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RESEARCH ARTICLE

BMP-9 and TGF-β3 Synergistically Regulate Chondrogenic Pathways in Bovine Synovial Fluid-Derived Mesenchymal Stem Cells (BSF-MSCs) in Transwell Co-Culture with Chondrocytes

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A B S T R A C T

The prevalence of osteoarthritis (OA), a degenerative condition affecting joints, has significantly risen in recent years. The primary pathogenic characteristic of OA encompasses the destruction of articular cartilage. Synovial fluid-derived mesenchymal stem cells (SF-MSCs) are considered a promising stem cell resource for healing cartilage defects due to their inherent multilineage differentiation capacity. Our study aimed to assess the chondrogenic differentiation potential of BSF-MSCs in a transwell co-culture system with chondrocytes. SF-MSCs were isolated, cultured, and characterized for multilineage differentiation potential. SF-MSCs were cultured with varying combinations of chondrogenic growth factors, including BMP-9 (10ng/mL) and TGF-β3 (10ng/mL), over 21 days in a transwell co-culture system along with chondrocytes. At the end of the experiment, the cells were analyzed for morphological assessments, immunofluorescence staining for COL-II, Alcian blue staining for glycosaminoglycan deposition, and real-time PCR for chondrogenic marker-specific markers including COL2A1, SOX9, ACAN, and for hypertrophic markers COL10A1. The results of real-time PCR revealed significant upregulation of COL2A1 ($P<0.001$), SOX9 ($P<0.05$), and ACAN ($P<0.05$) in experimental groups. These findings highlight the role of BMP-9 and TGF-β3 in enhancing chondrogenesis through BSF-MSCs and chondrocyte interactions.

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INTRODUCTION

Osteoarthritis (OA) is a prevalent degenerative disease in veterinary patients (Anderson *et al.,* 2020) leading to the gradual deterioration of the articular hyaline cartilage in synovial joints. Due to limited pharmaceutical and surgical treatment options in veterinary medicine for OA, stem cell therapy especially involving mesenchymal stem cells (MSCs) (Tsai *et al.,* 2014), represents a powerful therapeutic approach.

MSCs offer a promising therapeutic avenue due to their inherent ability to differentiate into various lineages, including chondrogenic, osteogenic, and adipogenic, and strong immunomodulatory properties (Kong *et al*., 2017). Therapeutic stem cells obtained from various tissues including bone marrow, synovial fluid, and adipose tissue emerge as a promising prospect in the realm of cartilage-associated defects and osteoarthritis therapeutics (Zayed *et al*., 2017; Zhu *et al*., 2021). Among all MSC sources, SF-MSCs are particularly attractive for cartilage regeneration owing to their accessibility, high proliferative capacity, and solid chondrogenic potential (Bertolino *et al*., 2023) and have been preferred in the treatment of tendon and ligament damage (Leal *et al*., 2024), osteoarthritis (Mariñas *et al*., 2018) and rheumatoid arthritis (Sarsenova *et al*., 2021). It was shown that SF-MSCs are crucial for maintaining tissue homeostasis (Liu *et al*., 2022). These cells have a significant ability to produce cartilage both *in vivo* and *in vitro* (Neybecker *et al*., 2018), making them one of the most promising candidates for cell-based therapies.

The chondrogenic differentiation of MSCs can easily be influenced by various intrinsic and extrinsic factors, with co-culture systems providing a platform to study

cell-cell interactions. Essential growth factors and cytokines released from chondrocytes in the co-culture system can affect MSCs via paracrine, juxtracrine, and gap junction signaling pathways (Peng *et al*., 2023) and positively affect cartilage repair for enhanced chondrogenic differentiation (Hubka *et al*., 2014; Zhang *et al*., 2017; Meng *et al*., 2021; Chan *et al*., 2022). This effect can easily be created in transwell co-culture, where synovial stem cells and chondrocytes could mimic the natural joint structure (Kubosch *et al*., 2016).

Additionally, exogenous growth factors such as transforming growth factor-beta 3 (TGF-β3) and bone morphogenetic protein-9 (BMP-9) are considered potent inducers of chondrogenesis (Hennig *et al*., 2007; Luu *et al*., 2007; Cheng *et al*., 2016; Kovermann *et al*., 2019; Gasson *et al*., 2021; Padmaja *et al*., 2022). BMP-9 has been shown as a powerful inducer of chondrogenesis in both immature and mature chondroprogenitor cells (Gardner *et al*., 2023). Additionally, TGF-β3 has been demonstrated a crucial role for cartilage development with sustaining the equilibrium between chondrogenic differentiation and chondrocyte hypertrophy (Du *et al.*, 2023). Even if they have been known for their chondrogenic differentiation potential separately, the combined effects of BMP-9 and TGF-β3 with SF-MSC on chondrogenesis remain insufficiently studied.

This study aims to elucidate the chondrogenic differentiation potential of BSF-MSCs using a transwell co-culture system with chondrocytes mimicking the joint structure and to evaluate the roles of TGF-β3 and BMP-9.

MATERIALS AND METHODS

Ethical approval: All procedures, methods of animal sacrifice, tissue management, and disposal of abattoir materials were reviewed and approved by the Ethical Committee of Afyon Kocatepe University (AKÜHADYEK-46-19; 30.04.2019), TURKIYE

Bovine synovial fluid-derived mesenchymal cells (BSF-MSCs) culture: Bovine synovial fluid-derived mesenchymal stem cells (BSF-MSCs) were isolated as described in our previous study by Akgün *et al*. (2022). Briefly, synovial fluid was harvested individually from the metatarsophalangeal joints of freshly collected hind limbs of one-year-old six bovines brought from the local abattoir in Afyonkarahisar, Turkiye within 30 minutes of slaughter. Approximately 3–5mL of synovial fluid was collected from each joint under sterile conditions within the biosafety cabin. The synovial fluid was diluted 1:2 with high-glucose (HG) Dulbecco's Modified Eagle Medium (HG-DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, South America) and centrifuged at 300g for 10 minutes. After discarding the supernatant, the cell pellet was suspended in HG-DMEM (Sigma-Aldrich, USA) containing 10% FBS (Biowest, South America), 1% L- Glutamine (Gibco, UK), 1% pen-streptomycin (Gibco, UK), 0.1% amphotericin B (Biochrom, Germany) and cultured in 25cm^2 culture flasks (Biochrom, Germany) containing 5mL culture medium in 5% CO_2 incubator at 37°C. After reaching 70-80% confluence, the cells were passaged. In the 4th passage, the cells were seeded in a 6-well plate for Transwell co-culture.

Multilineage differentiation of BSF-MSCs: For both adipogenic and osteogenic differentiation, BSF-MSCs from P3 were seeded into four well culture plates (1.96 cm²/well) at a density of 9×10^3 cells/cm^2 each and allowed to grow in HG-DMEM containing 10% FBS, 1% L-Glutamine, 1% pen-streptomycin, and 0.1% amphotericin B till cells reached 70% confluence.

Adipogenic differentiation: On day 3rd, cells were washed. The medium was replaced with adipogenic differentiation medium, containing low glucose (LG)-DMEM supplemented with 10% FBS, 0.5mM 3-isobutyl-1- methylxanthine (IBMX) (Sigma, Germany), 100µM indomethacin (Cayman Chemical Company, USA), 1µM dexamethasone (Sigma, Belgium), 10µg/ml insulin (Sigma-Aldrich, Germany), 1% L- Glutamine, 1% penicillin-streptomycin and 0.1% amphotericin B. Adipogenic differentiation was performed over 21 days, with the medium replaced every three days. On day 21, the cells were fixed in 4% neutral buffered formalin for 10 minutes and then stained with Oil-Red O (5mM) (Sigma-Aldrich, USA) to observe oil droplets.

Osteogenic differentiation: To stimulate osteogenic differentiation, BSF-MSCs at day 3rd were cultured with media containing α -MEM (Sigma-Aldrich, USA) supplemented with 10% FBS, 0.1µM dexamethasone, 0.05mM ascorbic acid (Dr. Ehrenstorfer GMbH, Germany), 10mM ß- glycerophosphate (Gibco, UK, 1% L-glutamine, 1% penicillin-streptomycin and 0.1% amphotericin B. The medium was changed every 3rd day After 21 days, cells were fixed with 4% paraformaldehyde and stained with 80mM Alizarin Red S (Merck, Germany) to observe calcium deposition.

Isolation and culture of bovine chondrocytes: Bovine chondrocytes were isolated from the bovine metatarsophalangeal joints (n=6), of collected same hind limbs that were isolated for synovial fluid. The cartilage was dissected into tiny pieces and washed thrice with phosphate buffer saline (PBS; Sigma, Germany) containing 2% penicillin-streptomycin. Minced cartilage was first incubated with 0.3% collagenase Type II (Sigma, USA) in HG-DMEM for 25 minutes, followed by 24-hour incubation in 0.06% collagenase Type II in HG-DMEM at 37°C. The dissociated tissue was strained through a 70µM cell strainer followed by centrifugation at 400 g for ten minutes. The cell pellet was suspended in 3mL HG-DMEM containing 10% FBS, 0.1% amphotericin B, and 1% pen-streptomycin and seeded into 25cm^2 flasks at 37°C in 5% CO_2 incubator. On 70-80 % confluence, the cells were harvested for transwell co-culture with BSF-MSCs.

Transwell co-culture of BSF-MSCs and bovine chondrocytes: BSF-MSCs at P4 were seeded at 15,000 cells/cm^2 density in 6-well plates. Concurrently, chondrocytes from P1 were co-cultured at the same seeding density on 0.4µm pore-sized transwell inserts (Millipore, USA), placed on top of the BSF-MSCs monolayer to facilitate indirect cell-cell interactions. Chondrogenic differentiation was conducted in experimental groups as illustrated in Table 1. The chondrogenic medium consisted of HG-DMEM with 5%

FBS, 50mM L-ascorbic acid (Sigma, Belgium), 0.1mM dexamethasone, 1% ITS-Premix (Gibco, USA), 0.35mM proline (Sigma, Belgium), 1% non-essential amino acid (Lonza, Belgium) and 1mM sodium pyruvate (Lonza, Belgium), 1% penicillin-streptomycin and 0.1% amphotericin B.

Experimental design: The experimental design for the co-culture of BSF-MSCs and bovine chondrocytes is provided in Table 1. Following 21 days of the chondrogenic differentiation protocol, cells were fixed with 4% paraformaldehyde (Merck, Germany), washed with PBS, and stained with 1% Alcian Blue to visualize glycosaminoglycans. Cells were also characterized by immunofluorescence assays and Real-Time PCR analysis to assess specific markers of chondrogenic differentiation.

Immunofluorescence assays: For immunofluorescence, the cells after chondrogenic differentiation were fixed with 4% paraformaldehyde for 30 minutes, followed by incubation in 0.1% Triton-X solution for 5 minutes. Cells were then blocked with 10% normal goat serum (Abcam, UK) for 30 minutes, washed, and incubated with COL-II (1/100 dilution) as primary antibody (ab185430, Mouse monoclonal; Abcam, UK) in a humidified chamber overnight at 4°C. The next day, cells were washed with phosphate buffer saline (PBS) 3 times for 5 minutes and incubated in a secondary antibody (1/300 dilution) (ab150117, Goat anti-Mouse Alexa Fluor 488; Abcam, UK) for 1 hour at room temperature. Subsequently, cells were washed 3 times with PBS for 5 minutes each, mounted with DAPI fluoreshield mounting medium for 1 minute, and observed under an inverted fluorescence microscope (Zeiss Axio Observer Z1) was used for cell imaging.

Real-time PCR: All primers for the study were designed using Fast PCR 6.0 software. Detailed nucleotide sequences and Tm values of all primers used in this study are presented in Table 2. Undifferentiated BSF-MSCs were characterized for mesenchymal markers (CD44, CD90, CD73, and CD105), hematopoietic markers (CD45, and CD34), and chondrogenic markers (COL II, ACAN, SOX9, and COL-10A1). Similarly, all experimental groups were assessed for chondrogenic differentiation by analyzing the expression of COL II, ACAN, SOX9, and COL-10A1. Briefly, total RNA from cells was extracted using a commercial RNA isolation kit (TRIzol Reagent, Thermo 15596026, USA), quantified by nanodrop assay and cDNA was synthesized using, Maxima First Strand cDNA synthesis kit (Thermo Scientific K1672, USA), following the manufacturer's guidelines and stored at −80°C. Real-time PCR analysis was carried out using RealQ Plus Master Mix Green (Ampliqon, A323406, Denmark). Each sample underwent triplicate measurements, and these replicates were employed in the subsequent statistical analysis. To assess mRNA expression level changes of the targeted genes based on Ct values of amplification curves, Relative Expression Software Tool V2.0.13 was employed (Bernstein *et al*., 2009). The GAPDH gene served as the endogenous control (housekeeping gene), and normalization of other genes was conducted relative to the GAPDH gene level. The Real-Time PCR instrument was programmed to run at 95°C for 15 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, using the ABI StepOnePlus (Thermo Scientific) Real-Time PCR cycler.

Statistical analysis: Fold-change in gene expression ($2^{-\Delta\Delta}Ct$) of chondrogenic and BSF-MSCs specific markers at passages P0 and P3 were analyzed using One-Way ANOVA and student t-test respectively, using SPSS version 22.0. The normality of the data distribution was assessed using the Shapiro-Wilk test. Kruskal-Wallis test was applied as Post hoc non-parametric test and comparisons for the Kruskal-Wallis test were corrected using the Bonferroni method to account for multiple comparisons. Data visualization was performed using GraphPad Prism version 6.01. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Morphological characteristics of BSF-MSCs and chondrocytes: BSF-MSC colonies were observed to form by day 4 post-seeding into culture flasks. By day 7, BSF-MSCs proliferated, covering 70–80% of the flask surface. At passage 0 (P0), BSF-MSCs exhibited two distinct morphologies: fibroblast-like cells (Fig. 1A) and larger, flattened cells (Fig. 1B). Starting from passage 1 (P1), cells demonstrated rapid adherence to the culture surface, reaching confluency within 2–3 days. The percentage of fibroblast-like cells significantly increased in later passages. This characteristic fibroblast-like morphology was consistently maintained at passages P3, P4, and P6 (Fig. 2. A, B, C). The chondrocytes exhibited polygonal morphology at 80–90% confluency at P0 and P1 (Fig. 3. A, B). The cells started exhibiting more fibroblast-like shapes, especially in the later passages.

Multilineage differentiation potential of BSF-MSCs: The adipogenic and osteogenic differentiation potentials of BSF-MSCs at P3 were assessed. Following 21 days of culture in adipogenic differentiation medium, Oil Red O staining revealed the presence of lipid droplets, indicated by dark red staining (Fig. 4B), whereas no staining was observed in the control group cultured in standard medium (Fig. 4A). Similarly, osteogenic differentiation was confirmed by the presence of calcium deposits, visualized as orange-red staining using Alizarin Red S after 21 days in osteogenic differentiation medium (Fig. 4D). No staining was detected in the control group maintained in standard culture medium (Fig. 4C).

***In vitro* characterization of BSF-MSCs by Real-time PCR:** When comparing BSF-MSCs at P0 and P3, there was a significant fold increase in the expression of mesenchymal marker genes at P3 with CD44 (1.78±0.33), CD73 (1.20±0.33), and CD90 (1.36±0.33) compared to P0. In contrast, the fold increase in CD105 expression at P3 (1.02±0.16) was not statistically significant as shown in the Bar and Whisker Graph Figure. 7. (A, B, C, D). Additionally, a significant downregulation of certain markers was observed, indicating changes in the transcriptional profile between P0 and P3.

Chondrogenic differentiation potential of BSF-MSCs in transwell co-culture by Alcian blue staining: Cells cultured in 6-well plates with chondrogenic differentiation medium for 21 days (as detailed in Table 1) were evaluated for chondrogenic differentiation. In the negative control group (Group 1), no staining was observed with Alcian Blue (Fig. 5A). However, in the experimental groups, glycosaminoglycan accumulation was evident as intense bright blue staining as shown in Figure. 5 (B, C, D, E, F).

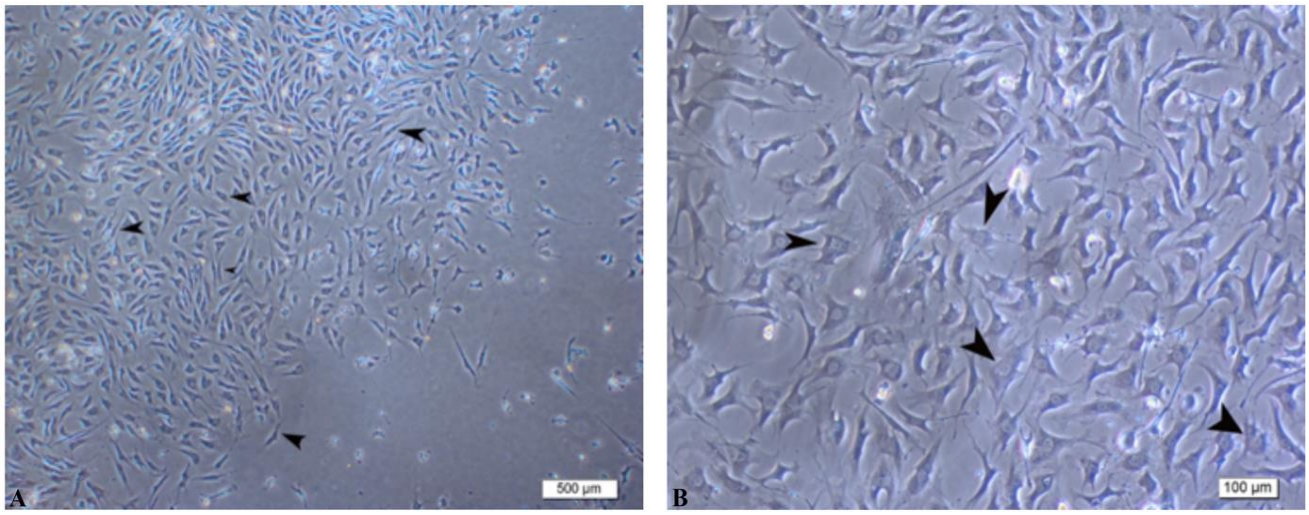

Fig. 1: Morphological characteristics of BSF-MSCs at passage 0 (P0): (A) BSF-MSCs exhibiting a fibroblast-like morphology (indicated by arrows) at Passage 0, Scale bar: 500µM. (B) Flat-shaped cells were also observed in the BSF-MSC population at Passage 0, as Scale bar: 100µM.

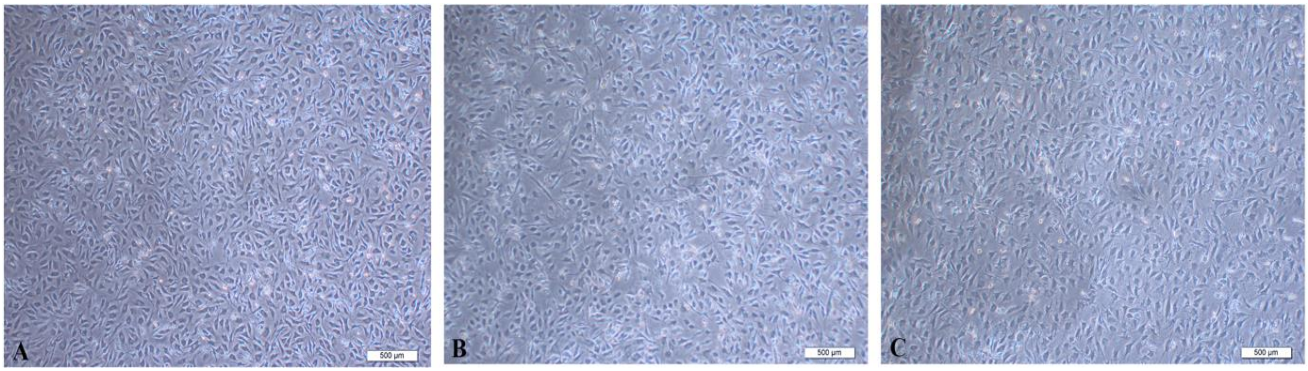

Fig. 2: Morphological characteristics of BSF-MSCs across different passages: BSF-MSCs exhibited fibroblast-like morphology at various passages. (A) Passage 3 (P3) (B) Passage 4 (P4), and (C) BSF-MSCs at Passage 6 (P6). Scale bar: 500µM.

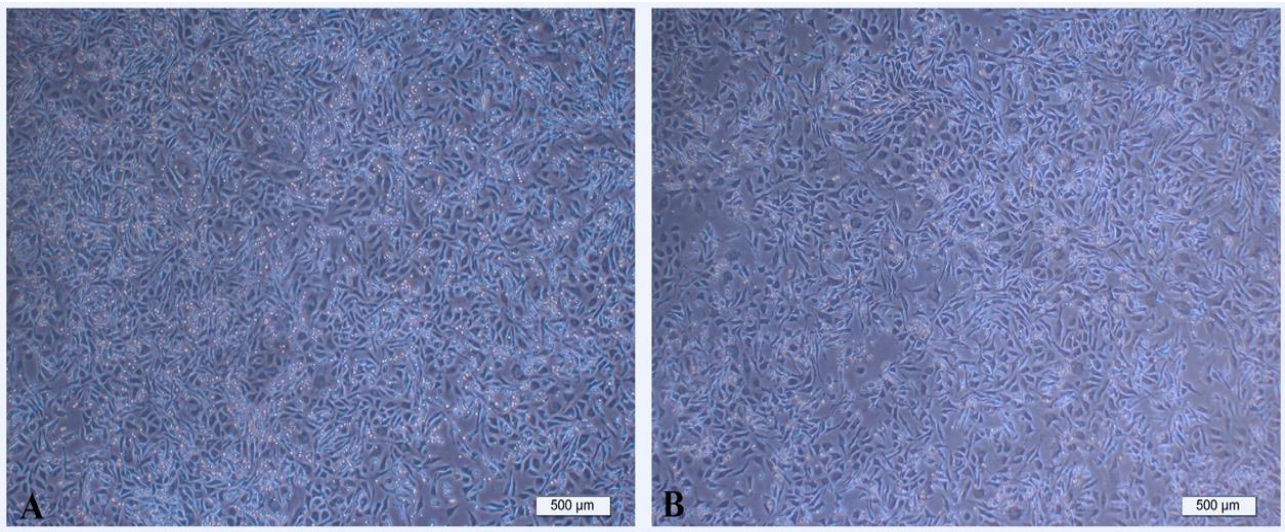

Fig. 3: Morphology of chondrocytes at P0: chondrocytes exhibiting polygonal morphology at 80–90% confluency. (A) Chondrocytes at passage 0 (P0), (B) Chondrocytes at passage 1 (P1). Scale bar: 500µM.

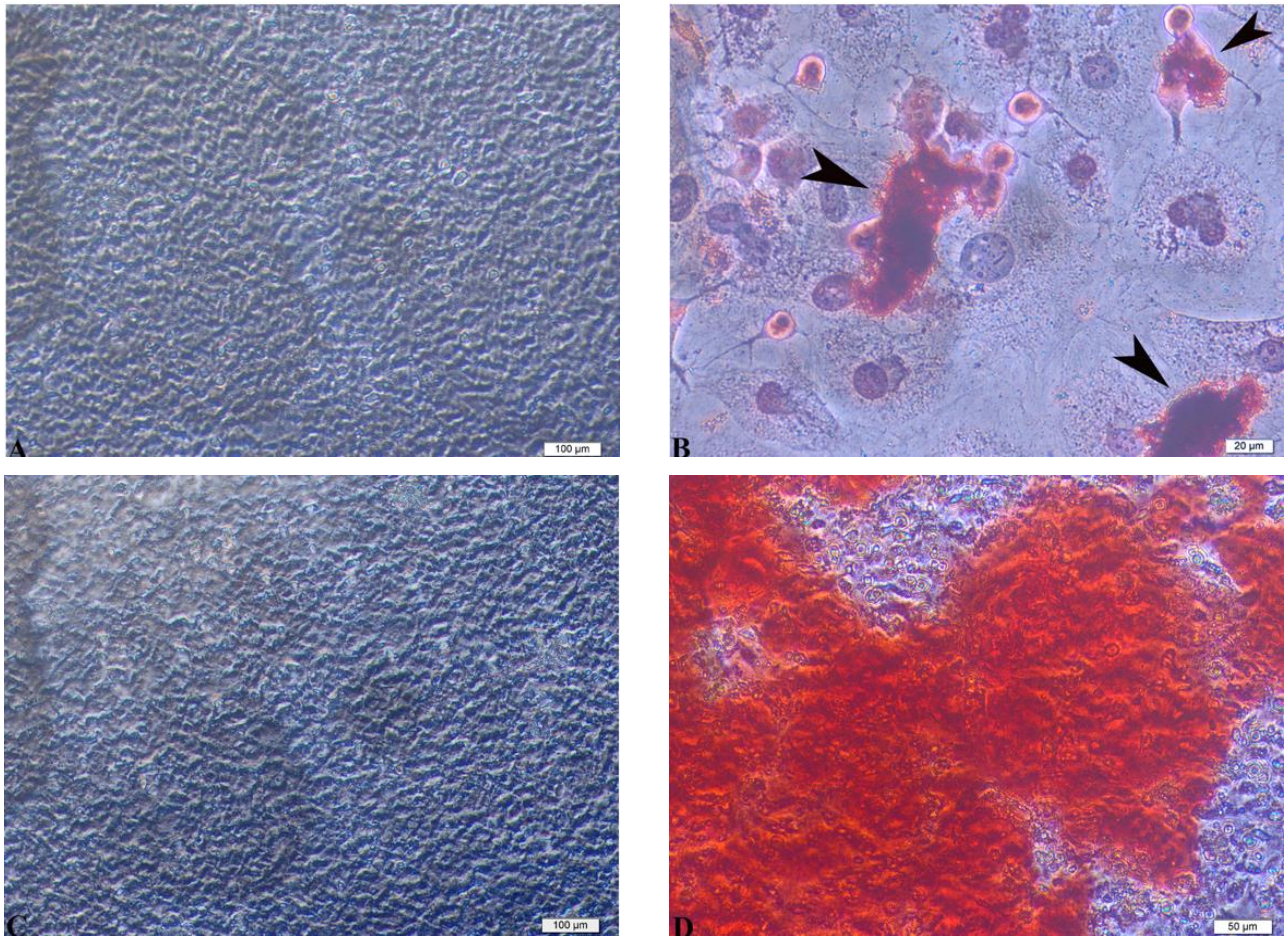

Fig. 4: Adipogenic and osteogenic differentiation of BSF-MSCs at day 21 (A) Negative control for adipogenic differentiation showing no lipid droplet formation. Scale bar: 100µM. (B) Lipid droplets in the cell cytoplasm, stained red, were observed as a result of adipogenic differentiation (indicated by arrows). Scale bar: 20µM. (C) Negative control for osteogenic differentiation with no calcium deposition. Scale bar: 100µM. (D) Calcium deposits stained orange-red, indicating successful osteogenic differentiation. Scale bar: 50µM.

Table 1: Experimental groups and culture conditions for chondrogenic differentiation protocol

Groups	Description
Group 1	Negative Control: BSF-MSCs cultured in standard cell culture medium.
Group 2	Positive Control: BSF-MSCs cultured in chondrogenic differentiation medium with 10ng/mL TGF-ß3.
Group 3	Transwell culture: BSF-MSCs and chondrocytes cultured in standard cell culture medium.
Group 4	Transwell culture: BSF-MSCs and chondrocytes cultured in chondrogenic differentiation medium with 10ng/mL TGF-ß3.
Group 5	Transwell culture: BSF-MSCs and chondrocytes cultured in a chondrogenic differentiation medium with 10ng/mL BMP-9.
Group 6	Transwell culture: BSF-MSCs and chondrocytes cultured in chondrogenic differentiation medium with 10ng/mL TGF-ß3 and 10ng/mL BMP-9.

Table 2: Real-time PCR genes, primer lists, and Tm values used in chondrogenic differentiation medium established groups and MSCs

Genes	Forward (5'→3')	Reverse (5'→3')	Tm (°C)
CD44	CAGCTGAGCCTGGCGCAGATCGA	GCCCTTCTATGAACCCGTACCTGC	60
CD73	GGAAAYCCCATTCTTCTCAACAGC	AATCAGRTTGCCCATGTTGCA	58
CD90	TGAACCCTACCATCGGCATCG	CCCACTTCTTTGTGTCACGGGTC	60
CD105	GGGGATGTGTCAAGGCTGGAGC	ACCAGTTTGGAGTCGTAGGCCA	60
CD34	TTGCACTGGTCACCTCRGGGA	ATAGCCCTGGCCTCCACCGTTCTC	60
CD45	TAAAYGGAGATGCAGGRTCAA	TCCACAACAGACACGTTGGGA	58
COL II	TTGGTGGAAACTTTGCTGCCCA	GGACCAGAAACACCGGGTTCAC	56
ACAN	GTGGTAAAAGGCATCGTGTTCC	TGGGATACCTCACAGTCTGATCG	64
SOX9	AGCTCTGGAGACTGCTGAACG	CTTGAAGATGGCGTTGGGCGA	64
COL10A1	TGCCACAAACAGCACTTTTGC	CCTCTCAGTGATACACCTTTACC	56
GAPDH	TGGGCAAGGTCATCCCTGAGC	TCCACAACAGACACGTTGGGA	60

Immunofluorescence analysis: Chondrogenic differentiation was further confirmed through immunofluorescence staining for COL-II. While Group 1 exhibited no detectable COL-II staining (Fig. 6. B, C), positive staining was observed in all other experimental groups, supporting successful chondrogenic differentiation as shown in Figure. 6. (E, F, H, I, K, L, N, O, R, S).

Real-Time PCR analysis of BSF-MSCs for chondrogenic differentiation potential: Real-time PCR analysis of chondrogenic differentiation was conducted across groups, as shown in Table 1. The fold increase in COL2A1, SOX9, ACAN, and COL10A1 gene expressions in Group 2 compared to the negative control group were 13.54, 6.57, 3.36, and 2.43, respectively. Statistically significant increases were observed in COL2A1 ($P<0.05$),

ACAN (P<0.05) and SOX9 (P<0.05) gene expressions compared to the negative control (Fig. 8A).

In comparison with the control group, significant upregulation in COL2A1, SOX9, and ACAN gene expressions was observed in Groups 3 and 4. Group 3 exhibited statistically significant increases in COL2A1 (P<0.05) and ACAN (P<0.05), while Group 4 showed significant increases in COL2A1 (P<0.01), SOX9 (P<0.05), and ACAN (P<0.05) (Fig. 8B, C).

In Group 5, which contained BMP-9, a statistically significant increase was noted for COL2A1 (P<0.01), SOX9 (P<0.01), and ACAN (P<0.05) gene expressions (Fig. 8D). Comparison between Group 3, without growth factors, and Group 5, containing BMP-9, revealed no statistically significant differences in chondrogenic markers, although COL2A1 gene expression in Group 5 was higher than in Group 4 containing TGF-ß3. The highest chondrogenic marker values were seen in Group 6, supplemented with both BMP-9 and TGF-ß3, with statistically significant increases in COL2A1 (P<0.001), SOX9 (P<0.001), and ACAN (P<0.05) (Fig. 8E). Notably, COL10A1 expression remained statistically unchanged (P>0.05).

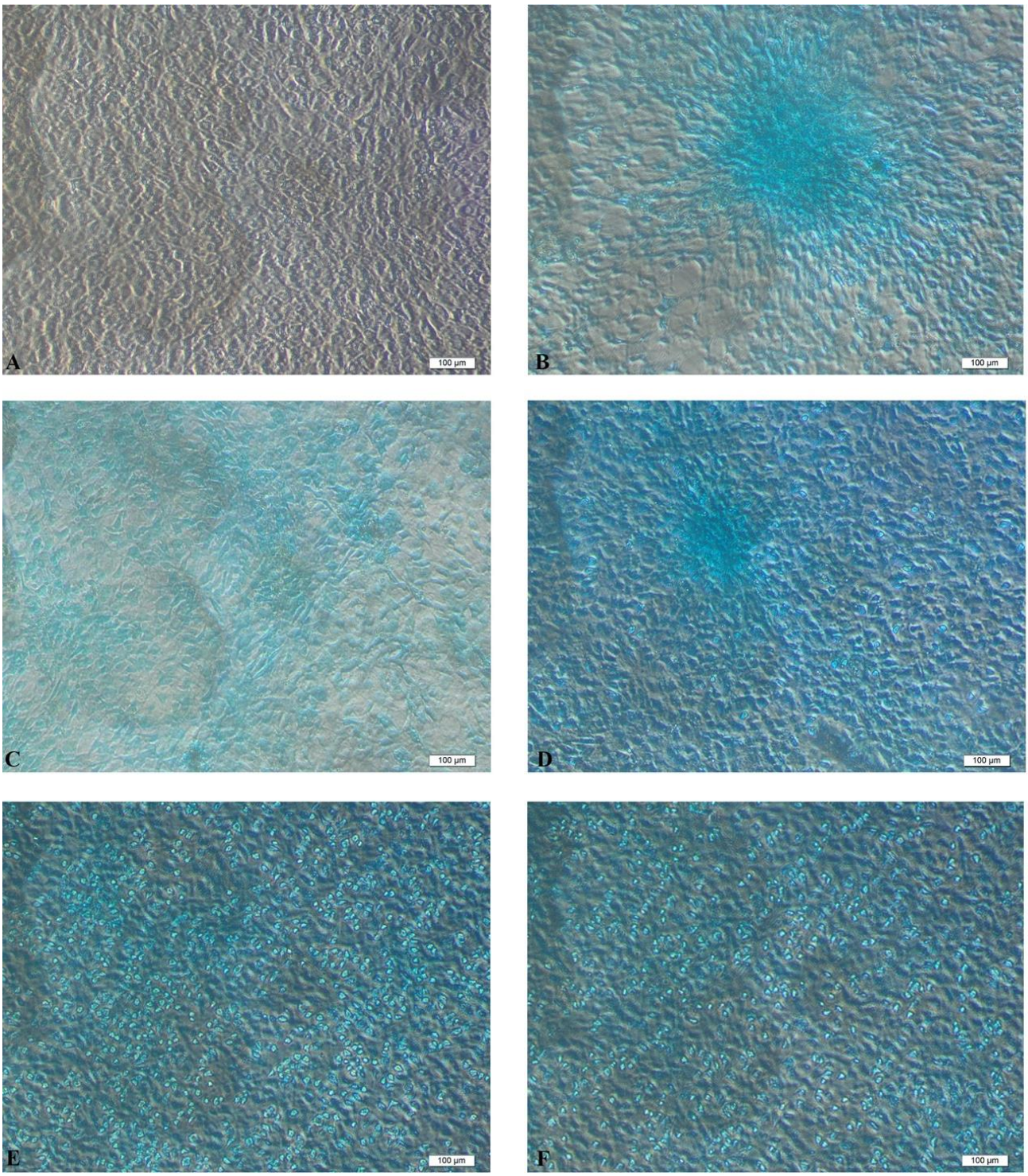

Fig. 5: Chondrogenic differentiation in transwell co-culture by Alcian blue staining: (A) No staining was observed in the negative control group. (B) Group 1, (C) Group 2, (D) Group 3, (E) Group 4, and (F) Group 5 show glycosaminoglycan accumulation, stained bright blue, indicating chondrogenic differentiation. Scale bar: 100µM.

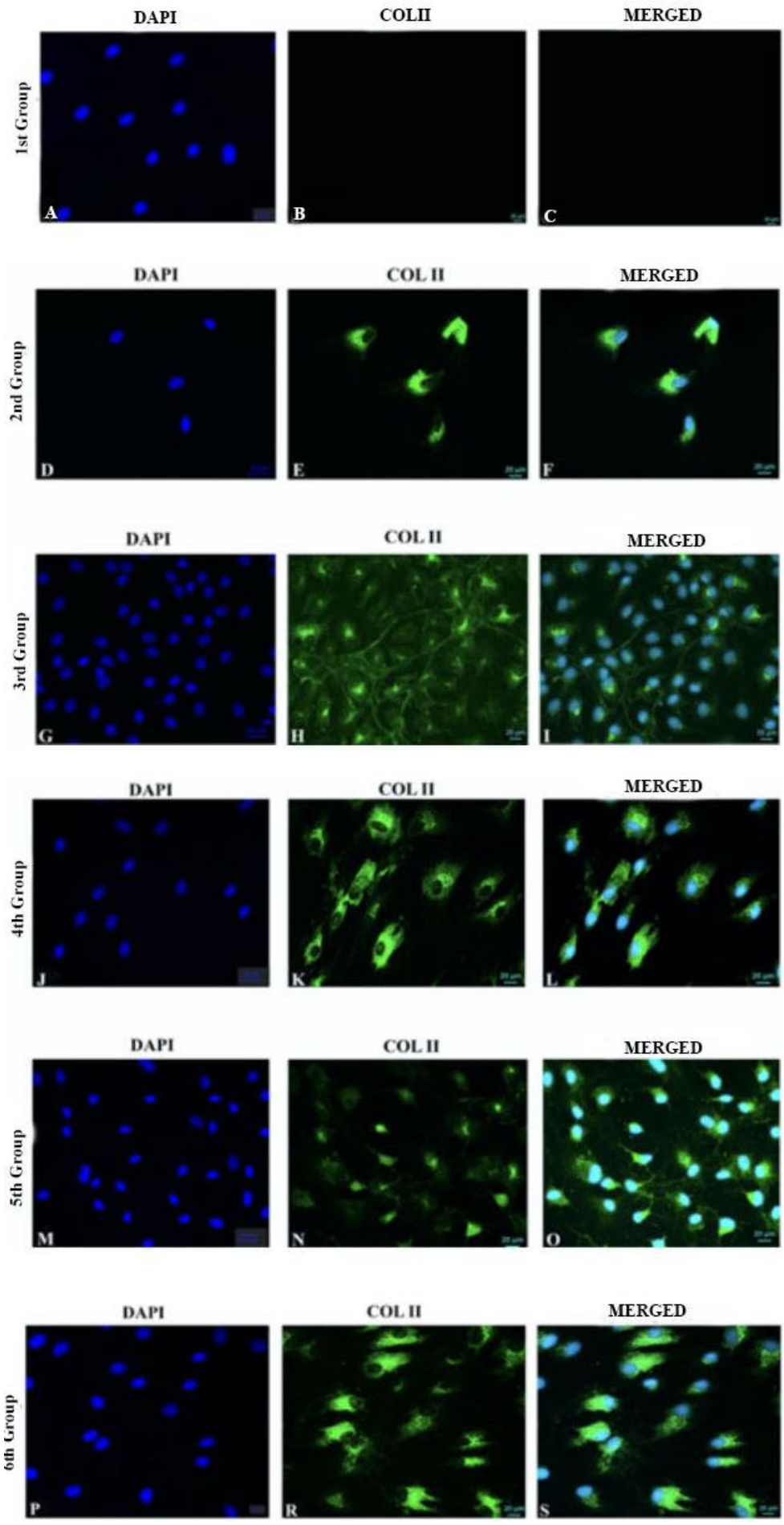

Fig. 6: COL-II expression in all groups at day 21 in transwell culture by immunofluorescence (IF). (A, D, G, J, M, P) Cell nuclei stained with DAPI. (B, E, H, K, N, R) COL II protein expression in the cytoplasm of cells. (C, F, I, L, O, S) Merged images showing both DAPI and COL II expression. Scale bar: 20μM.

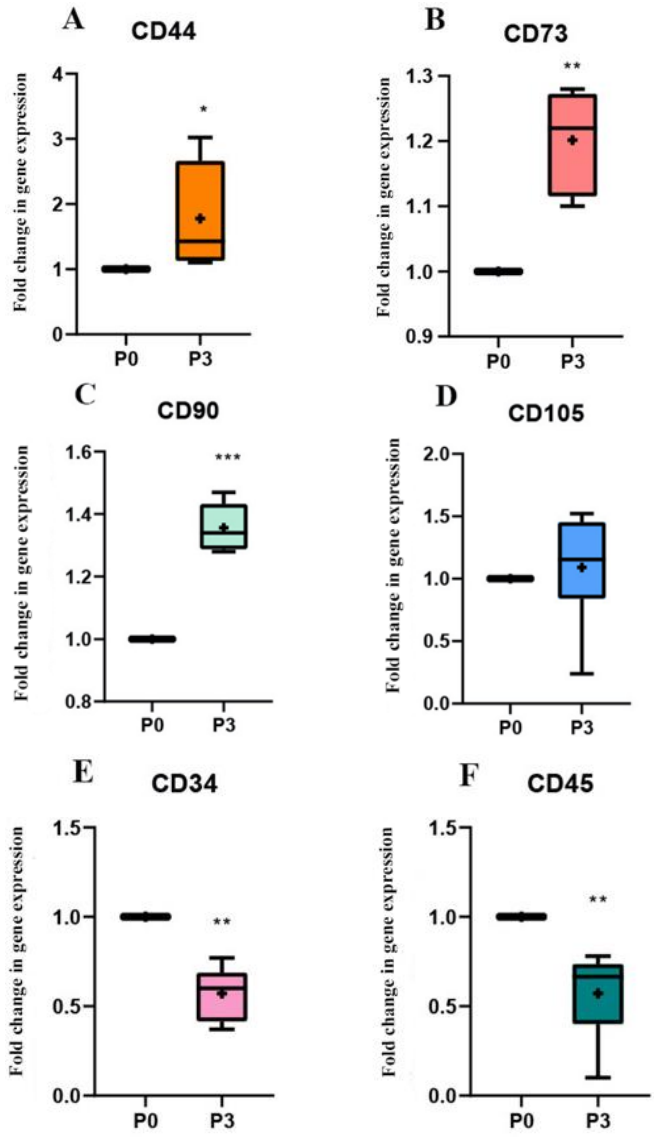

Fig. 7: Real-time PCR expression of BSF-MSC markers in terms of fold change (Bar and Whisker Graph). (A) CD44 expression is significantly higher at Passage 3 (P3) compared to Passage 0 (P0) (P<0.05). (B) CD73 is significantly upregulated at P3 (P<0.01) compared to P0. (C) CD90 shows significant upregulation at P3 (P<0.001) compared to P0. (D) No change was observed in CD105 gene expression between P3 and P0. (E, F) CD34 and CD45 levels are significantly lower at P3 compared to P0 (P<0.01). The data is presented as bar and whisker graphs, with statistical significance indicated). P≤0.05*, P≤0.01**, and P≤0.001***

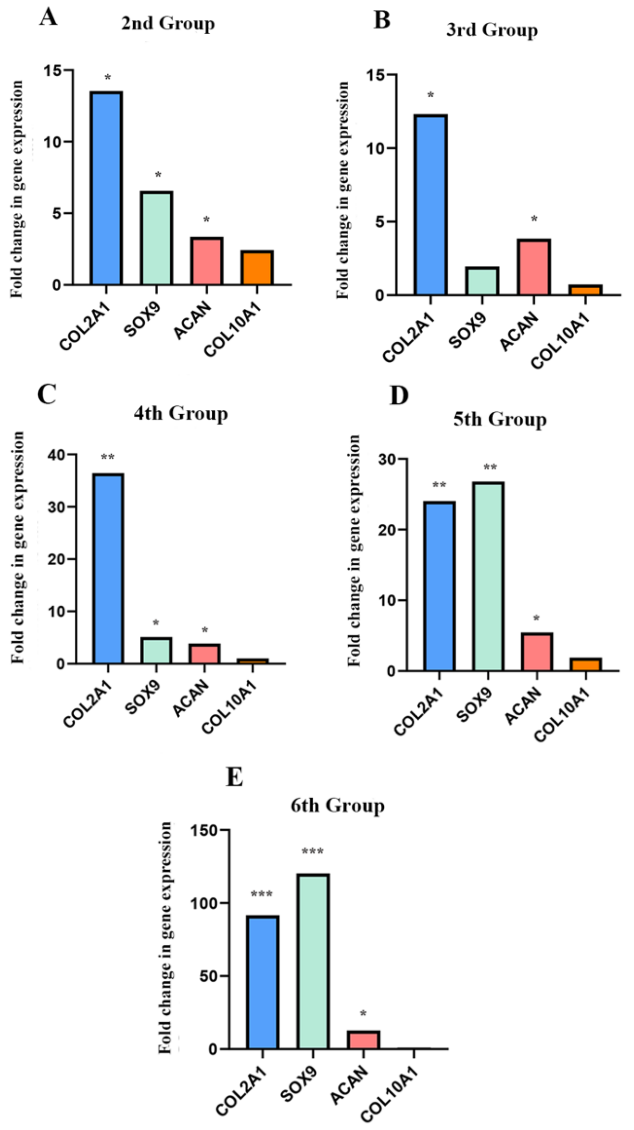

Fig. 8: Real-time PCR expression of chondrogenic differentiation markers in terms of fold change. (A) In Group 2, COL2A1 (P<0.05), SOX9 (P<0.05), and ACAN (P<0.05) were significantly upregulated compared to Group 1. (B) In Group 3, COL2A1 (P<0.05) and ACAN (P<0.05) were significantly upregulated compared to Group 1. (C) In Group 4, COL2A1 (P<0.01), SOX9 (P<0.05), and ACAN (P<0.05) were significantly upregulated compared to Group 1. (D) In Group 5, COL2A1 (P<0.01), SOX9 (P<0.01), and ACAN (P<0.05) were significantly upregulated compared to Group 1. (E) In Group 6, COL2A1 (P<0.001), SOX9 (P<0.01) and ACAN (P<0.05) were significantly upregulated compared to Group 1. The data is presented as bar and whisker graphs, with statistical significance indicated). P≤0.05*, P≤0.01**, and P≤0.001***

DISCUSSION

This study demonstrates the significant regulatory influence of chondrocytes on the chondrogenic differentiation potential of BSF-MSCs in a transwell co-culture system, emphasizing the role of cell-cell interactions. Morphological changes, Alcian Blue staining, immunofluorescence for COL-II, and real-time PCR analysis corroborate the differentiation outcomes. Our findings indicate that synovial fluid serves as a significant source of stem cells demonstrating diverse capacities for osteogenic, adipogenic, and chondrogenic differentiation. It was suggested that the transwell system facilitated indirect signaling, promoting a physiologically relevant environment for the chondrogenic differentiation potential of BSF-MSCs by chondrocytes exposed to TGF-ß3 and BMP-9 growth factors by regulation of chondrogenic pathways.

In our study, BSF-MSCs exhibited adherence to the culture dishes upon cultivation subsequently developed cellular niches, and maintained fibroblast-like morphology throughout passages, consistent with their mesenchymal origin. Similar to our findings, Harvanová *et al*. (2011) reported fibroblast-like morphology.

In our research, we investigated the gene expression profiles of MSC markers (CD44, CD73, CD90, and

CD105) and hematopoietic cell markers (CD34 and CD45) at P0 and P3 in BSF-MSCs before their usage for chondrogenic studies. The Real-Time PCR analysis showed that BSF-MSCs expressed CD44, CD90, CD73, and CD105 while downregulating CD34 and CD45 expressions which are consistent with the established MSC phenotype. This aligns with previous findings that MSCs do not express hematopoietic markers CD45 and CD34 (Hatakeyama *et al*., 2017; Li *et al*., 2020). Furthermore, CD44 and CD90 have been reported to play significant roles in chondrogenic differentiation (Jones *et al*., 2021). CD44, a hyaluronan receptor, is considered crucial for maintaining cartilage integrity and supporting extracellular matrix interactions (Jones and Crawford, 2014), while CD90 is linked to regulating differentiation and cellular adhesion (Krawetz *et al*., 2012). These molecular profiles confirm the chondrogenic potential of SF-MSCs and their suitability for cartilage regeneration.

It has been reported that the natural joint mechanism can be successfully mimicked in transwell co-culture systems, providing a physiologically applicable model, effective for studying chondrogenesis (Griffith *et al*., 2021). We believe that the design of our study, with chondrocytes placed on transwell inserts and SF-MSCs seeded at the bottom of the wells with varying chondrogenic differentiation mediums, leverages the advantages to optimize the outcomes of chondrogenic differentiation which is consistent with previous reports (Fischer *et al*., 2010). We assume that by combining chondrocytes and SF-MSCs in Transwell inserts, this system not only can enhance differentiation efficiency but also provide insights into the molecular crosstalk critical for cartilage regeneration as reported by Zhou *et al*., (2023). It was reported that this effect is particularly evident in transwell systems, where the suppression of TGF-β3-induced hypertrophy underscores the regulatory potential of chondrocyte-derived factors (Aung *et al*., 2011). Furthermore, comparative studies reported that cartilage matrix formation is significantly enhanced in co-culture systems involving chondrocytes and multiple stem cells, such as synovial membrane-derived stem cells, embryonic stem cells, or induced pluripotent stem cells, relative to monolayer cultures (Van *et al*., 2015). This superior matrix production highlights the synergistic interactions between chondrocytes and MSCs in co-culture environments. Additionally, chondrocytes secrete TGF-β isoforms that not only enhance chondrogenesis in MSCs but also upregulate key markers like SOX9, ACAN, and COL2A1, as observed in our study (Qing *et al*., 2011)

Moreover, the inclusion of BMP-9 and TGF-β3 in the transwell co-culture system further amplifies the differentiation potential, as evidenced by the significant upregulation of chondrogenic markers (SOX9, ACAN, and COL2A1) and suppression of undesirable hypertrophic changes and deposition of glycosaminoglycan by Alcian blue in our study. Furthermore, in our research, we formed treatment groups by adding the concentrations of TGF-β3 and BMP-9 growth factors either alone or together to the culture medium in the transwell co-culture system. TGF-β3 is known to induce chondrogenic differentiation of stem cells, while BMP-9 primarily activates hypertrophy (Wu *et al*., 2012). However, it has also been shown that TGF-ß could cause hypertrophy when added to a transwell culture consisting of synovial stem cells and chondrocytes (Jorgenson *et al*., 2018). There are also studies showing that BMP-9 induces chondrogenesis depending on time and dose while causing hypertrophy (Morgan *et al*., 2020). However, the combination of both growth factors, used at the concentration (10ng/mL), yielded a synergistic effect on chondrogenesis, as evidenced by the robust upregulation of COL2A1, SOX9, and ACAN which is consistent with previous findings by Padmaja *et al*. (2022). Importantly, we observed no significant changes in COL10A1 expression, indicating the absence of hypertrophic differentiation.

Our findings align with previous study reporting the chondrogenic potential of BMP-9 and TGF-β3 (Akgün *et al*., (2022). BMP-9 demonstrated superior efficacy in enhancing COL2A1 and SOX9 expression compared to TGF-β3 alone. However, their combination elicited the most pronounced response, highlighting their complementary mechanisms of action. Additionally, our prior investigations revealed that the addition of BMP-9 or TGF-β3 in transwell co-culture did not significantly upregulate hypertrophy markers such as MMP-13 and RUNX-2, further validating the stability of the chondrogenic phenotype (Akgün *et al*., 2022). We assume that both growth factors (10ng/mL BMP-9 and TGF-ß3) and chondrocytes have shown a synergistic effect on chondrogenesis in Transwell culture. Additionally, the interplay between growth factors and cell-cell interactions was critical in modulating chondrogenesis. Chondrocytes in the transwell system likely secrete additional cytokines and growth factors, reinforcing the differentiation of SF-MSCs. This reciprocal signaling mechanism highlights the importance of co-culture systems in mimicking native tissue environments.

However, to better understand the chondrogenic differentiation potential of BMP-9 and TGF-ß3, further studies could be established with the varied doses which could help delve deeper into the molecular crosstalk between these pathways and devising therapeutic strategies for significantly enhancing cartilage repair outcomes. Future studies should delve deeper into the molecular crosstalk between these pathways and explore additional factors that could further enhance chondrogenesis. Although this study highlights the potent chondrogenic potential of SF-MSCs, we suggest long-term *in vivo* studies to validate the true therapeutic potential of SF-MSCs before clinical therapies. Additionally, we believe that exploring the roles of other growth factors and signaling molecules during chondrogenesis could provide deeper insights into optimizing chondrogenesis.

Conclusions: SF-MSCs emerge as a promising source of stem cells for targeted therapies in cartilage defects with significant chondrogenic differentiation potential in a transwell co-culture system. Our results indicate that the chondrogenic potential of SF-MSCs can be enhanced by combinatorial effects of BMP-9 and TGF-β3 supplementation in co-culture systems influencing chondrogenic pathways. Our findings suggest that BMP-9 and TGF-β3 enhance chondrogenesis in vitro, requiring further in vivo validation.

Abbreviations: ACAN: aggrecan; BMP-9: bone morfogenetic protein-9; CHDR: chondrocytes; COL10A1: collagen type X; COL2A1: collagen type II; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; IBMX: 3-isobutyl-1- methylxanthine; HG-DMEM: high glucose-DMEM; ITS: Insulin Transferrin Selenium; lg-DMEM: low glucose-DMEM; PBS: phosphate-buffered saline; SFMSC: synovial fluid mesenchymal stem cells; SOX9: SRY-box transcription factor 9; TGF-β3: transforming growth factor-beta 3.

Conflict of interest: The authors declare no potential conflict of interest for this study.

Ethics approval and consent to participate: The methods of animal sacrifice, tissue management, and disposal of abattoir materials were reviewed and approved by the Ethical Committee of Afyon Kocatepe University (AKÜHADYEK-46; 30.04.2019), Turkey.

Consent for publication: Not applicable.

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Authors contribution: EEA and KA designed and directed the project. EEA and KA planned experiments. EEA and ME carried out the experiments. EEA drafted the manuscript. All authors helped with the research and analysis. ME and KA critically revised the manuscript and provided supervision. All authors contributed to the writing of manuscript and approved the final version.

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