

MicroRNA-221-mediated Control of Osteogenic Differentiation in Unrestricted Somatic Stem Cells Cultured on Nano-polycaprolactone Scaffolds Coated with Nano-hydroxyapatite

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Abstract

Stem cells are essential for engineered tissue, and controlling their differentiation is crucial. Our study explored the impact of using a bone tissue-engineering scaffold and anti-miR-221 transfection on unrestricted somatic stem cells (USSCs) for promoting osteogenic differentiation. Polycaprolactone (PCL) was used as a biodegradable scaffold material, and collagen type 1 nanofibers and nano-hydroxyapatite (HA) particles were incorporated into the scaffold to mimic the extracellular matrix for bone tissue engineering. Scaffold structure and cell morphology were assessed using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy and Scanning Electron Microscopy (SEM) analysis. The bone-differentiation potential of USSCs was assessed by measuring mRNA expression of osteogenic genes in the PCL/HA scaffold under varying growth medium compositions. Surprisingly, anti-mir-221 transfection was accompanied by increased osteogenic differentiation of USSCs when seeded on the PCL/HA scaffold in a basal growth medium. Importantly, the inclusion of osteogenic supplements in the growth medium significantly increased expression of the osteopontin (OPN) gene but decreased expression of osteocalcin (OCN). These observations demonstrated that the composition of the growth medium influences cell behavior and gene expression. Thus, the findings underscore the importance of miRNA studies and the optimization of growth medium compositions to provide more natural conditions for bone tissue regeneration and engineering.

Keywords: Anti-mir-221; Hydroxyapatite; Osteogenic differentiation; PCL; USSCs.

1. Introduction

Bone is a regenerative tissue that can heal itself, but severe injuries, including traumatic injury, congenital malformation, and bone tumor surgery, may require tissue transplantation [1]. The best material for bone

grafting is the patient's own bone, known as an autograft; however, it has limitations, including limited cell viability, stability, and volume across various surgical techniques [2]. Furthermore, the risks of disease transmission, morbidity at the donor site, and resource

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constraints are concerns with allograft transplantation [3]. Bone tissue engineering has emerged as a response to the constraints of both autograft and allograft bone using cells, growth hormones, and biodegradable scaffolds [1, 4]. The mechanical characteristics and structure of scaffolds are crucial for cell differentiation and proliferation as they must mimic the bone's extracellular matrix (ECM) to support cell growth and function effectively [5, 6]. Consequently, the ideal scaffold closely resembles these characteristics to aid in healing.

Porous scaffolds are produced using various methods, especially electrospinning, which is widely used in tissue engineering and biomaterials. This method produces polymer fibers with diameters ranging from 3 nm to over 5 μm , similar to those of the natural extracellular matrix [7]. Collagen and elastin, found in the ECM, have diameters of several tens to hundreds of nanometers [8]. Fibers with diameters between 100 and 500 nm are called nanofibers, and because of their increased surface-to-volume ratio, they have a better potential for cell attachment [9]. Additionally, modifying the surface of these nanofibers can improve their effectiveness and compatibility for medical use [10]. Therefore, electrospun polymers are highly porous and suitable for tissue engineering applications.

Poly-caprolactone (PCL) is a useful nanofibrous scaffold for bone tissue engineering. It is a synthetic, biodegradable, and biocompatible polymer that is particularly favored for its mechanical strength, low degradation rate, fast freezing behavior due to its calcium ions, and thermoelastic properties. Nevertheless, it lacks flexibility and cell attachment sites [11, 12]. Mixing PCL with other materials can improve cell adhesion, aiding in bone tissue formation.

Interestingly, non-stoichiometric hydroxyapatite (HA), the most stable form of calcium phosphate at biological pH, shares structural and compositional similarities with natural bone minerals. Its benefits include strong cell attachment, non-inflammatory properties, osteoconductive and osteoinductive properties, and non-toxicity, making it a popular choice for bone tissue engineering [13, 14]. Combining HA with PCL can create strong scaffolds suitable for this application. Previous studies have explored this combination [15].

Choosing the right stem cells is crucial for producing new bone. Mesenchymal stem cells (MSCs) are good for osteochondral tissue engineering because they can differentiate into osteoblasts and chondroblasts. Comparing umbilical cord blood stem cells to bone marrow stem cells reveals benefits such as lower immunogenicity, fewer ethical concerns, and greater reproducibility [16]. A type of stem cell called unconstrained somatic stem cells (USSCs), found in human umbilical cord blood, can grow and differentiate well without causing tumors. These novel CD45-negative stem cells retain their pluripotency even after differentiating into osteoblasts and other cells of the three germ layers [17]. Therefore, USSCs are promising for future cell-based therapies, as studies show they can differentiate into osteoblasts both *in vitro* and *in vivo* [18-20].

Both transcriptional and post-transcriptional mechanisms help regulate MSC differentiation into osteoblasts during osteogenesis. MicroRNAs (miRNAs), a type of RNA, target mRNAs to manage gene expression after transcription [21-23]. They play a key role in various cellular activities like differentiation, metabolism, apoptosis, and development. The mir-221 microRNA is linked to osteoblast development [24]. Research shows that blocking mir-221 improves USSCs' osteogenic development and promotes an osteogenic phenotype *in silico* [25]. In this study, we examined how mir-221 and a bone tissue scaffold influence USSC differentiation into osteoblasts. A scaffold was made by suspending HA nanoparticles in a PCL solution and using electrospinning. USSCs were transfected with anti-mir-221, and the levels of late osteogenic markers were measured using real-time PCR.

2. Materials and methods

2.1. Scaffold Fabrication

8 mL of chloroform (Merck, Germany) was poured into an appropriate Erlenmeyer flask. Then, 1.22 g of polycaprolactone (PCL average Mn 80,000) powder (Sigma-Aldrich) and 2 mL of dimethylformamide (DMF) (Sigma-Aldrich) were added, followed by a fit magnet. The flask was sealed and placed on a magnetic stirrer (KOMET-Germany) for 3-4 minutes at 100 rpm. Next, 0.244 g of HA nanopowder (Sigma-Aldrich) was

poured into a tube, and then 8.5 mL of chloroform and 1.5 mL of DMF were added.

The mixture was first placed in an ultrasonic system (Bandelin, Sonorex, Germany) for 30 seconds at 20 °C. A fit magnet was added, and the mixture was stirred on a magnetic stirrer for 115 minutes at 1000 rpm. Next, the contents were mixed again on the stirrer for 2-3 hours at 100 rpm to ensure full agitation. The mixture was then placed in an ultrasonic bath for an additional 30 seconds at 20 °C, followed by stirring for 5 minutes at 1000 rpm, resulting in a homogeneous suspension. The mixture was then loaded into two 5-mL syringes, each connected to an electrospinning pump with a 21-gauge needle. A rotating cylindrical stainless steel collector (10 cm in diameter) with an aluminum foil cover was used. The needle-to-collector distance was fixed at 45 cm, and the collector rotational speed was determined to be between 2500 and 3000 rpm. Simultaneously, two nozzles were used to increase the harvested nanofibers. The device setup was performed at a solution feed rate of 0.5 ml/h, a high-voltage potential of 20-22 kilovolts applied between the needle and the collector, a nozzle angle of 45 degrees from horizontal, and a five-hour operating time. Scaffolds were cut into circular shapes (diameter of 1.5 cm) and placed in a cell culture plate.

2. 2. Plasma treatment

To overcome the inherent hydrophobicity of PCL, oxygen plasma treatment was performed using a Diener Electronic-Nano UHP (Germany). Pure oxygen was introduced into a cylindrical quartz reactor at 0.4 mbar for 10 min. Plasma at 40 kHz was then applied to the reactor for 5 min. Finally, pure oxygen was applied for 10 min, and the samples were removed from the reactor.

2. 3. Scaffold hydrophobicity and porosity measurements

To measure the intensity of scaffold hydrophobicity before and after plasma treatment, a contact angle test was performed according to ASTM D724. Contact angles were measured using ImageJ analysis software (NIH, USA). To calculate porosity, the scaffold's ideal density was determined from the component densities and the proportions of each component in the mixture. The scaffold thickness was measured using a caliper. A precision balance also measured the weight of a piece of

scaffold. True density was calculated by volume and weight. Finally, porosity was calculated as the ratio of the true density to the ideal density.

2. 4. Sterilizing Scaffolds

The scaffolds were exposed to 70% ethanol and ultraviolet light. They were then immersed in ethanol for 3 hours and incubated in growth medium for one day. If contaminated, the medium's color will change.

2. 5. ATR-FTIR spectroscopy

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy (BOMEM-BM-102) was performed to confirm the presence of HA and COL on the surface of PCL scaffolds.

2. 6. Mechanical properties

The mechanical properties of the scaffold were measured using tensile testing equipment (SANTAM-SPM20-Iran). The scaffold was cut into 1 cm × 6 cm specimens, and the tensile test was conducted at a crosshead speed of 10 mm/min at room temperature and humidity. The stress-strain curve was plotted, and the mechanical properties were determined using the device's software.

2. 7. Scaffold and cell morphology

Scanning electron microscopy (SEM; CamScan MV2300-UK) was used to assess scaffold structure, fiber diameter, and cell attachment. First, the samples were gold-coated (BIO-RAD-E5200) and then inserted into the microscope. A 15 kV voltage was applied, and imaging was performed with a secondary electron detector. The diameters of the nanofibers were measured using ImageJ (NIH, USA) by analyzing 100 random fibers from each image. Cells need to be dehydrated before SEM imaging. For this purpose, after 7 days of culture, the scaffolds were rinsed with PBS, fixed in 2.5% glutaraldehyde for one hour, rinsed again, dehydrated in increasing ethanol concentrations, gold-coated, and then scanned by SEM.

2. 8. Cell culture

USSCs from Stem Cells Technology Research Centre-Tehran-Iran were grown in a specific medium that

included high glucose Dulbecco's modified Eagle's medium (DMEM-Gibco), supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics (penicillin/streptomycin). The cells were initially at a density of 10^4 cells/cm², and were incubated at 37 °C in a humidified incubator (Mettler) containing 5% CO₂. Every 48 h, half of the growth medium was removed and replaced with fresh medium.

2. 9. Cell seeding

Scaffolds were sterilized and placed in 4-well tissue culture polystyrene (TCPS). USSCs were suspended in growth medium containing PBS and trypsin, and cells were counted using a Neubauer hemocytometer. Approximately 20,000 cells were loaded into each well. Every 72 h, half of the growth medium was removed and replaced with fresh medium. The cells were cultured for 7 days in 3 groups as described in [Table 1](#).

2. 10. miRNA transfection

Cells were reverse-transfected with anti-mir-221 (inhibitor of mir-221, Qiagen) according to the manufacturer's instructions. The efficiency of miRNA transfection in USSCs was previously demonstrated by our research team [25], who used Lipofectamine 2000 (Invitrogen) to transfect miRNA (3-fluorescein-labeled, Exiqon) After transfection, cells were seeded on a scaffold and incubated for 7 days.

2. 11. Osteogenic differentiation

Some supplements induce osteogenic differentiation in stem cells, and the DMEM growth medium was supplemented with 250 nmol/L dexamethasone, 50 µg/mL ascorbic acid 2-phosphate, and 10 mmol/L β-glycerol phosphate to create the osteogenic medium [26].

2. 12. Real-time quantitative RT-PCR

Total cellular RNA was extracted using RNX-plus (Cinnagen, Iran). Scaffolds containing cells with two glass beads were placed in a vial, and RNX-plus was added. The vial was vortexed for 3 minutes to degrade scaffolds and lyse cells. The RNA was converted to cDNA according to the manufacturer's instructions using M-MuLV reverse transcriptase and random hexamers (Cinnagen, Iran).

For real-time PCR, 1µL of cDNA, 0.1µL of primers, 5 µL of master mix (Applied Biosystems), and 3.8µL of distilled water were combined in a real-time PCR vial. PCR was performed using a Step One instrument (Applied Biosystems), with data normalized to a housekeeping gene (HPRT). The thermal cycle was designed based on the primer properties ([Table 2](#)). Thermal cycles included 95 °C for 5 min, followed by 40 thermal cycles of denaturation at 94 °C for 25 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 20 seconds. The relative mRNA expression levels were quantified by $2^{-\Delta\Delta Ct}$ (Ct method).

Table 1. Description of different conditions of USSC cultivation.

Group	Transfection	Scaffold	Growth medium	Cell
1	-	PCL/nHA	DMEM high glucose	USSC
2	anti-mir-221	PCL/nHA	DMEM high glucose	USSC
3	anti-mir-221	PCL/nHA	High glucose DMEM + osteogenic supplements	USSC

Table 2. Oligonucleotide primers used for quantitative real-time PCR amplifications.

Gene	Primer sequences		Tm (°C)	Size (bp)
HPRT	Forward	CCT GGC GTC GTG ATT AGT G	58	125
	Reverse	TCA GTC CTG TCC ATA ATT AGT CC	58	
OPN	Forward	GAC CTG ACA TCC AGT ACC C	59	183
	Reverse	GTT TCA GCA CTC TGG TCA TC	58	
OCN	Forward	GCA AAG GTG CAG CCT TTG TG	60	80
	Reverse	GGC TCC CAG CCA TTG ATA CAG	63	

HPRT, Hypoxanthine guanine phosphoribosyl transferase; OPN, Osteopontin; OCN, Osteocalcin

2. 13. Statistical analysis

All experiments were performed at least three times, and data are expressed as mean $\pm$ standard deviation (SD). The student's t-test was used to determine whether the groups differed significantly. This calculation was performed using an online calculator (<http://www.graphpad.com/quickcalcs/>). The difference was significant if the P value was < 0.05 .

3. Results and Discussion

3. 1. Characterization of scaffold

To be effective, a tissue engineering scaffold must possess specific characteristics, such as high porosity and surface area. Electrospinning has emerged as an attractive technique for scaffold production due to its ability to produce fibers with diameters of 100-500 nm, known as nanofibers [9, 27]. A scaffold without beads and with interconnected pores was introduced, with predominantly aligned nanofibers on the nanoscale and a porous structure resembling the native ECM (**Figure 1A**). At higher magnification, aligned fibers and HA nanoparticles were more visible (**Figure 1B**). SEM analysis confirmed that the PCL surface became rough after HA coating. This observation is consistent with

previous research demonstrating that surface roughness can enhance cell adhesion and proliferation [4, 28]. Pore size and filament diameter measurements were performed in ImageJ on SEM images. As shown in **Figure 1B**, the diameter of approximately 75% of the nanofibers comprising the produced scaffold was less than 500 nm. The average nanofiber diameter was 399 ± 177 nm, and the diameter distribution shown in **Figures 1C** and **4** was well-defined. However, a limited number of thicker fibers were observed, but they were excluded as deviation data. Microfibers have a lower surface-to-volume ratio than nanofibers, leading to slower degradation. As a result, the presence of thicker fibers can improve the scaffold's strength while still maintaining the desired biological properties provided by thinner fibers. Therefore, the presence of thicker fibers can be considered an advantage, complementing the positive attributes provided by the thinner fibers. According to studies, a bone tissue-engineering scaffold composed of micro- and nanofibers has demonstrated effectiveness for bone regeneration [29]. Our design also featured smooth fibers with a good distribution at the nanoscale, along with a porous structure that mimics the cell ECM.

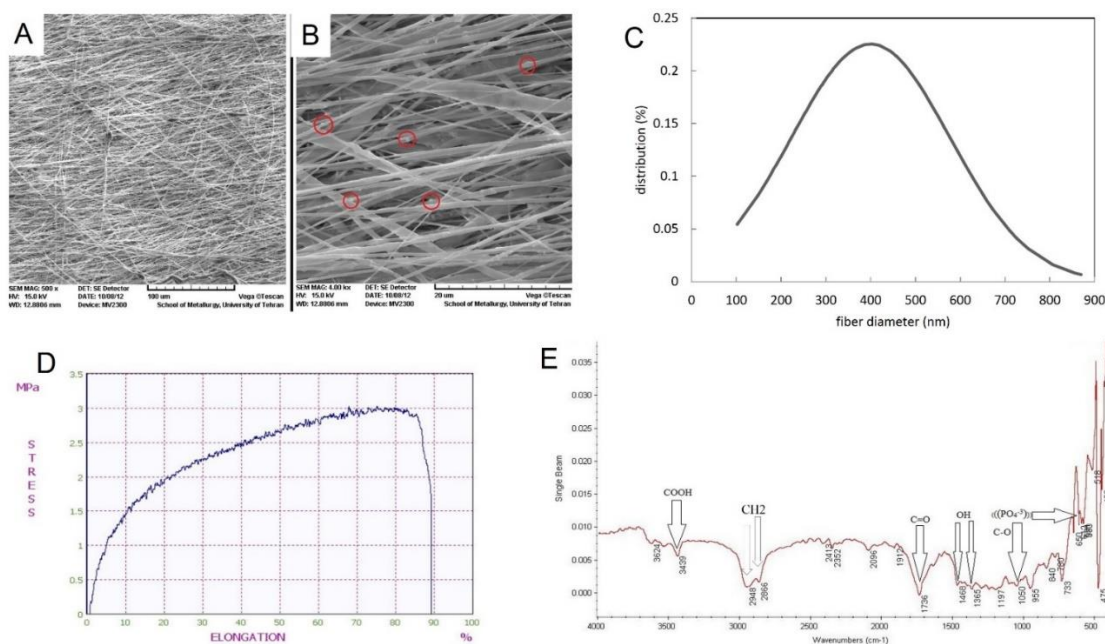


Figure 1. The morphology of the fabricated scaffold using Scanning Electron Microscope at magnification of 500x (A), and four kx (B). Red circles show HA nanoparticles. (C) The normal distribution curve of fiber diameter by the ImageJ software. (D) The stress-strain diagram of the PCL/HA scaffold showed an increase in its tensile strength. (E) ATR-FTIR spectrum of the PCL/nHA scaffold. It indicated that HA was successfully coated on the surface of the PCL scaffold.

PCL is a hydrophobic polymer that can limit cell growth due to its difficulty in penetrating the medium. Strategies to improve this include chemical modifications [30], plasma treatment [31], and hydrophilic coatings [32]. In our experiment, the scaffold showed strong hydrophobicity before plasma treatment, which decreased after plasma treatment.

The hydrophilicity of the PCL scaffold is relatively low, making it difficult for water to be thoroughly absorbed by the structure. To achieve this, we examined how inorganic HA affects the scaffold's water adsorption. The hydrophilicity/hydrophobicity of the scaffold was evaluated by measuring contact angles. Contact angles below 90° indicate hydrophilicity, and above 90° indicate hydrophobicity. Studies have shown that contact angles from 40° to 70° are ideal for cell attachment [33]. Before plasma treatment, the contact angle was $123.66^\circ \pm 0.86$, according to the D724-ASTM standard. After surface modification, it dropped to zero, showing full water absorption and confirming the scaffold's hydrophilic nature.

The scaffold from this study has a high porosity of about 89%. The nanofibrous structure of the scaffold is responsible for its high surface area [34]. Finally, the findings indicate that HA was successfully coated on the surface of the PCL scaffold.

The stress-strain curve of the PCL/HA scaffold is shown in **Figure 1D**, documenting a tensile strength of 3.45 ± 0.36 MPa and an elongation at break of 79.4 ± 8.7 %. Additionally, the best linear fit of the stress-strain curve yielded a compressive modulus [35]. The Young's PCL/HA scaffold modulus was calculated to be 0.16 ± 0.01 MPa, which was higher than the pure PCL scaffold (0.091). These results suggest an increase in the scaffold's tensile strength.

ATR-FTIR spectroscopy was performed to evaluate the functional groups of the scaffolds (**Figure 1E**). In the spectrum obtained from the PCL/HA scaffold, the characteristic peaks of all materials can be observed in the composite scaffold. As shown in **Figure 1E**, the main characteristic C=O band of the carbonyl ester group appeared at 1736 cm^{-1} , the C–O band stretching was visible at approximately 1050 cm^{-1} , and CH₂ asymmetric and symmetric tensions were at 2948 cm^{-1} and 2866 cm^{-1} , respectively, which is consistent with the presence of PCL [36]. The two peaks at 1365 cm^{-1} and 1468 cm^{-1}

were associated with the OH hydrophilic group on the surface, and the COOH surface hydrophilic group produced a peak at 3439 cm^{-1} . Further, the vibrations of the PO₄³⁻ ions in HA resulted in two distinct peaks at 630 cm^{-1} and 1050 cm^{-1} , indicating that HA was successfully coated on the surface of the PCL scaffold [37, 38].

3. 2. Morphology evaluation of cells on the scaffold

USSCs are preferred over bone marrow mesenchymal stem cells in osteogenic differentiation studies due to their high proliferative capacity, diverse differentiation potential, ease of collection, and suitability for long-term use, making them more advantageous in experiments [39]. To evaluate cell adhesion to the scaffold, cell morphology was analyzed by scanning electron microscopy (SEM) after 7 days (**Figure 2**). However, the HA-coated scaffold has a rough surface that helps cells attach, grow, and differentiate; the electron micrographs showed an uneven cell density. At low magnification, dark areas have many cells, while lighter areas have fewer. With higher magnification and light adjustments, distinct morphological changes were observed in the scaffold (**Figure 2D**). The scaffold surface was mostly covered with cells, with some areas having minimal density. Over time, more cells could fill the surfaces, leading to differences in cell density due to cell adhesion.

3. 3. Gene expression analysis

Osteoblast differentiation is a vital phase in bone formation, regulated by multiple signaling pathways and transcription factors that may be significantly affected by miRNAs [40]. Regulation can occur via several mechanisms, such as miRNA-206 inhibition of osteoblast differentiation by targeting the Connexin 43 (CX43) gene, a major gap junction protein in osteoblasts. In contrast, the downregulation of mir-206 promotes osteoblast differentiation [41]. Another relevant miRNA in the regulation of osteoblast differentiation is mir-221, whose target genes and pathways have been identified by in silico methods. The downregulation of mir-221 has been demonstrated to promote osteogenic differentiation in human stem cells [25]. This study examined the osteogenic differentiation capacity of USSCs cultivated on PCL/HA scaffolds, with mir-221 inhibition via anti-mir-221 transfection.

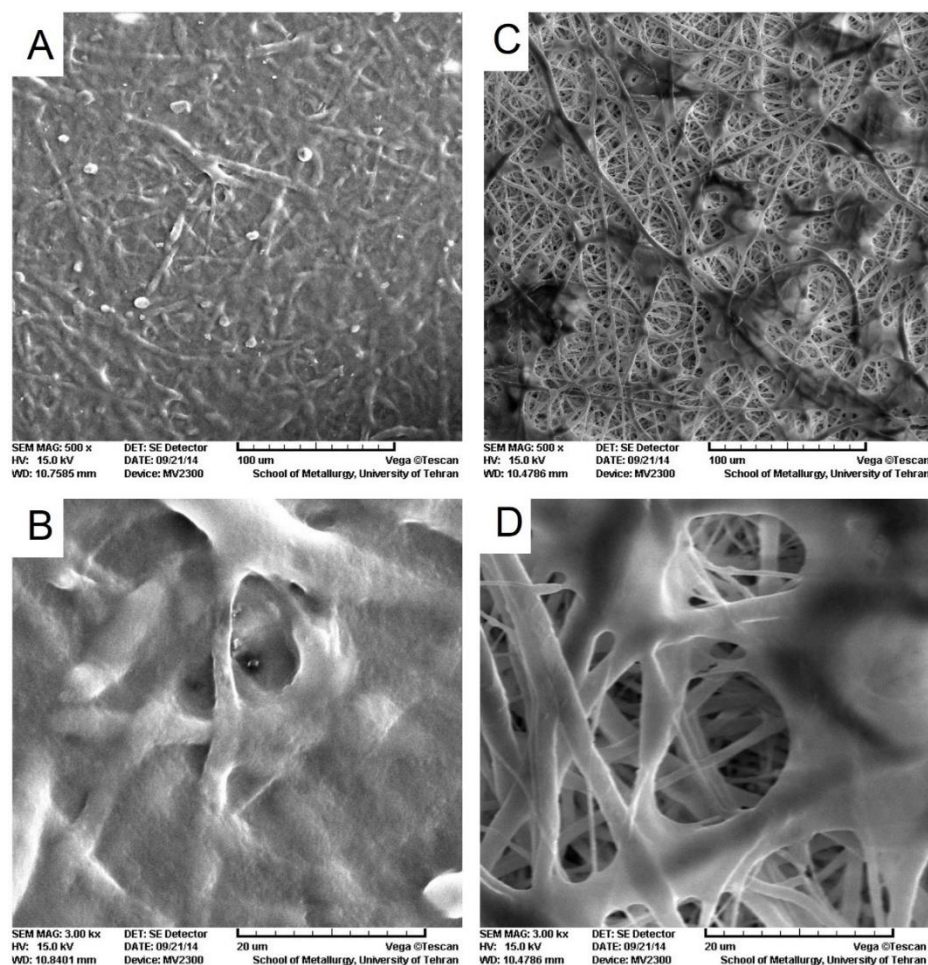


Figure 2. Morphology evaluation of cells onto the scaffold after 7 days of loading the cells using SEM analysis. Some areas were completely covered with cells (A, B), while some areas had a relatively low cell density (C, D). The images were taken at magnifications of 500x (A, C) and 3x (B, D).

The expression of specific transcription factors, cell-surface markers, and extracellular matrix proteins can track the differentiation of MSCs into mature osteoblasts. Initially, transcription factors trigger the expression of alkaline phosphatase (ALP) and Type 1 collagen. In more mature osteoblasts, the expression of Osteopontin (OPN) and Osteocalcin (OCN) increases, indicating the completion of osteogenic differentiation [40]. OCN and OPN are two non-collagenous proteins that play significant roles in the organization and deposition of the bone matrix as structural molecules [42]. To evaluate the bone-forming abilities of USSCs on a nanocomposite scaffold, real-time PCR was performed to assess the expression levels of OPN and OCN, as osteogenesis markers, and hypoxanthine guanine phosphoribosyl transferase (HPRT) as an endogenous

control under different cultivation conditions (**Figure 3A, B**). All three cultures were tested on the scaffold, as detailed in **Table 1**. In Sample 1, which served as the calibrator, the cells were cultured for 7 days in basal medium (high-glucose DMEM) without transfection. In Sample 2, the cells were cultured for 7 days in basal medium with anti-mir-221 transfection. In Sample 3, the cells were cultured for the same time in a growth medium (high-glucose DMEM + osteogenic supplements) with anti-miR-221 transfection.

Our results indicated that after 7 days, OPN expression increased significantly in USSCs transfected with anti-mir-221 in both basal medium (~1.5-fold) and particularly in osteogenic supplement medium (~2.90-fold) relative to the calibrator, while OCN expression showed a contrasting behavior which was a moderate

increase in the basal anti-mir-221 sample (~1.5-fold) but a decrease (~0.37-fold) in the osteogenic supplemented sample (Figure 3 and Table 2). Consequently, the OPN/OCN ratio was ~7.8 (log2 difference ~2.97), indicating a strong predominance of OPN expression under these conditions. These findings are consistent with previous research showing that members of the mir-221 family act as negative regulators of osteogenesis [43, 44]. For example, Fan et al. stated that mir-221-5p inhibition in mesenchymal stem cells from myeloma bone disease increased osteogenic differentiation by upregulating smad family member 3 (SMAD3) and activating the PI3K/AKT/mTOR signaling pathway [43]. Our data also demonstrated that treating USSCs with anti-miR-221 on the scaffold enhanced osteogenic differentiation, consistent with the literature. However, a change in the growth medium composition led to different outcomes. According to a study by Liu et al., alterations in the microenvironment significantly affect the function of specific miRNAs [45]. Comparisons with recent scaffold studies also found that scaffold composition is critical for enhancing stem cell osteogenic differentiation [46, 47]. Therefore, it is expected that modifying the cell environment could lead to significant changes in miRNA studies.

The low OCN expression may be due to cells having limited gene expression capacity during the 7-day

incubation. Additionally, anti-mir-221 transfection may significantly increase OPN expression, while cells cannot induce large amounts of OCN. It is noteworthy that osteoblasts produce OPN during the pre-mineralization stage of osteoblastic maturation and promote mineralization by enhancing ALP and OCN expression, supporting its essential role in early crystal nucleation [48]. On the other hand, it has been demonstrated that deletion of OCN genes markedly disrupts the structure of hydroxyapatite and collagen fibers, thereby confirming OCN's pivotal role in the late phase of bone matrix mineralization [49]. Therefore, its high expression is critical at the onset of bone differentiation, especially for the initiation of bone mineralization. OCN is undetectable in areas where crystal formation initially formed and is instead present in a fully mineralized matrix [48, 50]. Regarding these issues, the pronounced OPN/OCN ratio (~7.8) in anti-mir-221 + osteogenic medium suggests that anti-mir-221 strongly promotes expression of the pre-mineralization marker OPN, while the late-mineralization marker OCN remains suppressed at Day-7. This pattern is consistent with time-course studies on PCL/HA scaffolds, which show that OPN can increase earlier.

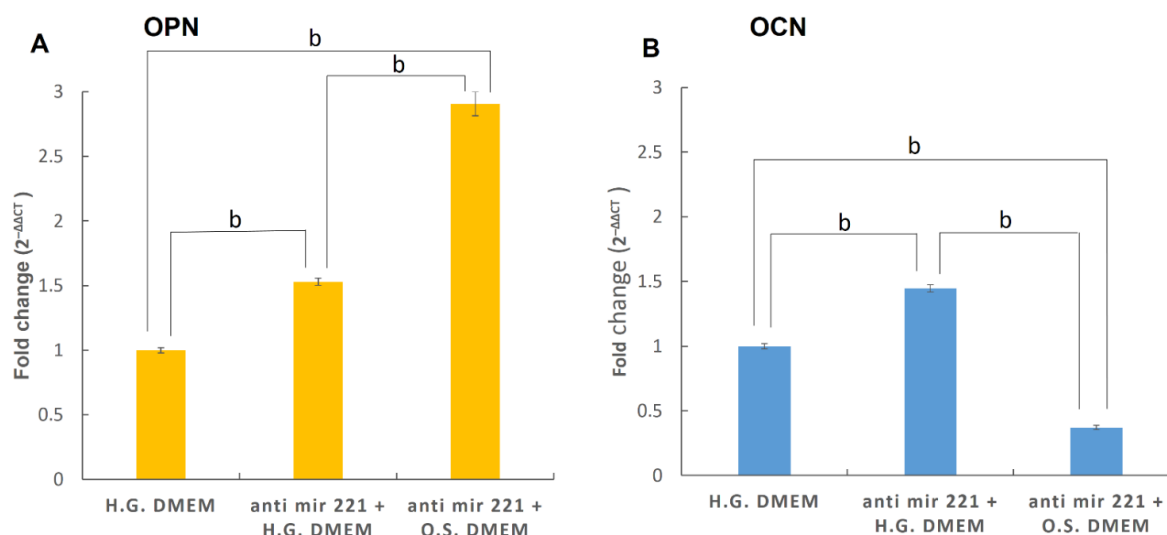


Figure 3. RT-qPCR analysis represented by the fold change in the mRNA level of osteogenic-related genes in USSCs cultured on scaffolds, Osteopontin, OPN (A), and Osteocalcin, OCN (B), relative to HPRT after 7 days (vs. Control group). A sample with cells cultured in simple growth medium (high-glucose DMEM, H.G. DMEM) on a nanocomposite scaffold was used as a calibrator. The expression levels were quantified using the $2^{-\Delta\Delta CT}$ method and then normalized to the housekeeping gene HPRT. b sign means shows the significant difference between the groups for each marker ($n = 3$) at $P < 0.05$.

In contrast, robust OCN upregulation typically requires prolonged culture (≥ 14 –21 days) to reach the mature osteoblast [51, 52]. In addition to insufficient 7-day incubation time, the observed decline in OCN expression may be due to complex interactions among mir-221 suppression, scaffold properties, and osteogenic supplement signals, in which early overactivation of some pathways shifts cell-fate timing. We therefore recommend measuring OPN/OCN, as well as additional osteogenic markers such as ALP activity and matrix mineralization (e.g., calcium deposition), at later time points (Day 14 and 21) to confirm the transition to matrix maturation and mineralization.

4. Conclusion

Taken together, suppressing mir-221 in USSCs on a bone tissue engineering scaffold without extracellular signaling induces osteogenic differentiation in cells. However, altering the components of the medium by adding osteogenic supplements may change the outcome. Research on miRNA should not be confined to two-dimensional environments. However, it should aim to provide more natural conditions, including a range of hormones and biochemical signaling molecules, to better mimic in vivo conditions. As a result, it is expected that the response of cells to a specific miRNA in vivo will differ greatly from the response in vitro.

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Conflict of interest

The authors declare no competing financial interest.

Authors' Contributions:

Shilan Nasri: Conceptualization, Supervision, Methodology, Project administration, Resources, Visualization, Writing-review and editing.

Zohreh Jahani: Co-corresponding author, Conceptualization, Methodology, Formal analysis, Investigation, Validation, Software, writing- original draft, Writing- review and editing.

Zahra Jahani: Resources, Writing- review and editing.

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Data availability

The data are available from the corresponding author upon request.

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Using artificial intelligence chatbots

There was no use of artificial intelligence in the making of this article.

References

1. Battafarano G, Rossi M, De Martino V, Marampon F, Borro L, Secinaro A, et al. Strategies for bone regeneration: from graft to tissue engineering. *International journal of molecular sciences*. (2021) 22(3):1128.
2. Smith C, Richardson S, Eagle M, Rooney P, Board T, Hoyland J. The use of a novel bone allograft wash process to generate a biocompatible, mechanically stable and osteoinductive biological scaffold for use in bone tissue engineering. *Journal of tissue engineering and regenerative medicine*. (2015) 9(5):595-604.
3. Josephson CD, Kuehnert MJ. Human tissue allografts: Responsibilities in understanding the path from donor to recipient. *Rossi's Principles of Transfusion Medicine*. (2022) :660-73.
4. Turnbull G, Clarke J, Picard F, Riches P, Jia L, Han F, et al. 3D bioactive composite scaffolds for bone tissue engineering. *Bioactive materials*. (2018) 3(3):278-314.
5. Williams DF. Challenges with the development of biomaterials for sustainable tissue engineering. *Frontiers in bioengineering and biotechnology*. (2019) 7:127.
6. Nikolova MP, Chavali MS. Recent advances in biomaterials for 3D scaffolds: A review. *Bioactive materials*. (2019) 4:271-92.
7. Zulkifli MZA, Nordin D, Shaari N, Kamarudin SK. Overview of electrospinning for tissue engineering applications. *Polymers*. (2023) 15(11):2418.
8. Sun B. The mechanics of fibrillar collagen extracellular matrix. *Cell Reports Physical Science*. (2021) 2(8).
9. Cho Y, Baek JW, Sagong M, Ahn S, Nam JS, Kim ID. Electrospinning and nanofiber technology: fundamentals, innovations, and applications. *Advanced Materials*. (2025) 37(28):2500162.

10. Chen Y, Dong X, Shafiq M, Myles G, Radacsi N, Mo X. Recent advancements on three-dimensional electrospun nanofiber scaffolds for tissue engineering. *Advanced Fiber Materials*. (2022) 4(5):959-86.
11. Gharibshahian M, Salehi M, Beheshtizadeh N, Kamalabadi-Farahani M, Atashi A, Nourbakhsh M-S, et al. Recent advances on 3D-printed PCL-based composite scaffolds for bone tissue engineering. *Frontiers in bioengineering and biotechnology*. (2023) 11:1168504.
12. Yang X, Wang Y, Zhou Y, Chen J, Wan Q. The application of polycaprolactone in three-dimensional printing scaffolds for bone tissue engineering. *Polymers*. (2021) 13(16):2754.
13. Du M, Chen J, Liu K, Xing H, Song C. Recent advances in biomedical engineering of nano-hydroxyapatite including dentistry, cancer treatment and bone repair. *Composites Part B: Engineering*. (2021) 215:108790.
14. Krishani M, Shin WY, Suhaimi H, Sambudi NS. Development of scaffolds from bio-based natural materials for tissue regeneration applications: a review. *Gels*. (2023) 9(2):100.
15. Jiao Z, Luo B, Xiang S, Ma H, Yu Y, Yang W. 3D printing of HA/PCL composite tissue engineering scaffolds. *Advanced Industrial and Engineering Polymer Research*. (2019) 2(4):196-202.
16. Seyedjafari E, Soleimani M, Ghaemi N, Sarbolouki MN. Enhanced osteogenic differentiation of cord blood-derived unrestricted somatic stem cells on electrospun nanofibers. *Journal of Materials Science: Materials in Medicine*. (2011) 22:165-74.
17. Adamiak M, Madeja Z, Zuba-Surma EK. Cord Blood Stem Cells. *Adult Stem Cell Therapies: Alternatives to Plasticity*: Springer. (2014) :35-51.
18. Zhou L, McDonald C, Yawno T, Jenkin G, Miller S, Malhotra A. Umbilical cord blood and cord tissue-derived cell therapies for neonatal morbidities: current status and future challenges. *Stem cells translational medicine*. (2022) 11(2):135-45.
19. Hoang DM, Pham PT, Bach TQ, Ngo AT, Nguyen QT, Phan TT, et al. Stem cell-based therapy for human diseases. *Signal transduction and targeted therapy*. (2022) 7(1):272.
20. Otori-Morita Y, Niibe K, Limraksasin P, Nattasit P, Miao X, Yamada M, et al. Novel mesenchymal stem cell spheroids with enhanced stem cell characteristics and bone regeneration ability. *Stem cells translational medicine*. (2022) 11(4):434-49.
21. Sun D, Chen Y, Liu X, Huang G, Cheng G, Yu C, et al. miR-34a-5p facilitates osteogenic differentiation of bone marrow mesenchymal stem cells and modulates bone metabolism by targeting HDAC1 and promoting ER- α transcription. *Connective Tissue Research*. (2023) 64(2):126-38.
22. Iaquinta MR, Lanzillotti C, Mazziotta C, Bononi I, Frontini F, Mazzoni E, et al. The role of microRNAs in the osteogenic and chondrogenic differentiation of mesenchymal stem cells and bone pathologies. *Theranostics*. (2021) 11(13):6573.
23. Zhao C, Gu Y, Wang Y, Qin Q, Wang T, Huang M, et al. miR-129-5p promotes osteogenic differentiation of BMSCs and bone regeneration via repressing Dkk3. *Stem cells international*. (2021) 2021:7435605.
24. Guelfi G, Capaccia C, Anipchenko P, Ciancabilla F, Oommen OP, Bufalari A, et al. Mimic miRNA and anti-miRNA activated scaffolds as a therapeutic strategy to promote bone, cartilage, and skin regeneration. *Macromol*. (2024) 4(2):165-89.
25. Bakhshandeh B, Ranjbar N, Abbasi A, Amiri E, Abedi A, Mehrabi MR, et al. Recent progress in the manipulation of biochemical and biophysical cues for engineering functional tissues. *Bioengineering & Translational Medicine*. (2023) 8(2):e10383.
26. Bakhshandeh B, Soleimani M, Ghaemi N, Shabani I. Effective combination of aligned nanocomposite nanofibers and human unrestricted somatic stem cells for bone tissue engineering. *Acta Pharmacologica Sinica*. (2011) 32(5):626-36.
27. Hiwrale A, Bharati S, Pingale P, Rajput A. Nanofibers: A current era in drug delivery system. *Heliyon*. (2023) 9(9).
28. Huang B, Vyas C, Byun JJ, El-Newehy M, Huang Z, Bártolo P. Aligned multi-walled carbon nanotubes with nanohydroxyapatite in a 3D printed polycaprolactone scaffold stimulates osteogenic differentiation. *Materials Science and Engineering: C*. (2020) 108:110374.
29. Sagadevan S, Schirhagl R, Rahman MZ, Ismail MFB, Lett JA, Fatimah I, et al. Recent advancements in polymer matrix nanocomposites for bone tissue engineering applications. *Journal of Drug Delivery Science and Technology*. (2023) 82:104313.
30. Khorramnezhad M, Akbari B, Akbari M, Kharaziha M. Effect of surface modification on physical and cellular properties of PCL thin film. *Colloids and Surfaces B: Biointerfaces*. (2021) 200:111582.
31. Velliangiri V, Vasu D, Ramkumar M, Jindal S, Gopinath P, Balraj B, et al. Development of Functional Amine Coatings on 3D-Printed PCL Scaffolds via Nonthermal Atmospheric Pressure Plasma for Tissue Engineering Applications. *Journal of Applied Polymer Science*. (2025) 142(26):e57097.
32. Toopghara SAH, Hwang Y-O, Kim YS, Kim YR, Zhang P, Park C, et al. Optimization of a porous polycaprolactone coating to enhance the mechanical properties and bioactivity of an additively manufactured hydroxyapatite scaffold. *Ceramics International*. (2025).
33. Majhy B, Priyadarshini P, Sen A. Effect of surface energy and roughness on cell adhesion and growth—facile surface modification for enhanced cell culture. *RSC advances*. (2021) 11(25):15467-76.
34. Suamte L, Babu PJ. Electrospun based functional scaffolds for biomedical engineering: A review. *Nano TransMed*. (2024) :100055.
35. Hassanajili S, Karami-Pour A, Oryan A, Talaei-Khozani T. Preparation and characterization of PLA/PCL/HA composite scaffolds using indirect 3D printing for bone tissue engineering. *Materials Science and Engineering: C*. (2019) 104:109960.
36. Ishwarchand W, Sarakar G, Swain BP. Investigation of optical properties, chemical network and electronic environments of polycaprolactone/reduced graphene

- oxide fiber nanocomposites. *Polymer Bulletin*. (2022) 79(10):8437-53.
37. Hossain MS, Ahmed S. FTIR spectrum analysis to predict the crystalline and amorphous phases of hydroxyapatite: a comparison of vibrational motion to reflection. *RSC advances*. (2023) 13(21):14625-30.
 38. Mystiridou E, Patsidis AC, Bouropoulos N. Development and characterization of 3D printed multifunctional bioscaffolds based on PLA/PCL/HAp/BaTiO₃ composites. *Applied Sciences*. (2021) 11(9):4253.
 39. Wang Q, Wang Y, Chang C, Ma F, Peng D, Yang S, et al. Comparative analysis of mesenchymal stem/stromal cells derived from human induced pluripotent stem cells and the cognate umbilical cord mesenchymal stem/stromal cells. *Heliyon*. (2023) 9(1).
 40. Ponzetti M, Rucci N. Osteoblast differentiation and signaling: established concepts and emerging topics. *International Journal of Molecular Sciences*. (2021) 22(13):6651.
 41. Narayan S. Small Molecules and Nanoparticles in Bone Healing: A Transformative Pathway in Tissue Engineering. *Regenerative Engineering and Translational Medicine*. (2025) :1-19.
 42. Shapiro IM, Landis WJ. The Role of Non-Collagenous Proteins and Other Matrix Molecules in Vertebrate Mineralization. *Mechanisms of Mineralization of Vertebrate Skeletal and Dental Tissues*: Springer (2024) :343-401.
 43. Fan F-Y, Deng R, Lai S-H, Wen Q, Zeng Y, Gao L, et al. Inhibition of microRNA-221-5p induces osteogenic differentiation by directly targeting smad3 in myeloma bone disease mesenchymal stem cells. *Oncology letters*. (2019) 18(6):6536-44.
 44. Bakhshandeh B, Hafizi M, Ghaemi N, Soleimani M. Down-regulation of miRNA-221 triggers osteogenic differentiation in human stem cells. *Biotechnology letters*. (2012) 34:1579-87.
 45. Liu Y, Liu W, Hu C, Xue Z, Wang G, Ding B, et al. MiR-17 modulates osteogenic differentiation through a coherent feed-forward loop in mesenchymal stem cells isolated from periodontal ligaments of patients with periodontitis. *Stem cells*. (2011) 29(11):1804-16.
 46. Taephatthanasagon T, Purbantoro SD, Rodprasert W, Pathanachai K, Charoenlertkul P, Mahanonda R, et al. Osteogenic potentials in canine mesenchymal stem cells: unraveling the efficacy of polycaprolactone/hydroxyapatite scaffolds in veterinary bone regeneration. *BMC Veterinary Research*. (2024) 20(1):403.
 47. Ebrahimi Z, Irani S, Ardeshtyrlajimi A, Seyedjafari E. Enhanced osteogenic differentiation of stem cells by 3D printed PCL scaffolds coated with collagen and hydroxyapatite. *Scientific Reports*. (2022) 12(1):12359.
 48. He T, Qin L, Chen S, Huo S, Li J, Zhang F, et al. Bone-derived factors mediate crosstalk between skeletal and extra-skeletal organs. *Bone Research*. (2025) 13(1):49.
 49. Xu Z, Yang C, Wu F, Tan X, Guo Y, Zhang H, et al. Triple-gene deletion for osteocalcin significantly impairs the alignment of hydroxyapatite crystals and collagen in mice. *Frontiers in Physiology*. (2023) 14:1136561.
 50. Carvalho MS, Cabral JM, da Silva CL, Vashishth D. Synergistic effect of extracellularly supplemented osteopontin and osteocalcin on stem cell proliferation, osteogenic differentiation, and angiogenic properties. *Journal of Cellular Biochemistry*. (2019) 120(4):6555-69.
 51. Stannitz S, Klimczak A. Mesenchymal stem cells, bioactive factors, and scaffolds in bone repair: from research perspectives to clinical practice, *Cells*. (2021) 10:1925.
 52. Bernardo MP, da Silva BC, Hamouda AE, de Toledo MA, Schalla C, Rütten S, et al. PLA/Hydroxyapatite scaffolds exhibit in vitro immunological inertness and promote robust osteogenic differentiation of human mesenchymal stem cells without osteogenic stimuli. *Scientific reports*. (2022) 12(1):2333.