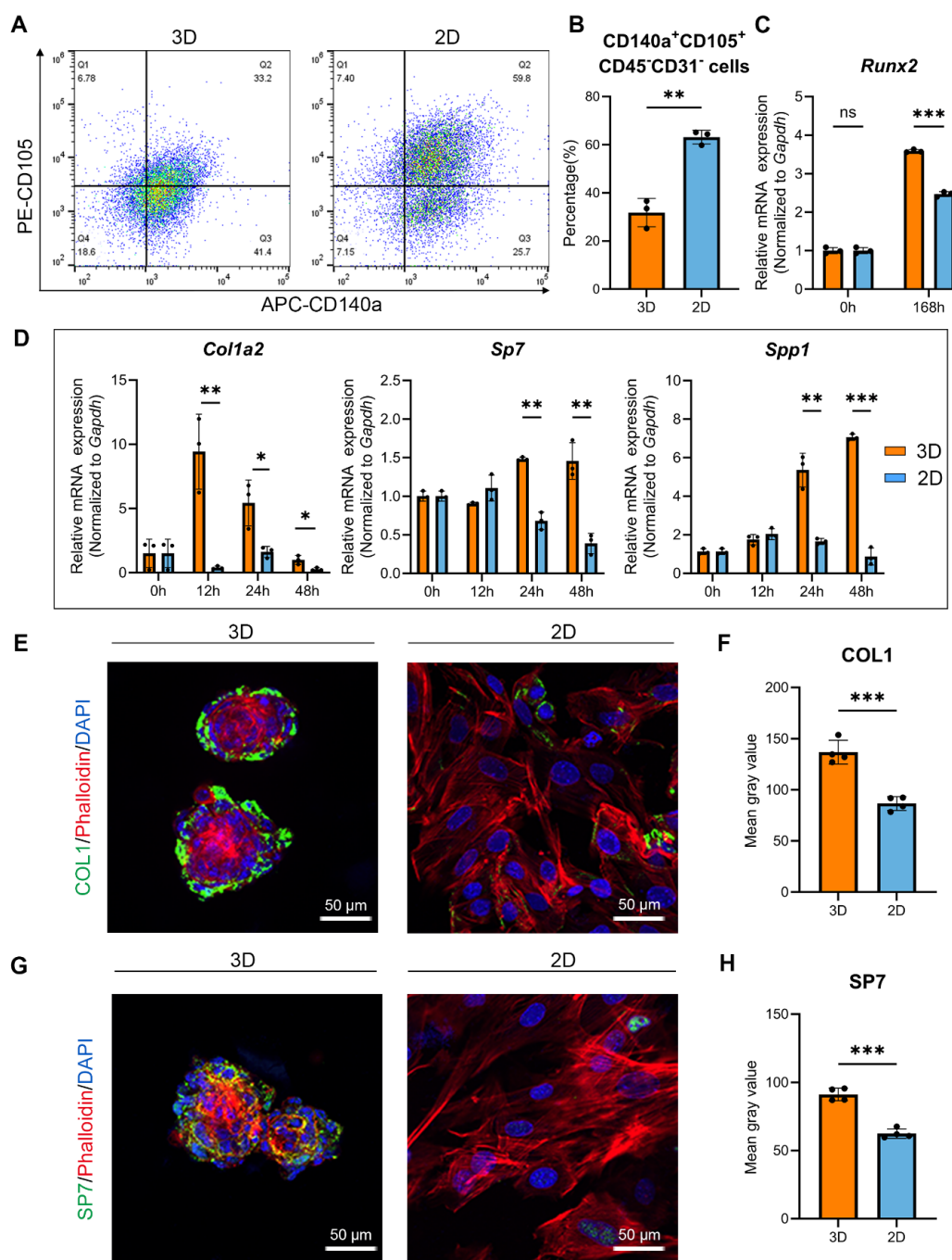


Figure 2. jB-PDC spheroids exhibit early osteogenic transcriptional responses under induction-free conditions. (A, B) Flow cytometric analysis of the proportion of the skeletal stem cell population (CD140a⁺CD105⁺CD45⁺CD31⁻) in 3D spheroids and 2D monolayer cultures after 48 h of *in vitro* culture ($n = 3$). (C) Relative mRNA expression level of the osteogenic transcription factor *Runx2* in jB-PDCs cultured as 3D spheroids or 2D monolayers after seven days ($n = 3$). (D) Relative mRNA expression levels of the osteogenic marker genes *Col1a2*, *Sp7*, and *Spp1* in jB-PDCs cultured as 3D spheroids or 2D monolayers after 48 h of *in vitro* culture ($n = 3$). (E) Representative confocal microscopy images showing COL1 (green) in 3D jB-PDC spheroids or 2D jB-PDC monolayers, with DAPI (blue) and phalloidin (red) counterstaining (scale bar = 50 μm, magnification = 40×). (F) Quantification of COL1 fluorescence intensity. (G) Representative confocal microscopy images showing SP7 (green) in 3D jB-PDC spheroids and 2D jB-PDC monolayers, with DAPI (blue) and phalloidin (red) counterstaining (scale bar = 50 μm, magnification = 40×). (H) Quantification of SP7 fluorescence intensity. For quantitative analyses in panels F and H, three random fields of view were analyzed from each independent replicate ($n = 3$). Statistical analyses between two groups were performed using Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate statistical significance. Abbreviations: 2D: Two-dimensional; 3D: Three-dimensional; COL1: Type I collagen; DAPI: 4',6-diamidino-2-phenylindole; jB-PDC: Jawbone periosteum-derived cell.

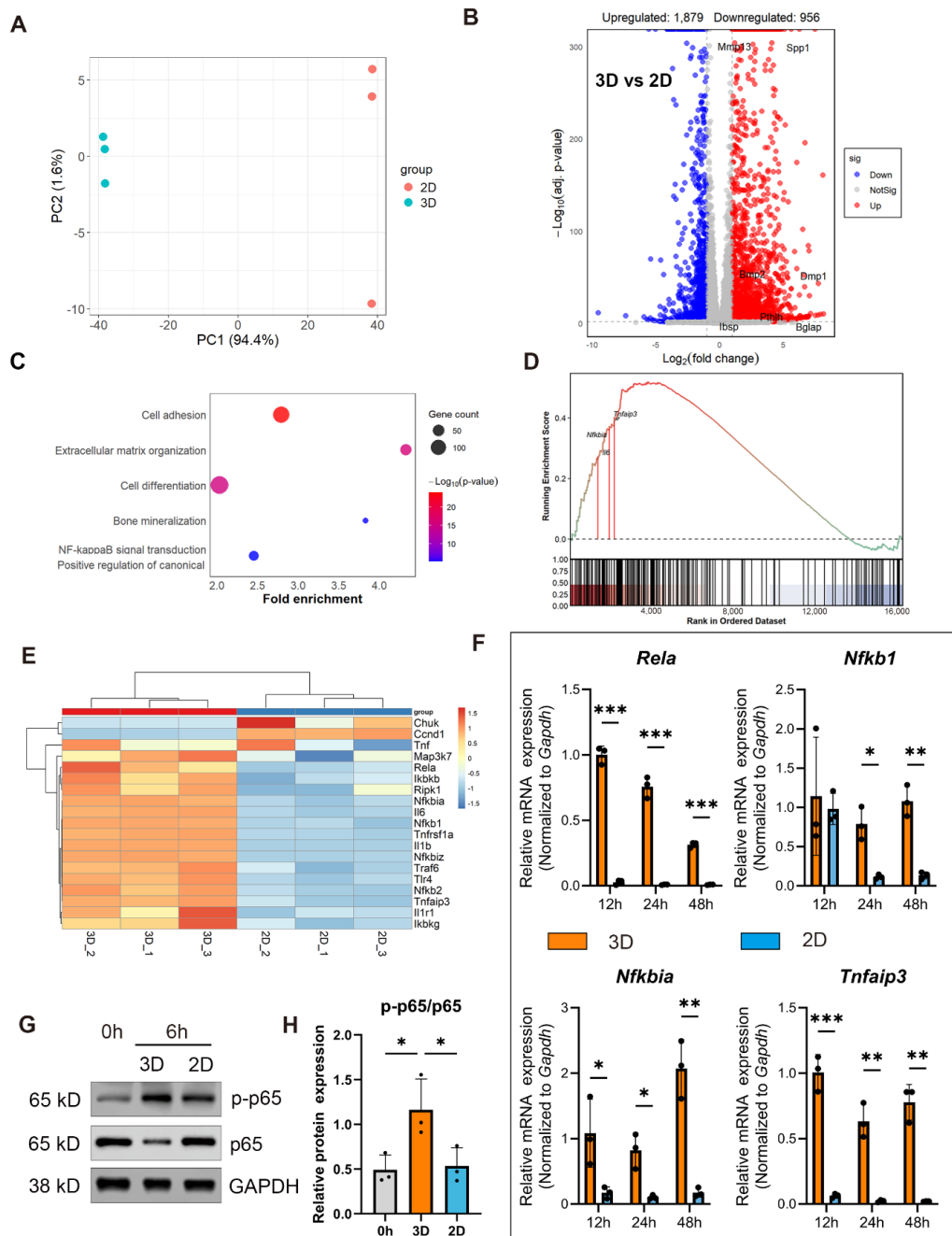


Figure 3. jb-PDC spheroids show activation of the canonical NF- κ B signaling pathway. (A) Principal component analysis of 3D spheroids or 2D monolayer cultures after 48 h of culture. (B) Volcano plot showing differentially expressed genes identified by bulk RNA sequencing of jb-PDCs cultured as 3D spheroids or 2D monolayers for 48 h. A total of 1,879 upregulated genes (red dots) were defined as those with $Q < 0.05$ and $\log_2(3D/2D) > 1$, and 956 downregulated genes (blue dots) as those with $Q < 0.05$ and $\log_2(3D/2D) < -1$. Genes with $|\log_2 FC| < 1$ or $Q \geq 0.0085$ are shown in gray. (C) Gene Ontology biological process enrichment analysis of upregulated genes in 3D jb-PDC spheroids. (D) Gene Set Enrichment Analysis showing enrichment of the NF- κ B pathway in 3D spheroids. (E) Heatmap showing the expression of significantly enriched NF- κ B pathway genes in 3D spheroids versus 2D cultures. (F) Quantitative real-time polymerase chain reaction of the NF- κ B pathway genes (*Rela*, *Nfkb1*, *Nfkbia*, and *Tnfrsf3*) in 3D spheroids versus 2D cultures ($n = 3$). (G) Western blot analysis of phosphorylated p65 and total p65 before (0 h) and at 6 h after spheroid formation ($n = 3$). (H) Densitometric quantification of the ratio of phosphorylated p65 to total p65. Statistical comparisons between two groups were performed using Student's *t*-test, and comparisons among multiple groups were performed using one-way analysis of variance. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate statistical significance.

Abbreviations: 2D: Two-dimensional; 3D: Three-dimensional; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NF- κ B: Nuclear factor κ -light-chain-enhancer of activated B cells; jb-PDC: Jawbone periosteum-derived cell.

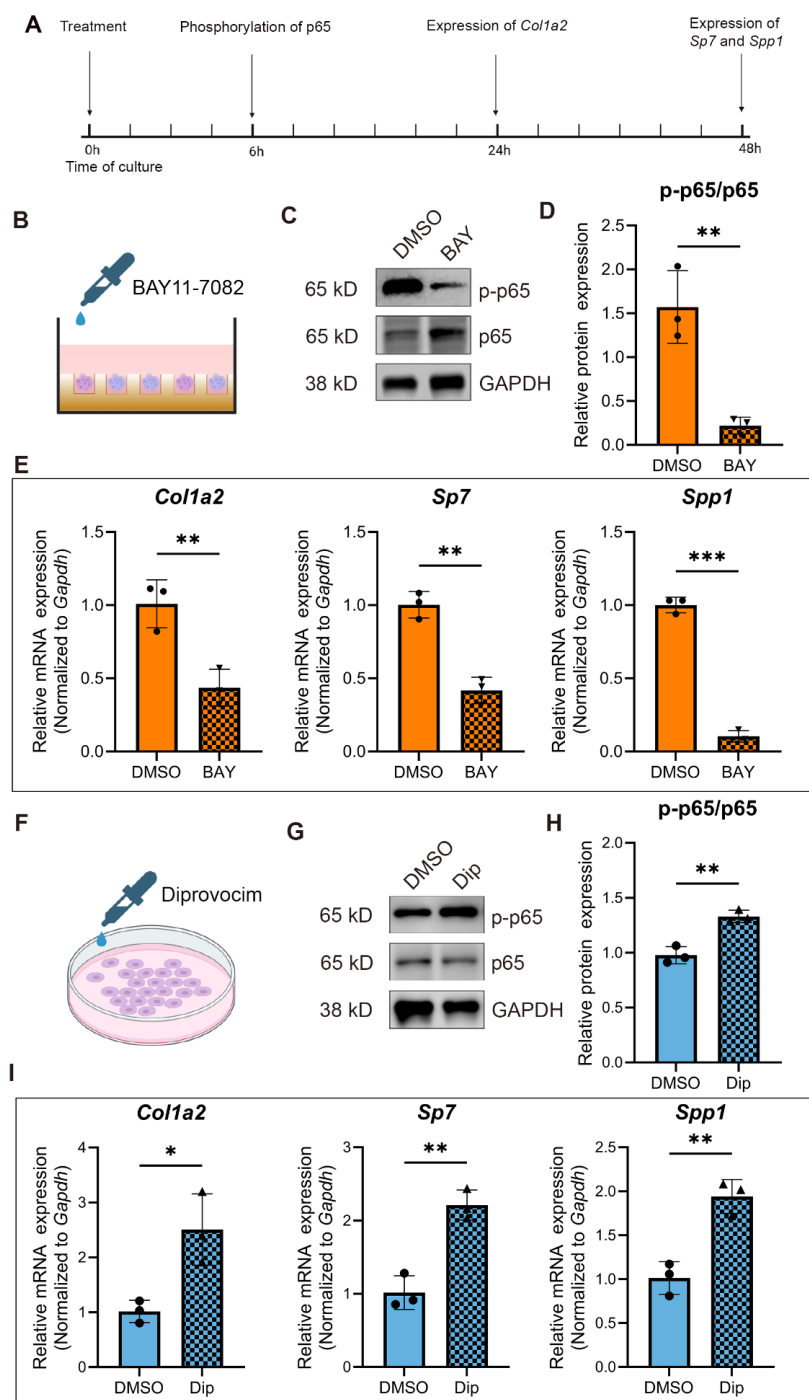


Figure 4. NF-κB signaling mediates spontaneous osteogenic responses in mouse jb-PDC spheroids. (A) Schematic diagram of the experimental treatment and sample collection time points. (B) Pharmacological inhibition of the NF-κB pathway with 1 μM BAY11-7082. (C) Western blot analysis of p-p65 and total p65 in 3D jb-PDC spheroids after 6 h of treatment. (D) Densitometric quantification of the ratio of p-p65 to total p65. (E) Relative mRNA expression levels of the osteogenic marker genes *Col1a2*, *Sp7*, and *Spp1* in 3D jb-PDC spheroids after 48 h of culture. (F) Pharmacological activation of the NF-κB pathway with 500 nM diprovocim. (G) Western blot analysis of p-p65 and total p65 in 2D jb-PDC monolayers cultured for 6 h. (H) Densitometric quantification of the ratio of p-p65 to total p65. (I) Relative mRNA expression levels of the osteogenic marker genes *Col1a2*, *Sp7*, and *Spp1* in 2D jb-PDC monolayers after 48 h of culture. Created in BioRender. Liu, Y. (2026) <https://BioRender.com/ols9fpv>. Statistical analyses between two groups were performed using Student's *t*-test. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 indicate statistical significance. Abbreviations: 2D: Two-dimensional; 3D: Three-dimensional; BAY: BAY11-7082; DMSO: Dimethyl sulfoxide; Dip: Diprovocim; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; jb-PDC: Jawbone periosteum-derived cell; p-p65: Phosphorylated p65.

markedly downregulated following p65 inhibition (Figure 4E).

We further introduced the NF- κ B activator diprovocim (500 nM) to 2D monolayer cultures (Figure 4F) to confirm the role of the NF- κ B pathway in regulating osteogenic gene expression. After 6 h of stimulation, we observed an increase in p65 phosphorylation (Figure 4G and 4H), confirming the activation of NF- κ B signaling. The elevation of *Col1a2* was observed after 12 h, accompanied by upregulation of *Sp7* and *Spp1* after 24 h (Figure 4A and 4I). Collectively, these results demonstrate that activation of the NF- κ B signaling pathway promotes osteogenic gene expression in jb-PDC spheroids.

3.5. F-actin disassembly activates NF- κ B-mediated osteogenic gene expression in jb-PDC spheroids

During spheroid formation, we observed marked remodeling of the F-actin architecture (Figure 1E). Hence, we hypothesized that F-actin disassembly within spheroids contributes to the activation of NF- κ B signaling, thereby facilitating osteogenic gene differentiation. To test this hypothesis, we treated the spheroids with jasplakinolide (50 nM), an actin filament stabilizer that preserves F-actin architecture. Bright-field images and live/dead staining confirmed that the addition of jasplakinolide (50 nM) did not significantly affect spheroid formation and cell viability within spheroids (Figure S6). Notably, the jasplakinolide-treated spheroids were substantially larger and exhibited more prominent intracellular actin filaments (Figure 5A).

Western blotting results further revealed a significant reduction in p65 phosphorylation in the jasplakinolide-treated group (Figure 5B and 5C), indicating suppression of NF- κ B signaling. This suppression of NF- κ B signaling was accompanied by significant downregulation of osteogenic marker genes *Col1a2*, *Sp7*, and *Spp1* (Figure 5D).

Taken together, these findings suggest that upon spheroid formation, jb-PDCs undergo F-actin disassembly, which is associated with increased p65 phosphorylation and subsequent transcriptional activation of osteogenesis-related genes (Figure 5E).

4. Discussion

Three-dimensional spheroids have emerged as a promising approach for studying ossification and bone regeneration. This technology preserves spatial cellular architecture, cell-cell interactions, and biomechanical cues that are often absent in conventional monolayer cultures.²⁸ However, it remains unclear whether a 3D spheroid system can trigger an early osteogenic response in a physiologically relevant, induction-free condition.

In this study, we developed a scaffold-free, self-assembling spheroid system using mouse jb-PDCs. Our

main finding is that, in the complete absence of exogenous osteogenic inductive factors, 3D spheroid formation leads to a rapid upregulation of key osteogenic markers *Col1a2*, *Sp7*, and *Spp1* within 48 h. The findings suggest that this induction-free osteogenic response is associated with a defined mechanotransduction axis, beginning with F-actin disassembly upon spheroid formation, which subsequently promotes NF- κ B signaling, as evidenced by increased p65 phosphorylation.

In the present study, the sequential upregulation of *Col1a2*, *Sp7*, and *Spp1* is consistent with features of the early phase of osteogenic differentiation. Osteogenic differentiation is a tightly regulated temporal process, during which mesenchymal progenitors progress through proliferation, osteogenic commitment, matrix maturation, and mineralization stages.^{29,30} During the commitment stage of *in vitro* 2D culture (days 0–7), *Col1a2* is upregulated as one of the early signals associated with extracellular matrix synthesis in osteoblast progenitors,^{31,32} followed by the expression of *Sp7*, *Spp1*, and *Bsp* in pre-osteoblasts.^{32–34} Typically, this characteristic cascade takes 3–4 weeks to occur in traditional 2D osteogenic cultures, which rely on supraphysiological concentrations of synthetic inducers over extended periods. In contrast, the findings of the present study demonstrate that 3D self-assembly alone is sufficient to induce an early osteogenic response. We observed a significant upregulation of osteogenic markers *Col1a2* at 12 h, *Sp7* at 24 h, and *Spp1* at 48 h. Notably, *Runx2*, the canonical “master switch” of osteogenesis³⁵, undergoes upregulation in the 3D group after seven days, indicating that osteogenic commitment may occur at a later stage.

The findings reveal that the activation of NF- κ B signaling promotes the spontaneous early upregulation of osteogenic markers in 3D spheroids, as evidenced by increased phosphorylation of the transcriptionally active p65 subunit and the upregulation of *Nfkb1a*, *Tnfaip3*, *Nfkb1*, and *Rela*. Among these genes, *Nfkb1a* and *Tnfaip3* are established direct transcriptional targets of canonical NF- κ B, and the upregulation of *Rela* and *Nfkb1* likely represents secondary feedback effects.³⁶ A previous study utilizing proteomic analysis revealed significant enrichment of the tumor necrosis factor- α /NF- κ B axis in a 3D osteogenic model,³⁷ which aligns with our findings. The NF- κ B signaling pathway is known to regulate immune responses, inflammatory reactions, cell proliferation, apoptosis, and development.³⁸ Upon activation of its canonical pathway, NF- κ B dimers (p50/p65) are released from inhibitory complexes and rapidly translocate into the nucleus to initiate transcription of target genes.³⁸ The role of NF- κ B in osteogenic differentiation has been controversial in previous studies. Some research indicates that NF- κ B inhibits osteogenesis in mesenchymal progenitors,^{39–41} while other studies demonstrate its supportive role in

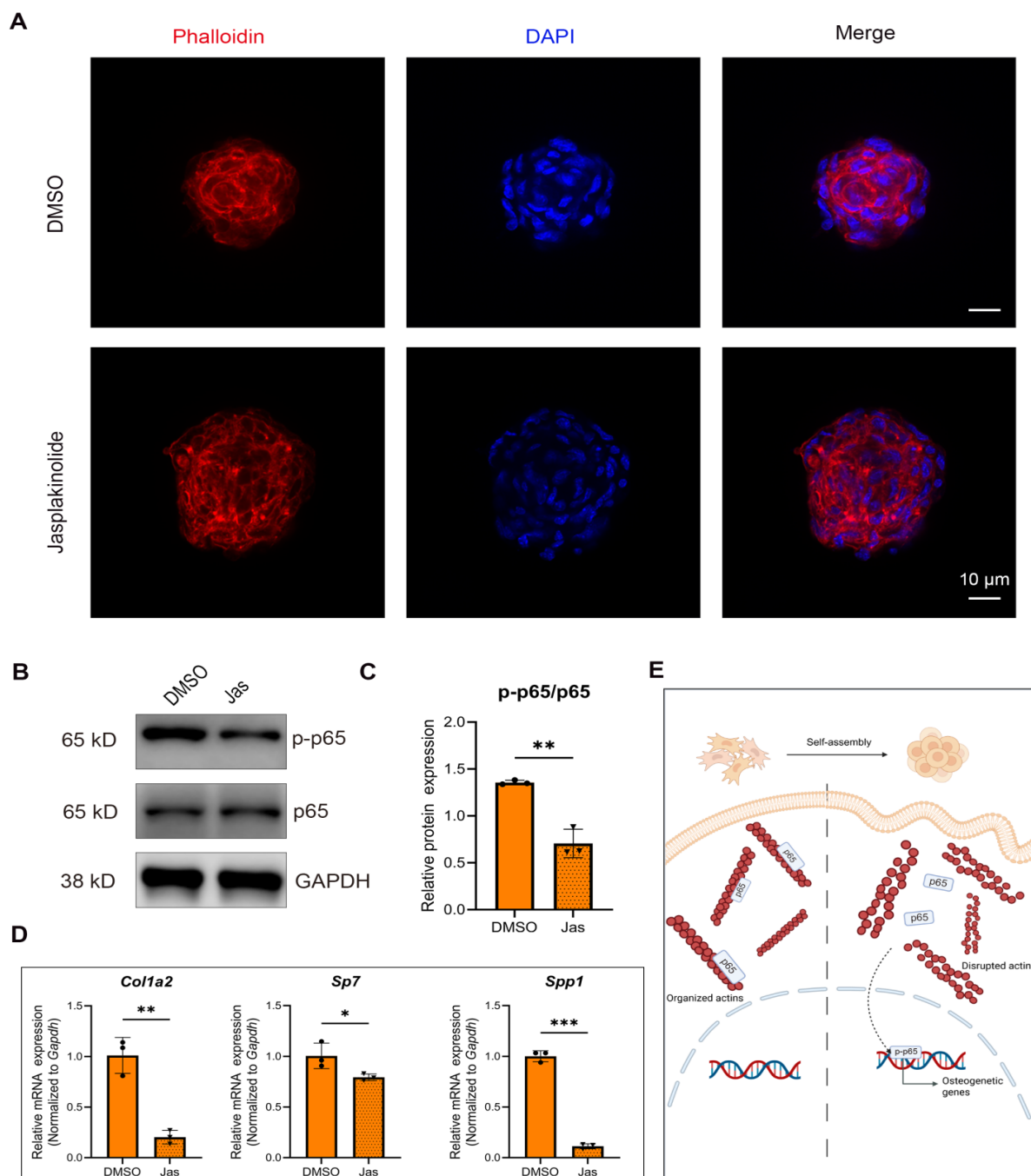


Figure 5. F-actin remodeling promotes NF- κ B-mediated osteogenic gene expression in mouse jb-PDC spheroids. (A) Representative confocal microscopy images of jb-PDC spheroids cultured with or without actin filament stabilizer (50 nM jasplakinolide) for 48 h, showing F-actin organization by phalloidin staining (red) and nuclei by DAPI staining (blue) (scale bar = 10 μ m, magnification = 40 $\times$). (B) Western blot analysis of p-p65 and total p65 in jb-PDC spheroids cultured with or without 50 nM jasplakinolide for 6 h. (C) Densitometric quantification of the ratio of the p-p65-to-total p65. (D) Relative mRNA expression levels of the osteogenic marker genes *Col1a2*, *Sp7*, and *Spp1* in jb-PDC spheroids after culture with or without 50 nM jasplakinolide for 48 h. (E) Schematic diagram illustrating the proposed mechanism by which spheroid formation promotes early osteogenic gene expression through F-actin remodeling and subsequent activation of the NF- κ B pathway. Created in BioRender. liu, Y. (2026) <https://BioRender.com/158p9au>. Statistical analyses between two groups were performed using Student's *t*-test. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 indicate statistical significance.

Abbreviations: 2D: Two-dimensional; 3D: Three-dimensional; BAY: BAY11-7082; DMSO: Dimethyl sulfoxide; Dip: Diprovocim; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NF- κ B: Nuclear factor κ -light-chain-enhancer of activated B cells; Jas: Jasplakinolide; jb-PDC: Jawbone periosteum-derived cell; p-p65: Phosphorylated p65.

osteogenic progression.^{42,43} This discrepancy likely arises from differences in cell types, microenvironmental context, and the spatiotemporal dynamics of NF- κ B activation.

Additionally, the findings reveal that the activation of the NF- κ B signaling pathway is associated with actin cytoskeleton remodeling. The p65 subunit of NF- κ B can directly bind to F-actin, concentrating at focal adhesions and stress fibers in adherent cells.⁴⁴ Previous studies have shown that disruption of the actin cytoskeleton leads to NF- κ B activation.^{45,46} In contrast to cells adhering to planar rigid substrates in 2D culture, cells within 3D spheroids experience reduced stress fiber formation due to altered extracellular matrix distribution and reduced mechanical tension.^{47,48} In the present study, the pharmacological stabilization of F-actin using jasplakinolide significantly reduced p65 phosphorylation and attenuated the osteogenic gene expression program. These results support a mechanistic link between 3D morphological reorganization, F-actin remodeling, and early osteogenic gene expression. Moreover, our findings align with emerging evidence that actomyosin-generated forces can remodel F-actin architecture, regulating the accessibility of mechanosensitive signaling proteins,⁴⁹ thereby converting mechanical cues into biochemical signals.

There are multiple differences between 3D spheroid and 2D monolayer cultures that may collectively influence gene expression and signaling. Cell density is one of the most evident differences. To exclude this confounder, we established a smaller 3D spheroid group with the same cell density as the 2D group. Even at identical cell density, the 3D spheroids still exhibited significantly higher mRNA levels of osteogenesis-related genes than the 2D cultures, indicating that cell density is not the primary driver of the observed responses. In this study, the spheroid diameter was approximately 50 μ m, well within the effective diffusion limit.⁵⁰ No obvious central necrosis was observed within the 48-h window, suggesting that cell viability was not substantially compromised. Additionally, the morphological changes and aggregation observed within 3D spheroids are consistent with cytoskeletal remodeling, including F-actin reorganization.⁵¹

Taken together, this study provides evidence that self-assembly of jb-PDCs into spheroids can induce an early osteogenic response within 48 h. We identified the F-actin/NF- κ B signaling axis as a potential mechanistic pathway underlying this response.

However, we acknowledge several limitations in our study. First, although we observed upregulation of the osteogenesis-related genes *Col1a2*, *Sp7*, and *Spp1* within 48 h, and *Runx2* at 7 days, our self-assembled spheroids should be viewed as an early-stage simplified system with

spontaneous osteogenic responses, rather than a fully mature bone organoid.

Our model provides a useful platform for capturing early osteogenic responses in a controlled 3D context and for supporting high-throughput screening and mechanistic studies. This system may be further developed toward more advanced organoid-like models, with the addition of other cell types or the integration of microfluidic technologies to increase multi-cellular complexity and spatial compartmentalization. Second, our pharmacological inhibition experiment indicated a mechanistic association between actin filament stabilization and downstream signaling. However, quantitative measurements of cytoskeletal tension and spheroid mechanical properties are needed to fully elucidate the underlying mechanotransduction mechanisms.

Overall, this study offers new insights into how biophysical cues from 3D spheroids can trigger endogenous osteogenic gene expression, highlighting the jb-PDC spheroids as a unique platform for dissecting the mechanobiology of osteogenic initiation and for developing biomimetic strategies for craniofacial bone repair.

5. Conclusion

In summary, this study demonstrates that self-assembly of mouse jb-PDCs triggers rapid osteogenic gene expression within 48 h under induction-free culture conditions. We identified the F-actin/NF- κ B signaling axis as a key pathway associated with these early osteogenic responses. These findings reveal that the physical event of 3D condensation is associated with an early osteogenic priming program. Our findings offer new insights into how biophysical cues govern the early phase of bone formation.

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Conflict of interest

The authors declare they have no competing interests.

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Writing—review & editing: All authors

Ethics approval and consent to participate

All animal experimental procedures were approved by the Animal Ethics Committee of the School of Stomatology, Wuhan University, China (Approval No. Wp20230204).

Consent for publication

Not applicable.

Availability of data

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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