

Scaffold Geometry Governs Mechanobiological Responses to Combined Biophysical Stimulation in Human Bone Tissue-Like Models

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Abstract

Advanced *in vitro* platforms replicating the native bone microenvironment are essential for optimizing regenerative therapies. In this study, we introduced two bone tissue-like models composed of human bone marrow-derived mesenchymal stem cells seeded onto two types of 3D-printed polylactic acid scaffolds, designed to mimic the morphological features of the trabecular bone of the iliac crest (PLA600) and those of the ulna, tibia, and femur (P3S3). To replicate the physiological environment, the bone tissue-like models were cultured within an automated multi-physical stimuli bioreactor that combines tunable fluid flow-induced shear stress, intermittent hydrostatic pressure, and pulsed electromagnetic field stimulation. We investigated how scaffold geometry influences bone marrow-derived mesenchymal stem cells mechanosensing and physical cue processing under intermittent hydrostatic pressure (15 kPa peak, 2 h/day), pulsed electromagnetic field stimulation (75 Hz, 1.5 mT, 4 h/day), and their sequential combination for 10 days. Bone marrow-derived mesenchymal stem cells exhibited a geometry-dependent sensitivity to the single stimuli, where cells within the P3S3 scaffolds were predominantly responsive to intermittent hydrostatic pressure, whereas those in the PLA600 scaffolds were highly sensitive to pulsed electromagnetic field stimulation. Notably, the geometry-dependent response gaps were mitigated by the sequential combination of intermittent hydrostatic pressure and pulsed electromagnetic field stimulation. We hypothesize that piezoelectricity and inductive coupling drive this synergy, initiating early osteogenesis via different, yet complementary molecular pathways. These findings establish a mechanistic rationale for designing personalized biophysical therapies tailored to specific bone microenvironments and anatomical sites.

1. Introduction

Trabecular bone is a highly mechanosensitive and metabolically active tissue, whose microarchitecture and composition vary between anatomical locations and with age ¹. Together with whole bone geometry, the microarchitectural and compositional variations are primary determinants of bone strength and fragility ². Although the mechanical strength of bone relies on an interplay of multiple factors, the specific effects of microarchitecture are the most frequently studied. Studies have shown that microarchitecture plays a crucial role in bone fragility, independent of bone density ³.

Another critical factor contributing to bone strength or fragility is mechanical stimulation.

Bone continuously adapts its microarchitecture and composition in response to mechanical loading, such as weight-bearing exercise ⁴, as well as its opposite, mechanical unloading, such as bed rest or spaceflight ⁵. This bone adaptation process follows three core principles outlined by Charles H. Turner (1998), which are: (1) dynamic loading, which means that bone responds to movement and activity, not stillness; (2) quick response, which reflects that even brief periods of stress can trigger bone growth or repair; and (3) adaptation to routine, in which bone adjusts to regular patterns of stress, becoming less sensitive to familiar loads ⁶.

Mechanical stimuli encompass the mechanical properties of the extracellular matrix, and mechanical stimuli intrinsic to bone tissue including fluid flow-induced shear stress (FSS), hydrostatic pressure (HP), compression, and tension⁷. Specifically, mechanical loading generates interstitial fluid flow through poroelastic flow within the lacunar-canalicular network and via intramedullary pressurization^{8,9}. While the former primarily generates flow within the bone matrix, the latter induces significant flow at both the endosteal surface and within the tissue¹⁰. This mechanical environment, characterized by FSS and HP¹¹, is particularly influential during bone healing, which naturally relies on the recruitment of circulating mesenchymal stem cells (MSCs) to the injury site. While the mechanisms of MSC homing are not fully understood, studies have demonstrated that the biochemical and mechanical microenvironments synergistically induce and promote osteogenesis. Thus, the biochemical microenvironment, composed of cytokines and chemokines, and the mechanical microenvironment, characterized by mechanical cues, both play significant roles in this process¹².

Historically, the impacts of these forces have been studied through various *in vitro* studies examining different types of mechanical loading (compressive pressure, tensile strain, and fluid shear stress)^{13,14} and *in vivo* studies examining various types of movement (interfragmentary, tensile, or shear)¹⁵. Moreover, exercise intervention programs have been shown to reduce bone loss or increase bone mass regardless of gender, age, and initial bone health status, which is driven by the interplay between cellular responses to hormonal stimuli and mechanical load stimuli¹⁶. Furthermore, today, biophysical stimuli are being recognized as a “fourth pillar”, alongside scaffolds, cells, and bioactive molecules, in bone tissue engineering¹⁷. This fundamental understanding has also driven the clinical translation of exogenous physical stimuli to non-invasively manipulate and accelerate bone healing^{18,19}.

However, findings from *in vitro* models have not been fully validated *in vivo*, due to the significant differences among these models. Thus, identifying mechanical stimuli-responsive molecular targets and developing clinical therapies targeting these molecules requires further research to bridge this gap, and thus, standardize mechanical parameters²⁰. Consequently, accurate knowledge of site-specific bone architecture and its local mechanical environment is required to build advanced *in vitro* technologies. As such, bioreactors that can accurately replicate the *in vivo* bone mechanical environment and optimize MSC-mediated bone tissue regeneration are considered promising tools, especially when they can host 3D bone tissue-like constructs. While existing *in vitro* investigation platforms cannot satisfy all the requirements to fully replicate the native bone tissue environment, significant progress has been made in the design and application of bioreactors that enhance artificial bone tissue grafts^{21,22}, and advance fundamental bone biology research^{23–25}.

In line with the movement towards more representative 3D bone tissue-like constructs and bioreactors that better mimic the mechanical environment of bone tissue, the primary

objective of our study was to generate basic knowledge on how human bone marrow-derived MSCs (BM-MSCs) respond to a single or a combination of physical stimuli when cultured in 3D bone tissue-like scaffolds replicating the architecture of healthy trabecular bone of two different anatomical positions. We employed an automated, perfusion bioreactor, previously described by the authors^{26,27}, which was upgraded to deliver combinations of FSS, intermittent HP, and pulsed electromagnetic field (PEMF) stimulations. We utilized two 3D-printed polylactic acid (PLA) scaffolds designed to mimic the human trabecular bone architecture in physiological conditions: PLA600, resembling the morphological features of the iliac crest, and P3S3, resembling the morphological features of long bones (ulna, tibia, and femur). Cell-seeded scaffolds were cultured under continuous perfusion and exposed to FSS alone or combined with intermittent HP simulating physiological loading at 15 kPa peak for 2 h/day; PEMF stimulation (1.5 mT, 75 Hz) for 4 h/day; and the combination of intermittent HP and PEMF stimulation for 10 days. To evaluate the early transcriptional responses of human BM-MSCs to single and combined physical cues, gene expression analysis was conducted.

2. Results

2.1 Validation of cell viability and infiltration within P3S3 scaffolds cultured under perfusion

Building upon our previous findings regarding the PLA600 scaffolds²⁶, our first objective was to evaluate the ability of the P3S3 scaffolds to ensure cell viability and infiltration under continuous, direct perfusion within the bioreactor. Our results showed that the P3S3 scaffold architecture supported the cytocompatibility of BM-MSCs and deep cellular infiltration within its porous structure when cultured within the bioreactor. This validation is essential to demonstrate that the transition to the P3S3 scaffold architecture maintained robust cell support for long culture periods established in our previous work with the PLA600 scaffolds²⁶.

First, the resazurin reduction assay (**Supplementary Fig. 1**) revealed that the metabolic activity of BM-MSCs cultured within the P3S3 scaffolds under continuous DP was significantly higher ($\approx 12,000$ RFU) in comparison to static culture ($\approx 5,500$ RFU) ($p < 0.05$). These quantitative findings were confirmed by the qualitative live/dead assay (**Supplementary Fig. 2**), which showed a higher density of live cells (green fluorescence) throughout the scaffold's core, with negligible dead cells (red fluorescence). This dense internal cell infiltration was further confirmed by SEM analysis (**Supplementary Fig. 2**), in which P3S3 scaffolds cultured under continuous DP exhibited confluent cell sheets at deep depths within the scaffolds.

2.2 Impact of intermittent HP on the mechanotransduction within BM-MSCs cultured in two different trabecular bone tissue-like scaffolds

Prior to analyzing the transcriptional profiles, cell viability was assessed to ensure sample quality (see **Supplementary Fig. 3**). As shown in Fig. 1 and Fig. 2, there is a geometry-dependent response of BM-

MSCs to intermittent HP (15 kPa peak, 2 h/day for 10 days). After 10 days of culture, there was a more evident osteogenic shift in the cells cultured within the P3S3 scaffolds, characterized by a $\approx$ 2-fold change in ALP. Moreover, compared to the PLA600 scaffolds, BMP signaling was initiated in the P3S3 group, with observed fold changes of $\approx$ 2- and 1.5 for BMP4 and BMP6, respectively. Interestingly, MMP3 was upregulated in the P3S3 and the PLA600 groups with $\approx$ a 2-fold change, while the expression of MMP6 was not modified in either.

Another key finding is the significant activation of the YAP/TAZ mechanosensing switch in the P3S3 group. The concurrent increase of $\approx$ 2-fold change in the expression of YAP1, TAZ1, and CTGF can suggest that the P3S3 geometry enhances the cells' ability to sense and respond to intermittent HP. Regarding the additional membrane-bound and intracellular signaling targets evaluated in our study, the Piezo1, MAPK1, and JAK1 genes were upregulated by $\approx$ 1.5-, 2-, and 2-fold change, respectively, in the P3S3 group.

Concurrently, slight upregulation of SNAI1 ($\approx$ 1.5-fold change) and TWIST2 ($\approx$ 2-fold change) were detected in the P3S3 group. Screening for inflammatory markers showed no elevated expression of pro-inflammatory targets in either the P3S3 or PLA600 groups under intermittent HP stimulation.

2.3 Impact of PEMF stimulation on the mechanotransduction within BM-MSCs cultured in two different trabecular bone tissue-like scaffolds

Prior to analyzing transcriptional profiles, cell viability was assessed to ensure sample quality (see **Supplementary Fig. 3**). Similar to biomechanical stimuli, our results showed that the biological efficacy of biophysical stimuli is intrinsically tied to the scaffold's microarchitecture, since within the PLA600 scaffolds, BM-MSCs showed divergent responses when exposed to PEMF stimulation (75 Hz, 1.5 mT, 4 h/day for 10 days) in comparison to the P3S3 group. While previous research has established the general osteogenic potential of PEMF stimulation, our results indicate that the PLA600 scaffolds are significantly more sensitive to this specific stimulus than the denser P3S3 scaffolds.

As shown in Fig. 3 and Fig. 4, in the PLA600 scaffolds, PEMF stimulation elicited a significant upregulation of OPN ($\approx$ 2.2-fold change). BMP signaling was also more pronounced in the PLA600 group, except for BMP4, which was upregulated similarly in both groups ($\approx$ 1.5-fold change in both groups). For the PLA600 group, the values were $\approx$ 3- and 1-fold change for BMP2 and BMP6, respectively. Furthermore, the PLA600 group showed a massive upregulation of MMP3 ($\approx$ 24-fold change) and a significant upregulation of MMP9 ($\approx$ 4-fold change).

Regarding the mechanosensitive pathways, only in the PLA600 group, PEMF caused upregulations of MAPK1 ($\approx$ 2-fold change), YAP1 ($\approx$ 1.5-fold change), and TAZ1 ($\approx$ 1.3-fold change). Finally, neither the PLA600 nor the P3S3 group exhibited a marked upregulation of the EMT-like state, cell motility, and the inflammatory cascade.

2.4 Synergistic effects of consecutive intermittent HP and PEMF stimulation on the mechanotransduction within BM-MSCs cultured in two different trabecular bone tissue-like scaffolds

Prior to analyzing transcriptional profiles, cell viability was assessed to ensure sample quality (see **Supplementary Fig. 3**). While BM-MSCs cultured within the P3S3 scaffolds responded better to the biomechanical stimulation provided as intermittent HP with a peak of 15 kPa for 2 h/day for 10 days, and those cultured within PLA600 scaffolds responded better to the biophysical stimulation provided as PEMF stimulation (75 Hz, 1.5 mT) for 4 h/day for 10 days, the sequential exposure of cells to intermittent HP and PEMF stimulation, generated response in cells cultured within both scaffolds, PLA600 and P3S3.

As presented in Fig. 5 **and** Fig. 6, early osteogenesis was initiated in BM-MSCs, with a ≈ 2.5 -fold change in RUNX2 in both groups. Moreover, our results showed a ≈ 2 -fold change in ALP only in the P3S3 group, and a ≈ 4 -fold change in COL1A1 only in the PLA600 group. Yet, no changes were observed in the expression levels of the late osteogenic genes OCN and OPN in both groups. Regarding BMP signaling, there were significant differences between the two groups, since the P3S3 group showed ≈ 3.4 -, 6-, and 4-fold changes in the BMP2, BMP4, and BMP6 genes, respectively, whereas the PLA600 group showed ≈ 1.5 - and 1.3-fold changes in the BMP4 and BMP6 genes, respectively. Similar to BMP signaling, the cells exhibited a different pattern of gene expression related to matrix remodeling, with only the P3S3 group showing $\approx$ a 7.4-fold change in the MMP3 gene, and the PLA600 group showing $\approx$ a 3-fold change in the MMP9 gene.

Our results also demonstrated that BM-MSCs cultured within the PLA600 scaffolds showed upregulations in motility and EMT-associated genes with upregulations in TGFB1 (≈ 3 -fold change), SNAI1 (≈ 6.3 -fold change) and TWIST2 (≈ 2.5 -fold change). On the other hand, lower upregulations were observed in the P3S3 group, with $\approx$ a 1.5-fold change in TGFB1 and $\approx$ a 1.7-fold change in TWIST2. A similar pattern emerged when comparing the proinflammatory marker profiles of the PLA600 and P3S3 groups. Specifically, we observed upregulations in IL6 (≈ 2 -fold change) and PTGS2 (≈ 1.6 -fold change) in the P3S3 group only, and an upregulation in NFKB1 (≈ 1.7 -fold change) in the PLA600 group only.

The observed molecular shifts are directly related to the mechanosensing capacity of BM-MSCs and the downstream intracellular signaling pathways they trigger. In the PLA600 group, mechanotransduction markers YAP1 (≈ 2 -fold change) and CTGF (≈ 1.8 -fold change) increased more than in the P3S3 group (≈ 1 -fold change for both). On the other hand, Piezo1, MAPK1, and JAK1 were more upregulated in the P3S3 group (≈ 2.5 -, 1.5-, and 1.8-fold changes, respectively) than in the PLA600 group (≈ 1.8 -, 1-, and 1-fold changes, respectively).

3. Discussion

The primary objective of our study was to evaluate how the physical microarchitecture of 3D scaffolds modulates the mechanotransduction cascades of BM-MSCs cultured in the presence of different types of physical stimuli and their sequential combination. By utilizing a customized, automated and

parallelized multi-physical stimuli perfusion bioreactor, we successfully demonstrated that scaffold architecture alters cell fate.

Our initial validations confirm that the P3S3 scaffold architecture effectively facilitated nutrient exchange and metabolic waste removal under flow conditions, which is crucial for overcoming the diffusion limitations observed in static 3D cultures²⁸. Furthermore, the increased metabolic activity further supports the role of FSS in stimulating cell proliferation²⁹.

When subjected to intermittent HP (15 kPa peak, 2 h/day for 10 days), BM-MSCs responded in a geometry-dependent manner. The osteogenic shift and activation of BMP signaling in the P3S3 group aligns with the concepts established by Long et al. (2025), who introduced and validated the role of scaffold architecture-induced stress stimulation (SASS) in the recruitment and behavior of bone cells, specifically BM-MSCs, osteoblasts, and osteoclasts. The authors showed that the scaffold architecture determines the mechanical microenvironment of bone cells through the dynamic compression and recovery of the scaffolds³⁰. The hypothesis was also tested under dynamic perfusion in a bone-on-a-chip model, where Kim et al. (2025) showed that geometric parameters modulate the wall shear stress pre-osteoblasts experience, which in turn alters infiltration, ALP activity, calcium deposition, and collagen formation³¹. Interestingly, the isolated upregulation of MMP3, combined with the absence of osteogenic commitment in the PLA600 group, may suggest that the specific geometry of the PLA600 scaffolds, when subjected to a 15 kPa peak HP, creates a mechanical environment that favors an active ECM remodeling phase rather than immediate osteogenic commitment.

Clinically, it has been previously proved that “prescribing” the correct exercise program is crucial because different programs can either promote or reduce bone mass. Furthermore, the bone mass in lower extremities can be maintained by weight-bearing and periodic high-intensity loading exercises³². However, clinical studies still did not reach a consensus on the pattern of changes in basal bone remodeling markers in relation to long-term exercise due to the small sample sizes, age of the participants, and time of sample collection. The latter are all factors that hinder reaching a consensus on expected changes in bone formation and resorption markers in relation to physical activity³³. Furthermore, clinical studies have demonstrated that brief, low- or high-intensity exercise may not have an immediate effect on bone mass, and in certain cases, may even cause a decrease in bone formation markers that can persist to 3 to 5 days after long duration (< 2 h) high-intensity exercise³³. Our results align with clinical studies, since the 10-day exercise simulation regimen altered the expression only of the bone formation biomarker, ALP, in the bone tissue-like model mimicking long bones (P3S3 group), but not in the one mimicking the iliac crest (PLA600 group). Moreover, a longer period of culture is required to stabilize a bone remodeling state specific to each bone tissue-like model. Thus, are the effects of physical activity on bone formation in various anatomical regions contributing to the results obtained in clinical studies?

At the molecular level, the P3S3 group uniquely showed an activation of the YAP/TAZ mechanosensing switch. The role of YAP/TAZ nuclear accumulation in driving osteogenic differentiation is well-

documented in bone tissues that operate in extremely stiff mechanical environments due to substrate rigidity and mechanical loading ³⁴. The concurrent upregulation of Piezo1, MAPK1, and JAK1 genes within the P3S3 group further confirms the activation of a comprehensive mechanotransduction axis. It is well stated in the literature that Piezo1 acts as a mechanosensory receptor that translates HP into biochemical signals, specifically activating the ERK/p38 MAPK pathways to induce BMP2 expression, which shifts the differentiation of mesenchymal stem cells toward osteoblasts ³⁵. The simultaneous upregulation of JAK1 further supports this osteogenic shift, since it has been identified as a vital mediator of extracellular mechanical signals and a necessary regulator for the sustained differentiation and matrix mineralization of MSCs as part of the JAK/STAT axis ³⁶. Moreover, the slight upregulation of SNAI1 and TWIST2 genes in the P3S3 group may suggest a regulated maintenance of the mesenchymal progenitor state, which prevents the premature onset of the RUNX2-driven mineralization. Both SNAI1 ³⁷ and TWIST2 ³⁸ act as inhibitors of MSC differentiation in bone tissue, as well as key modulators of RUNX2 function. This concurrent, slight upregulation is consistent with the dual role of TGF- β signaling in early osteogenesis. TGF- β signaling promotes the proliferation and chemotaxis of osteoprogenitor cells, while simultaneously utilizing these transcription factors to prevent premature maturation and mineralization ³⁹.

Conversely, the PLA600 group showed a more pronounced response of PEMF stimulation (75 Hz, 1.5 mT, 4 h/day for 10 days) compared to the P3S3 group. The exclusive upregulations in OPN, BMP2, MMP3, and MMP9 may suggest a powerful activation of ECM remodeling pathways, which aligns with the importance of a dynamic balance between mineralization and remodeling in natural bone healing and regeneration ⁴⁰. The upregulation of MMP3 further characterizes this remodeling state. MMP3 is often linked to the inflammatory states and catabolic effects seen in osteoarthritis ⁴¹. However, in a tissue engineering context, its role may be more nuanced, in which cells may initiate an early-stage matrix remodeling to prepare the cells for osteogenic commitment.

This differential response to PEMF based on scaffold architecture aligns with clinical evidence suggesting that various anatomical sites respond differently to PEMF stimulation. For instance, the commercial devices of Orthofix, Inc. that are approved by the US Food and Drug Administration (FDA) deliver different PEMF signals (burst frequency, pulse frequency, and magnetic field amplitude) based on the target anatomical site ⁴². Several clinical studies failed to prove the beneficial effects of PEMF stimulation on bone healing. Examples include acute scaphoid fractures ⁴³ and acute tibial shaft fractures ⁴⁴. However, it was able to accelerate bone healing and alleviate pain in femoral neck fractures ⁴⁵ and diaphyseal femoral fractures ⁴⁶. Despite the application of various treatment protocols, the latter may suggest that the response to PEMF stimulation is largely related to the anatomical site.

Our molecular analysis confirmed that cells actively translated these electromagnetic fields into biochemical signals. First, the MAPK signaling pathway not only transduces signals from growth factors and adhesion molecules, but also from their physical environment, and BMPs utilize the MAPK pathway, in addition to the Smad one, to activate RUNX2 and Distal-Less Homeobox 5 (Dlx5) ⁴⁷. Furthermore, the

activation of YAP/TAZ confirms that cells actively translated electromagnetic stimuli into biochemical signals via their physical microenvironment ⁴⁸.

The most striking finding of our study is the outcome of the sequential combination of intermittent HP and PEMF stimulation. This combination of stimuli helped cells overcome the limitations associated with single stimulation in both microarchitectural environments, since osteogenesis was initiated in both groups via the activation of RUNX2. Furthermore, the activation of TGFB1, paired with the subsequent upregulation in SNAI1 and TWIST2 exclusively in the PLA600 group, can indicate that the coupling of mechanical and physical stimulation has the potential to induce a “Type 2 EMT” program geared toward tissue regeneration. The latter is different from the “Type 1 EMT” program that governs embryogenesis and organ development, or “Type 3 EMT”, which drives oncogenesis and cancer progression ⁴⁹. Conversely, only the P3S3 group showed an upregulation in IL6 and PTGS2. While these markers typically indicate inflammation, prostaglandins and IL-6 are also potent, multifunctional regulators of bone metabolism since they can exert dual stimulatory and inhibitory effects on both bone formation and resorption ^{50,51}. For instance, Knippenberg et al. (2007) showed that different prostaglandins have different effects on adipose-derived MSCs and distinguished prostaglandin F2 α as a promoter of osteogenic differentiation ⁵². Thus, these upregulations in the P3S3 group likely reflect a coordinated signaling network driving osteoinduction rather than a purely chronic inflammatory response. The observed molecular shifts are directly related to the mechanosensing capacity of BM-MSCs and the downstream intracellular signaling pathways they trigger. In the PLA600 group, YAP1 and CTGF increased more than in the P3S3 group; whereas, in the P3S3 group, Piezo1, MAPK1, and JAK1 were more upregulated. Taken together, our results demonstrate that while sequential intermittent HP and PEMF stimulation initiates early osteogenic commitment in both groups via RUNX2 activation, the underlying molecular mechanisms and ultimate cellular fates differ significantly based on the scaffold architecture.

Notably, this is the first *in vitro* platform to combine intermittent HP simulating physiological loading with PEMF stimulation. While this novelty limits direct *in vitro* comparisons with existing literature, our findings align conceptually with clinical evidence exploring dual physical therapies. Some clinical trials were performed to investigate whether these dual therapies ameliorate bone-related diseases. For instance, randomized clinical trials have demonstrated that combining moderate-intensity aerobic exercise with either magnetic therapy in female osteoporotic cohorts ⁵³ or full-body pulsed electromagnetic fields in male osteoporotic cohorts ⁵⁴ significantly enhances new bone formation compared to exercise alone.

Ultimately, to explain why this dual stimulation successfully drives early osteogenic commitment across the architecturally different physical environments, we propose an electromechanical priming hypothesis. Since PLA exhibits low macro-scale piezoelectricity, it may generate transient electrical charges upon the application of mechanical stress (e.g., FSS and intermittent HP), which can slightly mimic the endogenous piezoelectricity of native bone derived from collagen fibers ⁵⁵. Furthermore,

PEMF stimulation exerts its effects via inductive coupling, wherein the time-varying magnetic field generates a secondary localized electric field within the biological microenvironment that modulates cellular behavior^{56,57}. Consequently, the sequential intermittent HP and PEMF applications can counteract the structural limitations of each scaffold geometry by directly altering both transmembrane potential and mechanosensitive signaling. This dual activation unlocks separate but equally effective downstream cascades, which trigger distinct osteogenic pathways that depend entirely on the structural properties of the scaffolds.

Despite these promising findings, we acknowledge some limitations of our study. First, the evaluation was restricted to a relatively short culture period, which captures early-stage gene expression dynamics rather than long-term protein synthesis and ECM mineralization. Consequently, our conclusions remain largely focused on transcriptional regulations. Second, given the complexity of the multi-physical stimuli system and the high number of baseline variables, the intermittent HP was evaluated at a fixed physiological loading level of 15 kPa, without assessing variations in FSS magnitudes. Third, the extensive number of experimental conditions hindered the performance of high-throughput multi-omics analyses, which could provide a more holistic understanding of the underlying global molecular patterns. Addressing these factors in future studies will be essential to elucidate the long-term osteogenic outcomes of this mechanical-physical stimulation regimen.

4. Materials and methods

4.1 3D printing and characterization of two biomimetic polylactic acid (PLA) scaffolds

The design and fabrication of the 3D-printed, biomimetic PLA scaffolds followed a previously established protocol^{58,59}. Briefly, virtual porogens (spheres) with diameters of 600 μm (for PLA600) and 300 μm (for P3S3) were randomly distributed within a cylindrical volume (10 mm diameter, 5 mm height) for preparing the scaffold computer-aided design (CAD) models. The resulting files were imported into ideaMaker (Raise3D Inc., Irvine, CA, USA) and sliced in the Z direction with a 0.25 mm step. Scaffolds were then fabricated by processing a PLA filament (FormFutura BV, The Netherlands) on a Raise 3D N2 printer (Raise 3D Inc., Irvine, CA, USA), with the nozzle temperature set to 205°C and the build plate temperature to 60°C. The physical characteristics of the resulting scaffolds that mimic the human trabecular bone architecture under physiological conditions are summarized in Table 1.

Table 1
Physical characteristics of PLA600 and P3S3 scaffolds.

Scaffold	Trabecular Spacing	Trabecular Thickness	Total Porosity	Anatomical Position
PLA600	531 μm	350 μm	60.34%	Iliac crest
P3S3	284 μm	258 μm	44.7%	Long bones (ulna, tibia, and femur)

4.2 Automated multi-physical stimuli bioreactor delivering tunable FSS, intermittent HP, and PEMF stimulation

Experiments were conducted using an upgraded version of a previously described perfusion bioreactor²⁷. Specifically, the multi-physical stimuli bioreactor was developed to deliver combinations of FSS, intermittent HP, and PEMF stimulation to bone tissue-like constructs in an automated, parallelized set-up. Briefly, the device consists of three independent units, each one composed of a 3D-printed culture chamber (CC), designed to house a 3D bone tissue-like construct press-fitted into a custom polydimethylsiloxane (PDMS) holder, and connected to a hydraulic circuit. Each hydraulic circuit is connected to a multichannel peristaltic pump (G100–1 J, Longer Precision Pump, China) and consists of a reservoir, oxygen-permeable tubing (Darwin Microfluidics, France), and, when intermittent HP is required, an automated solenoid pinch valve (MFLX98302-46, Masterflex, Cole Parmer, USA) is positioned downstream of the CC. The constructs housed within the CCs can be exposed to tunable combinations of FSS (range of flow rate $Q = 0.024\text{--}24\text{ mL/min}$) and intermittent HP. A custom Arduino-based controller allows for precise regulation of the HP stimulation period ($T = 0.25\text{--}30\text{ s}$) and duty cycle ($DC = 1\text{--}100\%$), enabling the delivery of bone tissue-like HP waveforms with peak pressure ranging from 3 to 35 kPa (Fig. 7A, 7B). In addition, to deliver PEMF stimulation to the cultured constructs, the bioreactor is equipped with a PEMF stimulator (IGEA Clinical Biophysics, Italy), consisting of a generator and two solenoids, between which the CCs can be placed (magnetic field intensity = 1.5 mT, frequency = 75 Hz) (Fig. 7C, 7D).

The mean permeability values of the PLA600 and P3S3 scaffolds were obtained using an adaptable test bench, previously described by the authors⁶⁰, resulting in $2.36 \times 10^{-10}\text{ m}^2$ and $2.14 \times 10^{-10}\text{ m}^2$, respectively. These values were used to calculate the FSS values developed within the constructs housed in the CC, depending on the flow rate imposed by the pump. Specifically, at $Q = 0.3\text{ mL/min}$, the FSS values developed within the scaffolds were 6.02 mPa for PLA600 scaffolds and 8.54 mPa for P3S3 scaffolds. The transport properties of the PLA600 and P3S3 scaffolds are summarized in Table 2.

Table 2
Transport characteristics of PLA600 and P3S3
scaffolds.

Scaffold	Permeability	FSS at 0.3 mL/min
PLA600	$2.36 \times 10^{-10} \text{ m}^2$	6.02 mPa
P3S3	$2.14 \times 10^{-10} \text{ m}^2$	8.54 mPa

4.3 P3S3 trabecular bone tissue-like model preparation and evaluation under perfusion

4.3.1 Cell culture conditions

Human telomerase reverse transcriptase (hTERT)-immortalized BM-MSCs of clonal line Y201, which was generated by James et al.⁶¹, were cultivated in basal medium that is low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, Inc.) supplemented with 15% fetal bovine serum (FBS, Gibco Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin/streptomycin, Gibco Thermo Fisher Scientific, Inc.), and incubated in a humidified atmosphere with 5% CO₂ at 37°C. The cells were passaged once they reached 80–90% confluency by enzymatic digestion using trypsin/ethylenediamine tetraacetic acid (trypsin/EDTA, Gibco Thermo Fisher Scientific, Inc.) before each assay.

4.3.2 Cell seeding on P3S3 and culturing within the perfusion bioreactor

P3S3 scaffolds were inserted into PDMS holders placed in a 6-well cell culture plate. To reduce the hydrophobicity of the scaffolds before cell seeding, they were washed with PBS (1X). Subsequently, two drops of 5 mg/mL PureCol™ EZ Gel solution (Merck, Italy) were added to the scaffolds to remove residual PBS from the pores. The scaffolds were then incubated at 37°C for 20–30 min to allow the polymerization of collagen, thereby minimizing cell loss. Cells were seeded directly onto the P3S3 scaffolds at a defined cell density of approximately 2×10^6 cells and a total volume of 150 µL, composed of 30 µL of basal medium and 120 µL of 5 mg/mL PureCol™ EZ Gel solution (Merck, Italy). The cell-seeded scaffolds were incubated for 4 h to facilitate cell infiltration and adhesion, then covered with basal medium and incubated overnight. After the overnight incubation, the cell-seeded scaffolds were cultured in two different culture conditions for 21 days: "Static", in which the samples were kept in the cell culture plate and the medium was changed every 3–4 days; and "Direct Perfusion", in which the samples were placed in the automated bioreactor and perfused unidirectionally at a flow rate of 0.3 mL/min with 50 mL of medium, and the medium was changed every 7 days. Samples were evaluated in triplicates (n = 3).

4.3.3 Cell metabolic activity assay

The resazurin reduction assay was performed to assess the cell metabolic activity of human BM-MSCs. Resazurin sodium salt (powder, Merck, Darmstadt, Germany) was used to prepare 1% resazurin solution in 1X PBS, which was diluted in basal medium to a final concentration of 0.015% (v/v) to prepare the working solution before each assay. On day 21 of culture, the cell-seeded scaffolds were removed from the CCs of the bioreactor and placed in a 12-well cell culture plate. 3 mL of resazurin working solution was added to each scaffold. Following a 3-h incubation period, 100 μ L aliquots were used for fluorescence quantification at excitation and emission wavelengths of 530 and 590 nm, respectively, using a microplate reader (Tecan GENios Microplate Reader, Spark, Tecan Trading AG, Männedorf, Switzerland). The results were averaged over three biological replicates ($n = 3$).

4.3.4 Cell viability assay

On day 21 of culture, the viability of human BM-MSCs seeded on the biomimetic P3S3 scaffolds was evaluated via the live/dead cell assay (LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells, Invitrogen Life Technologies). Briefly, the cell-seeded scaffolds were removed from the CCs of the bioreactor, placed in a 24-well cell culture plate, washed with PBS (1X), and then incubated in basal medium containing 2 μ M calcein-AM and 2 μ M ethidium homodimer-1 (EthD-1). After 15 min of incubation at 37°C and 5% CO₂ atmosphere, cells were imaged using a fluorescence microscope (THUNDER Imager 3D Cell Culture, Leica Microsystems) to visualize the green calcein AM that stains live cells and the red EthD-1 that stains dead cells.

4.3.5 Cell infiltration and distribution assessment

On day 21 of culture, the infiltration and distribution of human BM-MSCs were examined using a scanning electron microscope (SEM) (JSM-IT500, JEOL, Italy). For SEM analysis, the cell-seeded scaffolds were removed from the bioreactor CCs, placed in a 24-well cell culture plate, and gently washed with PBS (1X). Cells were then fixed with 2.5% glutaraldehyde at 4°C overnight. After cell fixation, the 2.5% glutaraldehyde was dispensed, and samples were washed with PBS (1X), dehydrated in graded alcohol of 70%, 90%, and 100% (twice) for 30 min each, covered by hexamethyldisilazane (HMDS) for 30 min, and then air-dried in a chemical fume hood. Finally, samples were coated with gold using a sputter coating machine (DII-29030SCTR Smart Coater, JEOL, Italy) before image acquisition.

4.4 Trabecular bone tissue-like model culturing within the multi-physical stimuli bioreactor under simulated rehabilitation programs

4.4.1 Cell seeding

Human BM-MSCs were seeded onto PLA600 and P3S3 scaffolds following the procedure outlined in *Section 2.3.2*. However, to account for the higher total porosity and average pore size of the PLA600 scaffolds, a different cell density and seeding volume were used. Specifically, each PLA600 scaffold was

seeded with 3×10^6 human BM-MSCs in a total volume of 200 μL composed of 30 μL of basal medium and 170 μL of 5 mg/mL PureCol™ EZ Gel solution (Merck, Italy).

4.4.2 Cell culture conditions

Cell-seeded scaffolds were cultured for 10 days under four conditions:

- *Direct Perfusion (DP)*: Continuous perfusion at $Q = 0.3 \text{ mL/min}$.
- *Rehabilitation Program 1 (RP1)*: Continuous perfusion ($Q = 0.3 \text{ mL/min}$) combined with intermittent HP (peak pressure = 15 kPa) for 2 h/day. HP was modulated via the pinch valves by imposing the following settings, depending on the adopted scaffold type:
 - PLA600 scaffolds: $T = 30 \text{ s}$; $DC = 70\%$.
 - P3S3 scaffolds: $T = 10 \text{ s}$; $DC = 70\%$.
- *Rehabilitation Program 2 (RP2)*: Continuous perfusion ($Q = 0.3 \text{ mL/min}$) combined with PEMF stimulation (1.5 mT, 75 Hz) for 4 h/day.
- *Rehabilitation Program 3 (RP3)*: Combined stimulation of RP1 and RP2 sequentially.

4.4.3 Cell metabolic activity assay

As described in Section 2.3.3.

4.4.4 Gene expression analysis

The expression of the genes of interest was assessed using reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from samples using TRIzol™ Reagent (Invitrogen Life Technologies) following the manufacturer's protocol, with an extended cell lysis step of 10 min. RNA yield and quality were determined spectrophotometrically using the NanoDrop One (Thermo Fisher Scientific Inc.). Subsequently, 1 μg of total RNA was reverse transcribed using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) on the Mastercycler X50s (Eppendorf AG). qPCR was performed using 1 ng/ μL of cDNA, SYBR™ Green Universal Master Mix (Applied Biosystems, Thermo Fisher Scientific, Inc.), and gene-specific primers at a final concentration of 2.5 μM . Reactions were run and monitored on a CFX96™ Real-Time System (Bio-Rad Laboratories) according to the manufacturer's standard cycling conditions. Cycle threshold (C_q) values for target genes were normalized to the reference gene, Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (YWHAZ), and relative gene expression was quantified using the $2^{-\Delta\Delta C_q}$ method^{62,63} relative to the control group, which is the DP group.

In addition to standard osteogenic markers (RUNX2, ALP, COL1A1, OCN, OPN), BMP signaling (BMP2, BMP4, BMP6), and matrix remodeling enzymes (MMP3, MMP9), we expanded our profile to investigate the following:

1. YAP/TAZ mechanosensing switch: We analyzed YAP1, WWTR1 (TAZ), and CTGF (CCN2) to assess the mechanosensing switch required for RUNX2 activation.
2. Intracellular signaling: We analyzed Piezo1, JAK1, and MAPK1 (ERK2) to investigate how physical stimuli were transduced through membrane-bound mechanosensors and downstream intracellular signaling cascades.
3. EMT-like state and motility: We tested whether the high MMP expression indicated a migratory “EMT-like” state rather than a secretory osteoblast phenotype, and for this purpose, we analyzed SNAI1 (Snail), TWIST2, and TGFB1.
4. Pro-inflammatory signaling: PTGS2 (COX-2), IL6, and NFKB1 were evaluated to determine if the physical stimuli induced a catabolic rather than an anabolic response.

Primer sequences for all analyzed genes are detailed in **Supplementary Table 1**.

4.5 Statistical analysis

All experiments were run with three biological replicates. Continuous variables were compared by the two-tailed t-test, and statistical significance was defined as a p-value < 0.05. The analysis was conducted using R (R Core Team, 2020) ⁶⁴, RStudio (RStudio Team, 2020) ⁶⁵, and the tidyverse package (Wickham, 2017) ⁶⁶.

Declarations

Conflict of interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, Farah Daou, Andrea Cochis, Stefano Gabetti, Diana Massai, Lia Rimondini; Methodology, Farah Daou, Stefano Gabetti, Andrea Cochis, Lia Rimondini; Investigation, Farah Daou, Shahd Alahmad, Stefano Gabetti, Beatrice Masante; Writing – Original Draft, Farah Daou, Stefano Gabetti; Writing – Review & Editing, all authors; Funding Acquisition, Cristina Bignardi, Diana Massai, Andrea Cochis, Lia Rimondini; Resources, Lia Rimondini, Diana Massai; Supervision, Andrea Cochis, Diana Massai, Cristina Bignardi, Lia Rimondini.

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Figures

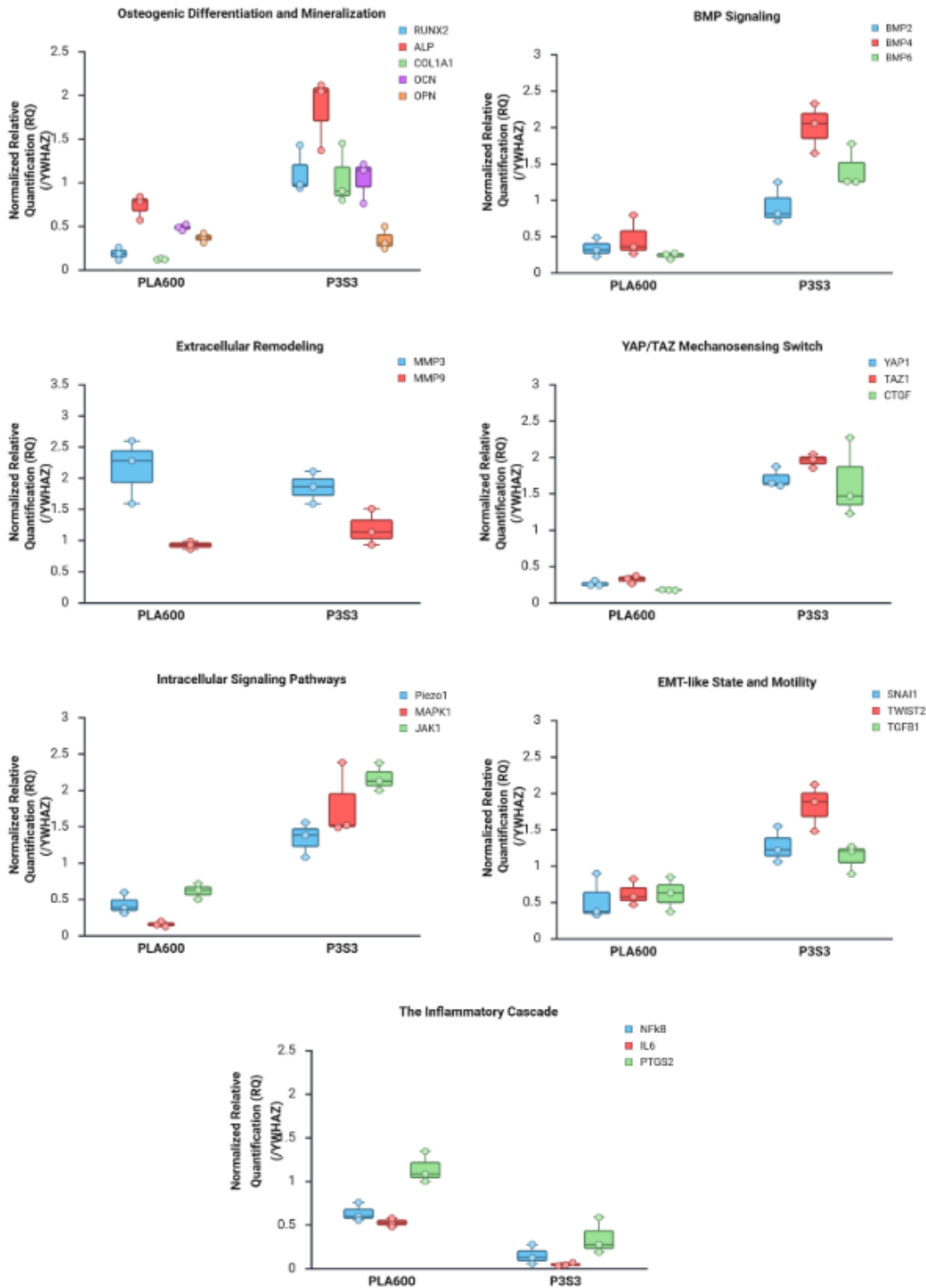


Figure 1

Comparative gene expression profiles of bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured within PLA600 vs. P3S3 scaffolds under perfusion and intermittent hydrostatic pressure (HP). Fold change values represent the expression levels of cells subjected to combined perfusion and intermittent HP (15 kPa, 2 h/day for 10 days), “RP1”, relative to control cells cultured under perfusion alone. Gene expressions are categorized by functional groups: osteogenic markers, BMP signaling,

matrix remodeling, EMT-like state and motility, YAP/TAZ mechanosensing switch, intracellular signaling, and the inflammatory cascade.

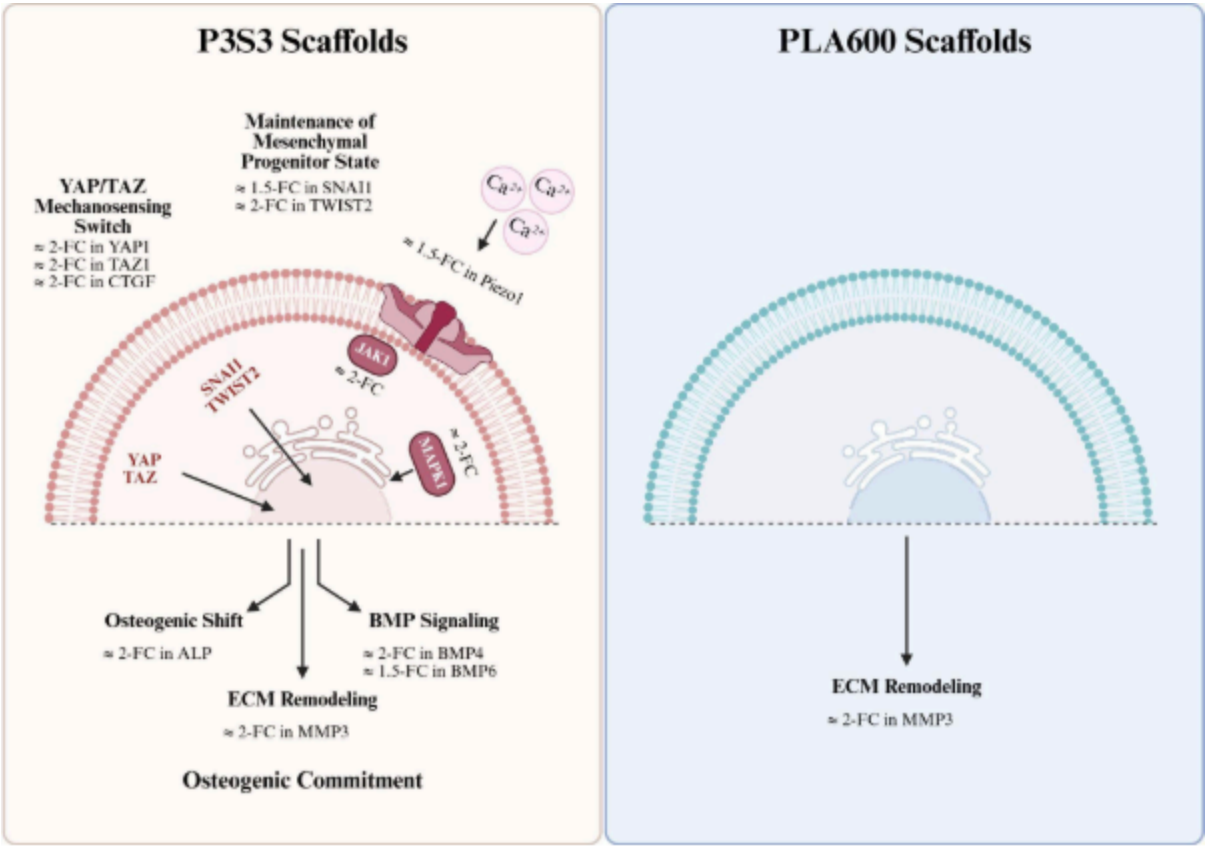


Figure 2

Schematic representation of geometry-dependent mechanotransduction and osteogenic commitment in bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured within PLA600 vs. P3S3 scaffolds cultured under perfusion and intermittent hydrostatic pressure (HP). Comparative cellular and molecular responses of BM-MSCs cultured within P3S3 (left) versus PLA600 (right) scaffolds subjected to combined perfusion and intermittent HP (15 kPa, 2 h/day for 10 days). Values indicate approximate fold changes ($\approx$ FC) in gene expression relative to control cells cultured under perfusion alone.

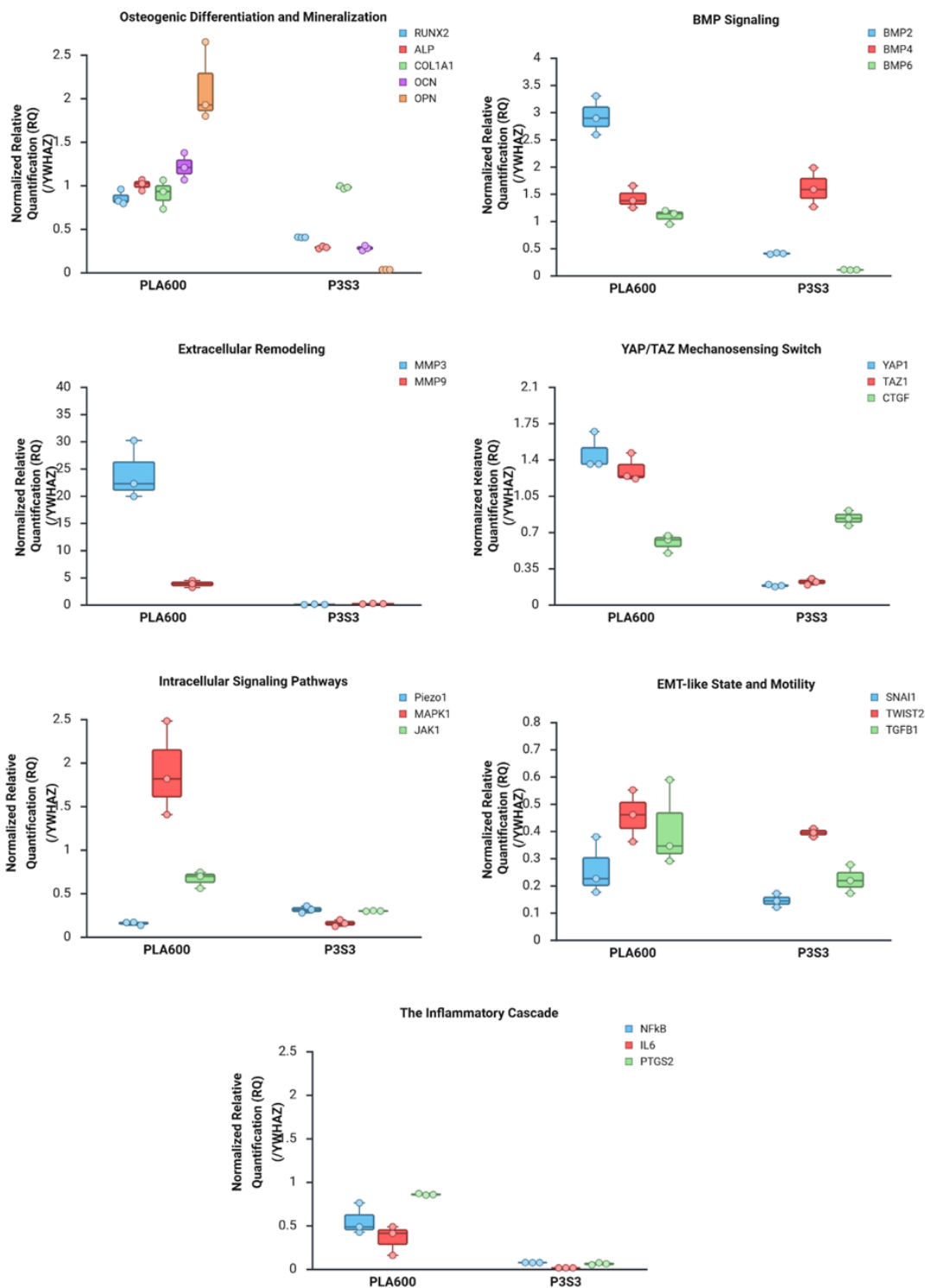


Figure 3

Comparative gene expression profiles of bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured within PLA600 vs. P3S3 scaffolds under perfusion and pulsed electromagnetic field (PEMF) stimulation. Fold change values represent the expression levels of cells subjected to combined perfusion and PEMF stimulation (75 Hz, 1.5 mT, 4 h/day for 10 days), “RP2”, relative to control cells cultured under perfusion alone. Gene expressions are categorized by functional groups: osteogenic

markers, BMP signaling, matrix remodeling, EMT-like state and motility, YAP/TAZ mechanosensing switch, intracellular signaling, and the inflammatory cascade.

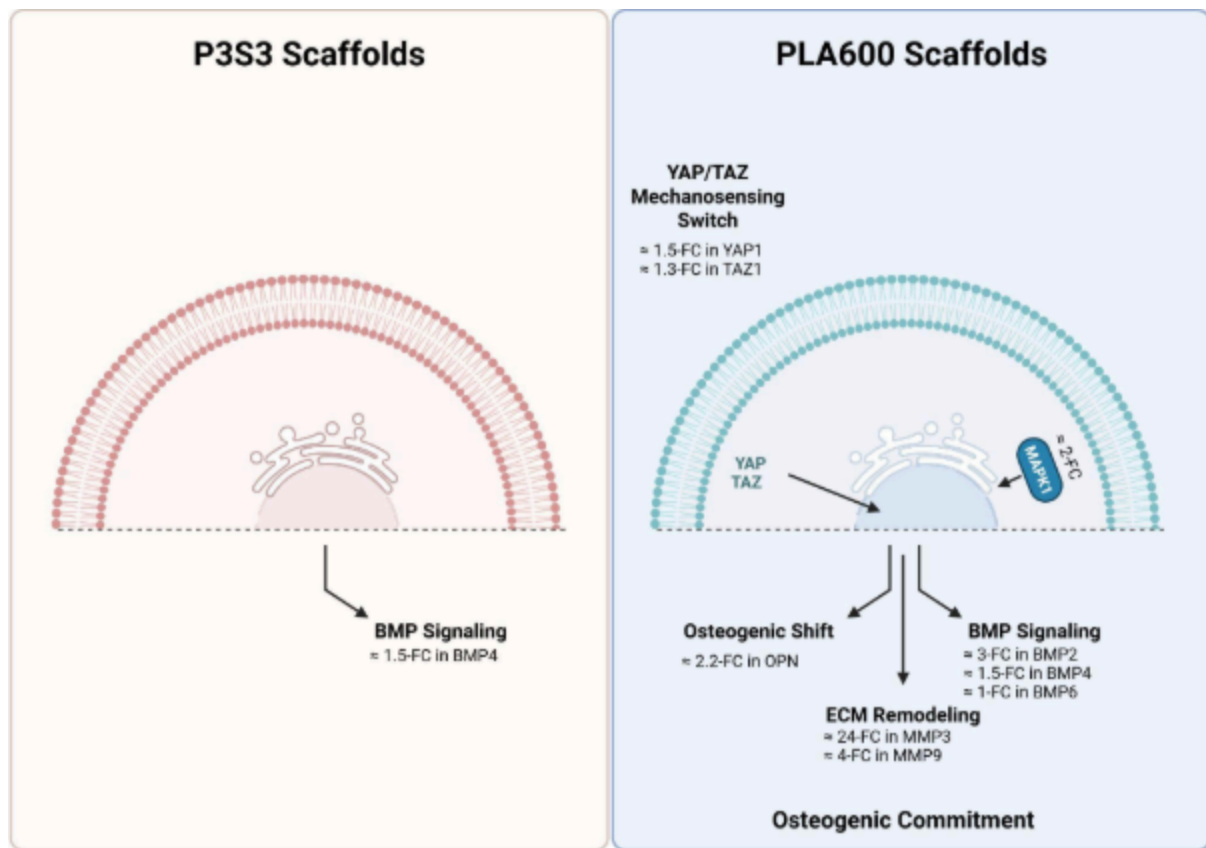


Figure 4

Schematic representation of geometry-dependent mechanotransduction and osteogenic commitment in bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured under perfusion and pulsed electromagnetic field (PEMF) stimulation. Comparative cellular and molecular responses of BM-MSCs cultured within P3S3 (left) versus PLA600 (right) scaffold architectures subjected to combined perfusion and PEMF stimulation (75 Hz, 1.5 mT, 4 h/day for 10 days). Values indicate approximate fold changes ($\approx$ FC) in gene expression relative to control cells cultured under perfusion alone.

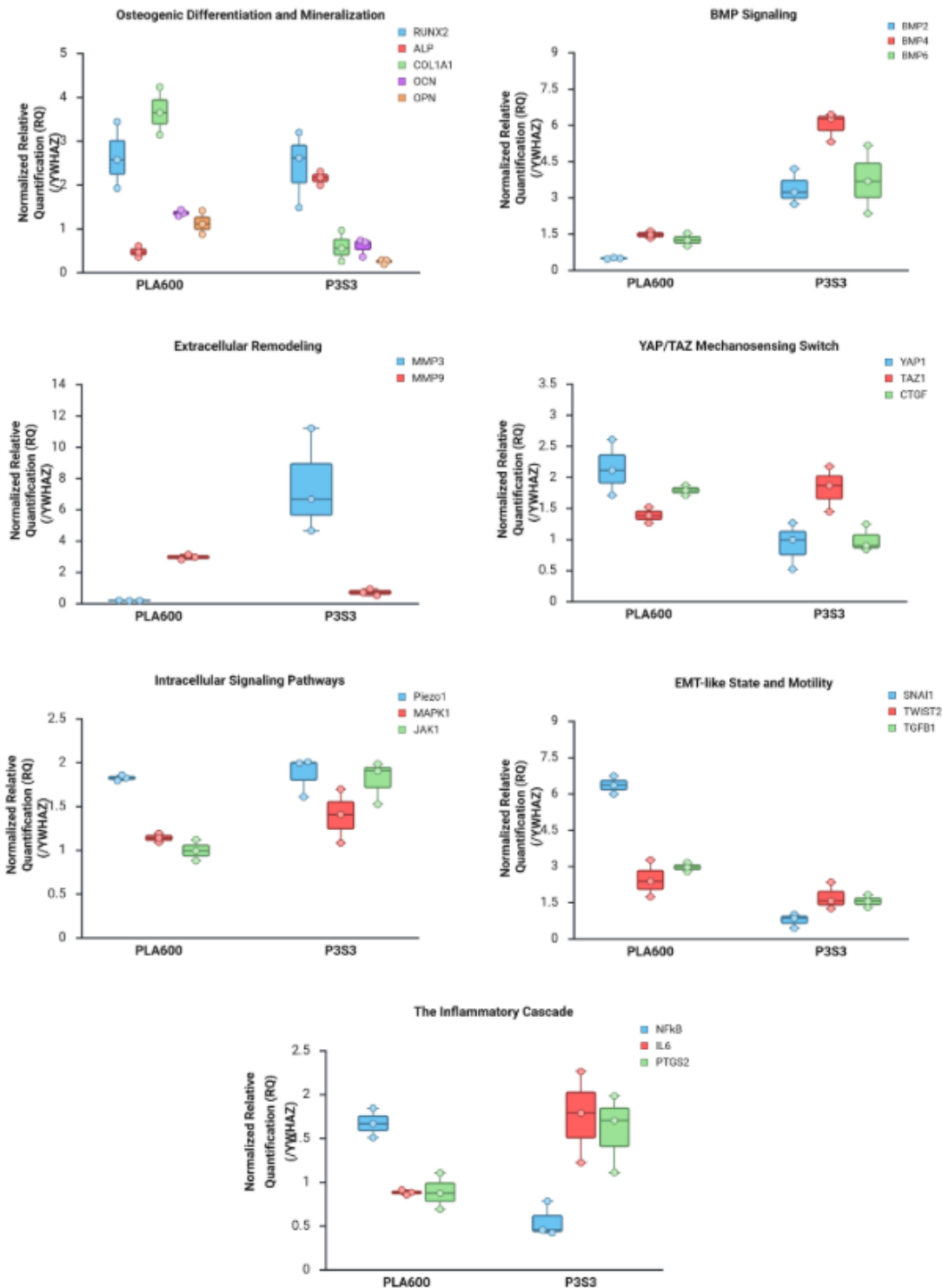


Figure 5

Comparative gene expression profiles of bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured within PLA600 vs. P3S3 scaffolds under perfusion, intermittent hydrostatic pressure (HP), and pulsed electromagnetic field (PEMF) stimulation. Fold change values represent the expression levels of cells subjected to combined perfusion, intermittent HP (15 kPa, 2 h/day), and PEMF stimulation (75 Hz, 1.5 mT, 4 h/day) for 10 days, “RP3”, relative to control cells cultured under perfusion alone. Gene

expressions are categorized by functional groups: osteogenic markers, BMP signaling, matrix remodeling, EMT-like state and motility, YAP/TAZ mechanosensing switch, intracellular signaling, and the inflammatory cascade.

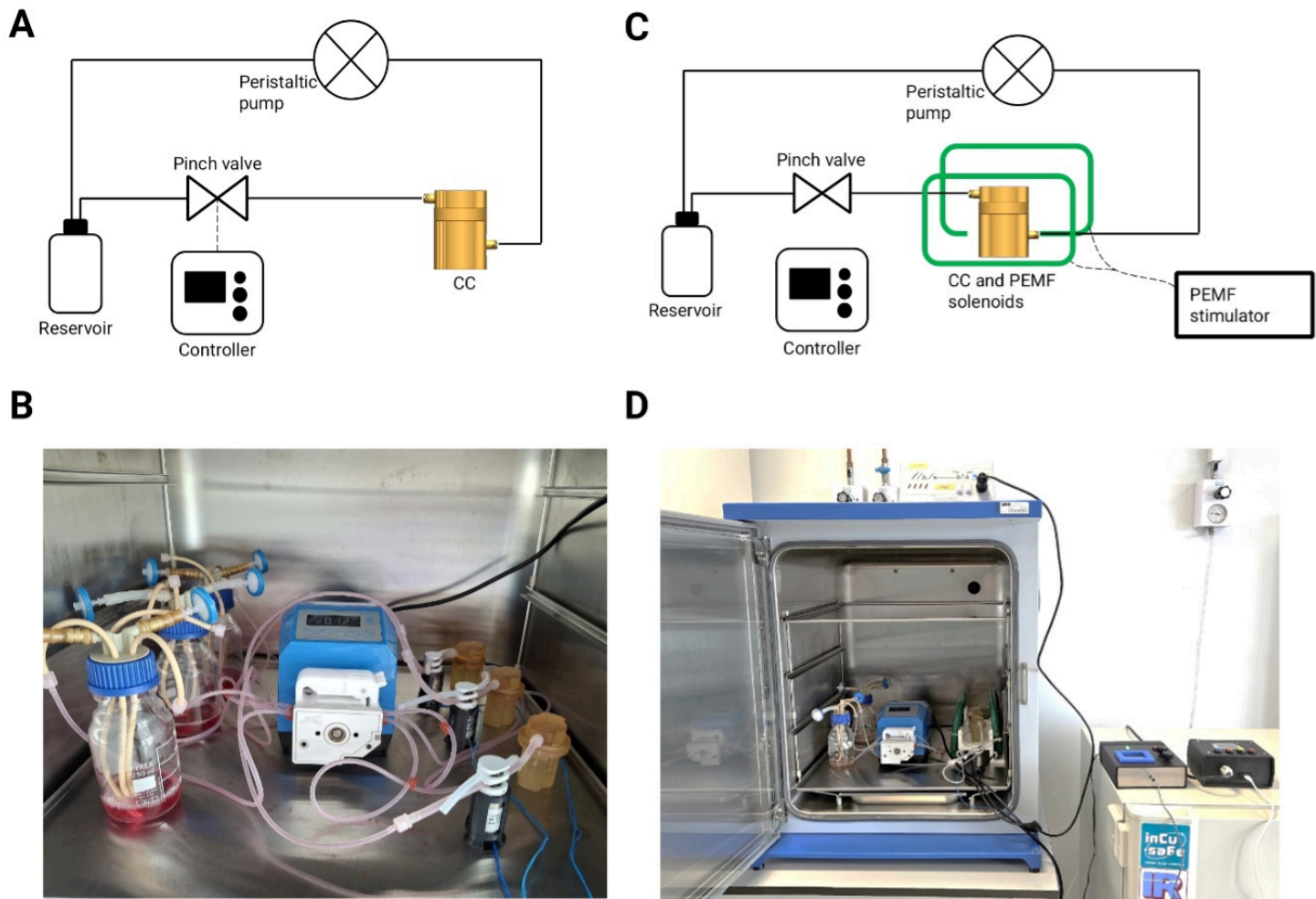


Figure 7

Configuration of the automated multi-physical stimuli bioreactor. (A) Schematic representation of the hydraulic circuit, featuring the culture chamber (CC), the pinch valve and its controller, the reservoir and the multichannel peristaltic pump. (B) Incubator-integrated parallelized bioreactor setup with pinch valves. (C) Schematic illustration of the integration of the pulsed electromagnetic field (PEMF) stimulator with the perfusion and intermittent HP bioreactor. (D) Full experimental set-up including FSS, intermittent HP, and PEMF units within the incubator.

Supplementary Files

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