

Research Article

# Fibrochondrogenic Differentiation Potential of Human Adipose-derived Mesenchymal Stem Cells in a Type I Collagen-based Meniscus Scaffold with Activated Platelet-Rich Plasma Stimulation *In-vitro*

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## Abstract

**Introduction:** Despite efforts to use scaffolds to treat meniscus tears, minimal progress has been made in facilitating meniscus regeneration and return of function. Our research objective was to develop a meniscus repair and regeneration implant by applying a resorbable scaffold in combination with cells and growth factors. We report here the results of using Platelet-Rich Plasma (PRP) as a source of growth factors to induce fibrochondrogenic differentiation of human Adipose-Derived Mesenchymal Stem Cells (hADSC) in a three-dimensional (3D) Type I collagen-based scaffold *in-vitro*.

**Methods:** Scaffold Preparation: Type I collagen scaffolds were prepared following a protocol previously published. Two different densities of scaffolds, High Density (HD) and Low Density (LD), were produced for *in-vitro* study. hADSC and PRP Preparation. hADSCs were cultured to the fifth passage to reach the desired number for experimentation. PRP was collected from human blood and activated. Cell Culture Procedure: Effects of PRP on hADSC proliferation and differentiation into fibrochondrogenic cells were examined in four scaffold groups: LD, HD, LD+PRP and HD+PRP. hADSCs were seeded onto scaffolds (n=5) at a concentration of  $2 \times 10^6$  cells/scaffold. 1% of PRP was added to the experimental media. Cellular proliferation was assessed at 1, 7, 14 and 21 days. Differentiation was measured using qRT-PCR on Days 14 and 21. qRT-PCR analysis of gene expression was completed with primers for COLLAGEN 1 and AGGRECAN. Data Analysis: ANOVAs were conducted (two-tailed tests) at the .05 significance level.

**Results:** Cellular proliferation of hADSCs seeded on each scaffold increased over time. Similar trend was observed for cells seeded on HD scaffolds with and without PRP. hADSC showed significant increase in cellular proliferation on the LD scaffolds at Days 1 and 7. At Day 21, PRP treatment and LD scaffold had a synergistic positive effect on Type I collagen gene expression. PRP did not elevate type I collagen gene in the HD group, the HD scaffold alone had the same level of type I collagen gene expression as LD+PRP. Aggrecan expression was elevated in the presence of

PRP in both the HD and LD scaffold groups, indicating enhanced fibrochondrogenic differentiation of hADSCs. Effective cell infiltration was observed across both HD and LD scaffolds with and without PRP treatment. HD scaffolds displayed larger cell clusters and more extensive cell migration over time compared to LD scaffolds. However, LD scaffolds resulted a more uniform cellular distribution than HD scaffolds.

**Conclusion:** Our study demonstrates that PRP can play an important role in directing hADSCs towards fibrochondrogenic differentiation in Type I collagen-based scaffolds *in-vitro*. Additionally, our study shows that collagen scaffold density can influence the spatial distribution and cellular behavior of infiltrated cells.

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**Keywords:** Meniscectomy; Platelet-Rich Plasma; Meniscus Repair; Collagen Meniscus Implants; Tissue Engineering; 3D Collagen Scaffolds; Stem Cells

## Introduction

The menisci of the knee are two (medial and lateral meniscus) crescent shaped pads of fibrocartilaginous tissue attached inferiorly to the fossae between the femoral condyles and the tibial plateaus. The cellular components of the meniscus are termed fibrochondrocytes, which are more fibroblast-like at the outer vascular region and more rounded, like the chondrocytes found in articular cartilage morphology in the inner and middle regions [1]. These cells interact with an Extracellular Matrix (ECM) component that is composed of collagen, Glycosaminoglycans (GAGs) and water. In contrast to articular cartilage, where collagen II is most abundant, collagen I is the predominant collagen type in the meniscus [2]. During weight-bearing movements, the menisci of the knee articulate with the femoral condyle to reduce and disperse forces across the joint surfaces. They also produce lubricating factors to minimize friction during movement and provide nutrients to the articular cartilage of the knee joint. Hence, each meniscus is critical to the joint's structural integrity, function and homeostasis. Damage to the meniscus leads to aberrant load distribution within the joint and pathological "wear and tear" changes to the articulating surfaces of the joint, which predispose patients to accelerated articular cartilage degeneration (i.e., accelerated osteoarthritis). Such alterations increase joint instability and progressively lead to a plethora of orthopedic sequelae associated with compensatory gait changes and altered movement patterns [3]. There are two broad categories of meniscus tears: acute and chronic, with the former being associated with a traumatic event and the latter associated with gradual wear and tear on the fibrocartilage structure.

Meniscus tears are among the most common orthopedic injuries with an incidence of roughly one million per year in the US alone [4]. Despite the recent advancements in the field of orthopedic medicine, which allow for longer health and activity spans, the prevalence of meniscus tears, either acute traumatic tears that occur more typically in younger patients or chronic overuse tears occurring in older patients, is increasing [3]. Hence, there is a need for effective, non-invasive means for treating meniscus injuries.

Healing of a meniscus tear depends on the amount of blood supply in the area. Blood is supplied to the meniscal tissue from the periphery to the center. Hence, the outer "red zone," which receives adequate blood supply is most likely to be healed by repair and regeneration without the need for surgical intervention. The middle "red-white zone" has moderate capacity to heal and finally the avascular "white zone" at the center of the meniscus, has the least capacity to heal properly [5].

Management of meniscus tears differs depending on a variety of factors ranging from the type of tear, the age and activity level of the patient and their symptoms. In any event however, the surgeon's priority is to try to preserve the meniscal tissue as much as possible. Acute symptomatic tears are typically managed conservatively with rest, ice, elevation and oral non-steroidal anti-inflammatory medications. Recent research has shown that supervised physical therapy focused on strengthening the muscle groups around the knee (particularly the quadriceps muscles) results in the same clinical outcomes as the most common operation for meniscus tears, an Arthroscopic Partial Meniscectomy (APM) [6-8]. Therefore, surgical management of meniscal tears is mostly indicated for patients who don't respond to conservative treatments, have diminished quality of life or have concurrent anterior cruciate ligament injuries. However, long term clinical studies showed that APM still pose greater risk of OA development and rapid chondrolysis with loss of joint space [9].

Intra-articular injections of Hyaluronic Acid (HA) and or corticosteroids are also commonly used in conjunction with physical therapy to accelerate the healing of damaged meniscus tissue or provide symptomatic relief [3]. Recently, cell-based tissue engineering and orthobiologics, which encompass all biological therapies used in orthopedic medicine, including scaffolds, cell-seeded and growth factors enriched scaffolds and Platelet-Rich-Plasma (PRP) have shown promise as augmentative treatments for promoting tissue regeneration and meniscus repair. Injections of mesenchymal stem cells (MSCs are typically derived from adipose tissue, bone marrow, synovial tissue or embryonic tissue) have shown the most promise of the purely cell based injectable orthobiologics for the purpose of regenerating native meniscus tissue [109].

PRP is an injectable orthobiologic, consisting of concentrated platelets and various growth factors, such as Fibroblast Growth Factor 2 (FGF2), Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor beta (TGFβ), which has the potential

to enhance the regeneration of native meniscus tissue [10]. There is a lack of large-scale clinical evidence demonstrating its efficacy in accelerating healing when administered in conjunction with physical therapy, surgery or both. However, a recent meta-analysis study found that PRP leads to a significant reduction in meniscectomy failure rates and improved overall functional outcomes in patients who received PRP with surgery compared to their non PRP treated counterparts [6].

Scaffold-based orthobiologic treatments (e.g., Collagen Meniscus Implants, CMI or Actifit) for meniscus repair after meniscectomy have been shown to restore the meniscal tissue and clinically reestablish knee function, reduce pain and prevent or delay the development of OA. CMI is one of the FDA-approved meniscus implants which was developed in the 1990s and it has more than 10 years of long-term clinical follow-up [11-15]. However, studies show some patients experienced decreased size and reduced mechanical function with the initial generations of implants [16]. Meniscal Allograft Transplantation (MAT) has also been clinically determined to help alleviate pain in young patients with symptomatic subtotal meniscectomy after 10 years post- surgery [17,18]. However, its chondroprotective effect is not comparable to intact meniscus [19]. There is currently no treatment modality that ideally restores the biomechanical function of the meniscus. Nevertheless, scaffolds offer structural support, mechanical stability and a suitable attachment site for seeded cells. This environment facilitates stem cell differentiation leading to the deposition of an extracellular matrix that closely resembles the native meniscus ECM, including proper collagen and glycosaminoglycan organization [20,21]. Efforts to improve the biomimetic and mechanical performance of biodegradable scaffolds for meniscus tear treatment must favor the right microenvironment to allow for meniscal tissue regeneration and repair. Hence, our goal is to combine the autologous stem cells and PRP with our novel meniscus scaffold to achieve a one-stage surgical procedure at the surgery site for a torn meniscus repair. We have developed a novel engineering method to fabricate the meniscus scaffolds which were designed to closely bio-mimic the native meniscus (the data were published in 2020 and 2022 ORS conference abstracts). The use of autologous stem cells (e.g., hADSCs) allows for one-stage surgical procedures and the avoidance of additional cell culture works. In the current study, we aimed to explore the potential of this approach *in-vitro*. Thus, two different densities of scaffolds along with PRP were tested to evaluate how cell-scaffold interaction could affect stem cell fate and which combination would be most effective at promoting the fibrochondrogenic potential of hADSCs. The preliminary *in-vitro* results reported here were based on the AlamarBlue assay and gene expression over a period of 21 days culture.

## Material and Methods

### *Scaffold Preparation*

Type I collagen from bovine deep flexor tendon was prepared in-house, using the purifying procedure developed by Li and Stone [22,23]. Type I collagen-based meniscus scaffolds were prepared as described previously: 7 g of purified Type I collagen fibers were swollen in 1L 0.07M lactic acid, pH 2.5 overnight at 4°C, followed by homogenization to produce a homogeneous dispersion of Type I collagen fibrils [23]. Two different densities of scaffolds were prepared by introducing different amounts of collagen dispersion (120 g for the high-density scaffolds and 80 g for the low-density scaffolds). Prior to engineering the collagen meniscus scaffolds, the pH of both dispersions was adjusted to 4.8 (the isoelectric point of the collagen) to reconstitute the collagen fibers. The reconstituted fibers were then de-gassed and wound on a mandrel to create fiber orientation in the circumferential direction. Afterwards, the wound collagen scaffolds were molded, dehydrated, lyophilized, crosslinked, rinsed, re-lyophilized, packaged and ETO sterilized. Finally, both High Density (HD) and Low Density (LD) meniscus scaffolds were stored on the shelf for further *in-vitro* studies.

### *Pore Size*

Scanning Electron Microscope (SEM) (Hitachi S-4800, Tokyo, Japan) images were taken and the pore structure from the radial cross-section of the scaffold was analyzed. Pore size was defined as the longest distance across a pore and was measured using ImageJ (version: 1.54 h, NIH, Bethesda, Maryland). At least 100 pores per image were randomly selected and measured. Average pore size was calculated.

### *Density and Porosity Measurement*

The water displacement technique was employed. The meniscus implant was cut in half along the circumferential direction to separate the inner and outer rims after freeze-drying. Scaffolds dry weights ( $W_{\text{collagen}}$ ) were measured immediately. The volumes of collagen ( $V_{\text{collagen}}$ ) can be calculated by using the collagen dry weight ( $W_{\text{collagen}}$ ) divided by collagen's density ( $1.41 \text{ g/cm}^3$ ). The scaffolds were then immersed in water at room temperature until being saturated (about 10 minutes). The

process was performed under the vacuum condition to ensure the air was completely removed. Subsequently, scaffolds wet weights ( $W_{\text{water+collagen}}$ ) were measured following the removal of excess water from the scaffold's surfaces using filter papers. The empty space of the scaffolds can be considered using the volumes ( $V_{\text{water}}$ ) occupied by water. The water volumes ( $V_{\text{water}}$ ) can be calculated by using scaffolds wet weights ( $W_{\text{water+collagen}}$ ) to subtract the collagen dry weight ( $W_{\text{collagen}}$ ) and water's density was assumed to be  $1 \text{ g/cm}^3$ . The density and porosity were then calculated according to Eq. 1 and 2.

$$\text{Eq 1. for Density: } \frac{\text{Scaffold Weight } (W_{\text{collagen}})}{\text{Scaffold Volume } (V_{\text{collagen}} + V_{\text{water}})}$$

$$\text{Eq 2. for Porosity: } \frac{\text{Empty Volume } (V_{\text{water}})}{\text{Scaffold Volume } (V_{\text{collagen}} + V_{\text{water}})}$$

#### *hADSC and PRP Preparation*

hADSCs were obtained from ATCC and cultured in Mesenchymal Stem Cell Basal medium supplemented with Mesenchymal Stem Cell Growth Kit for Adipose and Umbilical-derived (ATCC, Manassas, Virginia) and 1% antibiotic-antimycotic (ThermoFisher Scientific, Waltham, Massachusetts). Cells were plated in T150 cell culture flasks and incubated at  $37^{\circ}\text{C}$ , 21%  $\text{O}_2$  and 5%  $\text{CO}_2$  and passaged until the fifth passage for experimentation. RegenKit-BCT, a commercial PRP collection tube, was used to obtain leukocyte-poor-PRP. PRP was collected from human blood and spun once according to the manufacturer's centrifugation protocol (RegenLab, Brooklyn, New York). Briefly, 10 mL of blood was drawn into a Regenkit-BCT tube and spun for 5 minutes at 1500 g. Platelet-Poor-Plasma was carefully drawn from the tube into an empty 15 mL conical tube until 1 mL remained. The remaining plasma was gently mixed with the platelets sedimented on the surface of the gel separator in the tube to obtain Platelet-Rich-Plasma. One freeze-thaw cycle was used to allow for platelet activation before cell culture treatment. A final concentration of 1% PRP (by volume) was added directly to culture medium every other day during cell culture. PRP characteristics are summarized based on guidelines by Kon, et al., in-Table 1.

| PRP Type          | N1N2-N0N0-N1N0                               |
|-------------------|----------------------------------------------|
| MPV               | 7xfl                                         |
| Red Blood Cells   | $0.06 \times 10^6 \text{ cells}/\mu\text{L}$ |
| White Blood Cells | $2.6 \times 10^3 \text{ cells}/\mu\text{L}$  |
| Neutrophils       | $0.08 \times 10^3 \text{ cells}/\mu\text{L}$ |
| Lymphocytes       | $2.09 \times 10^3 \text{ cells}/\mu\text{L}$ |
| Monocytes         | $0.21 \times 10^3 \text{ cells}/\mu\text{L}$ |
| Eosinophils       | $0.00 \times 10^3 \text{ cells}/\mu\text{L}$ |
| Basophils         | $0.02 \times 10^3 \text{ cells}/\mu\text{L}$ |

**Table 1:** Summary of PRP characteristics.

#### *Cell Culture Procedure*

The effect of PRP on hADSC proliferation and differentiation into fibrochondrogenic cells was investigated. A total of four groups of scaffolds were studied, LD alone, HD alone, LD+PRP and HD+PRP. hADSCs were seeded onto scaffolds ( $n=5$ ) at a concentration of  $2 \times 10^6$  cells/scaffold. Cells in (2D) monolayer culture were used as positive control to normalize all measurements during experimentation. 1% of PRP was added to the experimental media. The media was changed every other day. Quantitative measurement of cell metabolism was performed using the AlamarBlue assay according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, Massachusetts). Since resazurin, the colorimetric indicator of cellular metabolism in AlamarBlue is a non-toxic reagent, repeated measurements were taken over time on the same sample for the following timepoints: Day 1, 7, 14, 21. After 4 hours of incubation with 10% (v/v) AlamarBlue added in culture media, absorbances were measured at 570 and 600 nm as reference wavelengths per the manufacturer's protocol with a standard spectrophotometer to determine the amount of AlamarBlue reduced for each sample. Absorbance values were corrected using absorbance of control

wells with no cells, with media only and media with AlamarBlue. Metabolic activity normalized to control was indicated as a percentage of AlamarBlue reduction using the following equations provided by the manufacturer's protocol.

$$\text{Eq 3: Percentage of AlamarBlue reduction} = \frac{[Alw - (Ahw \times Ro)] \text{ for test well}}{[Alw - (Ahw \times Ro)] \text{ for control well}}$$

$$\text{Eq 4: Ro} = \frac{(\text{Absorbance of AlamarBlue in media} - \text{Absorbance of media only}) \text{ at } 570 \text{ nm}}{(\text{Absorbance of AlamarBlue in media} - \text{Absorbance of media only}) \text{ at } 600 \text{ nm}}$$

Alw: absorbance at lower wavelength minus the media blank

Ahw: absorbance at higher wavelength minus the media blank

Ro: Correction factor

On Days 14 and 21, samples were additionally harvested for gene expression studies to assess cellular differentiation. Messenger RNA was collected from cells after lysis using the RNeasy Plus Mini kit (QIAGEN, Venlo, Netherlands). The reverse transcription of mRNA to cDNA was done using the iScript™ cDNA Synthesis Kit and a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California). The relative gene expression levels of the two ECM proteins (Aggrecan and collagen Type I) most predominantly associated with meniscus and cartilage matrix synthesis were measured by qPCR with QuantStudio™ 3 Real-time PCR System (Applied Biosystems, Waltham, Massachusetts) using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, Waltham, Massachusetts) following standard protocol. The relative fold change of each target primer was calculated using the 2-ΔΔCT method and normalized to the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase GAPDH. Target sequences for each primer are shown in Table 2.

| Primers         | Sequences                                   |
|-----------------|---------------------------------------------|
| TYPE I COLLAGEN | 5'-ATG CCA TCA AAG TCT TCT GCA A-3' Forward |
|                 | 5'-CTT-GGG-GTT-CTT-GCT-GAT-GTA-C-3' Reverse |
| AGGRECAN        | 5'-CTA-CAT-TGG-TGG-AAG-TGG-TCA-C3' Forward  |
|                 | 5'-CCA CTA-GCT-CTC-CCA-CTA-ATG-T-3' Reverse |
| GAPDH           | 5'-ACC-CAG-AAG-ACT-GTG-GAT-GG-3' Forward    |
|                 | 5'-GAG-GCA-GGG-ATG-ATG-TTC-TG-3' Reverse    |

**Table 2:** List of the primers and their sequences.

### Histological Study

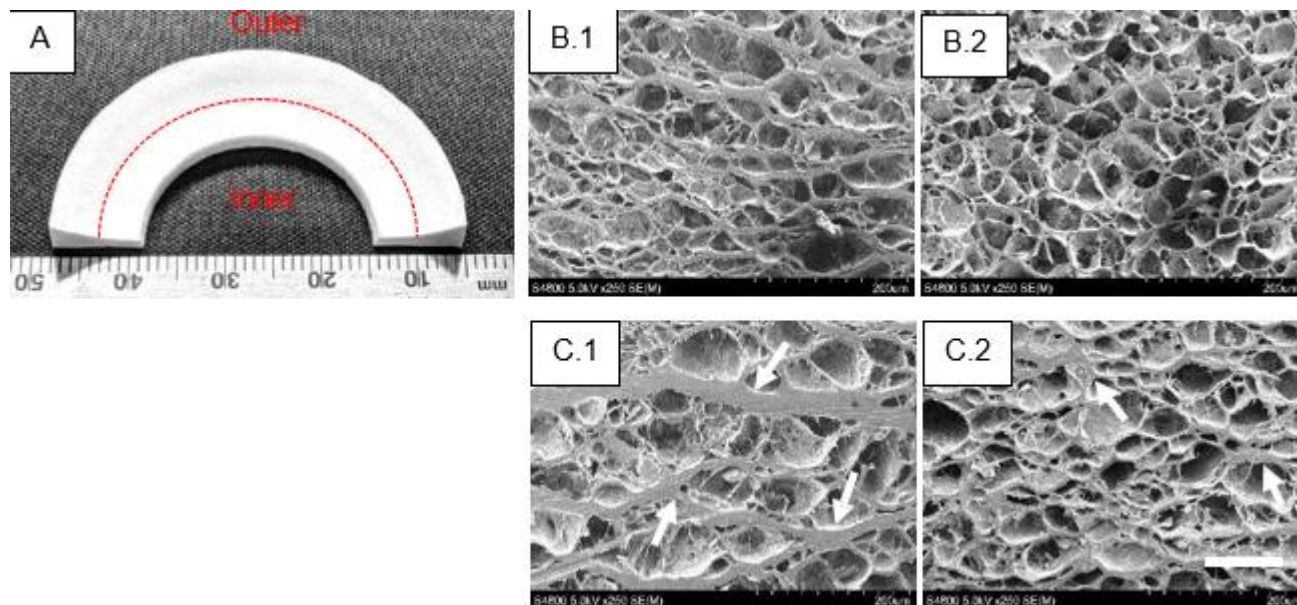
Scaffolds were fixed in 10% formalin (ThermoFisher Scientific, Waltham, Massachusetts) at Days 1, 7, 14, 21 for histology to evaluate cell infiltration and distribution within the scaffolds over time. All fixed samples were subsequently dehydrated before paraffin embedding. After paraffin embedding, sections were cut for each sample at 7 μm thick. Four sections from each group at each time point were stained with hematoxylin and eosin. Images of each section were taken for qualitative evaluation of cell migration within the scaffold overtime.

### Statistical Analysis

Shapiro-Wilk test was performed to determine normal distribution of data. An Analysis of Variance (ANOVA) was conducted with a two-factor repeated measures ANOVA where time was one factor and PRP treatment was another factor to test for the effect of PRP treatment of hADSCs on each scaffold over time. Similar analysis was conducted to test the effect of the LD and HD scaffold density in the absence or presence of PRP over time. Sidak's multiple comparison tests were performed to test for differences between groups. Statistical significance was set at the 0.05 alpha level for all tests. Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software Inc., San Diego, California).

## Results

### General Appearance of Meniscus Scaffold and Pore Structure



**Figure 1:** Meniscus scaffolds: an overall look (A) and SEM images (B and C); the LD inner rim (B.1) and the LD Outer rim (B.2); and the HD inner rim (C.1) and the HD outer rim (C.2). Scale bar indicates 100  $\mu\text{m}$ . White Arrow indicating thicker collagen fibers in HD scaffold inner and outer rim.

The general appearance of meniscus scaffold was shown in Fig. 1. The LD and HD scaffolds shared the same dimensions. Both had 4.5 cm in length (anterior to posterior horn distance), 1 cm in width (peripheral to inner rim distance), 0.2 cm in height at the inner rim and 0.7 cm at the outer rim. In general, the inner rim has thicker collagen fibers than the outer rim. The fiber thickness increased with the increase in scaffold density. However, pore structure remained similar sizes regardless of the locations or the density.

### Density Porosity and Pore Size Measurement

Table 3 shows the overall average density of HD scaffolds (25% increase) was significantly higher ( $p=0.0036$ ) than LD scaffolds. When comparing LD and HD scaffolds' partial density, HD scaffolds revealed a significant difference at the inner rim with a 35% increase ( $p=0.001$ ) and with a 23% increase at the outer rim ( $p=0.039$ ). The porosity for both LD ( $88.2\pm0.2\%$ ) and HD ( $86.2\pm0.9\%$ ) scaffolds did not show any statistical difference ( $P>0.05$ ) as well as the pore sizes. Both groups have similar pore sizes at inner and outer rims.

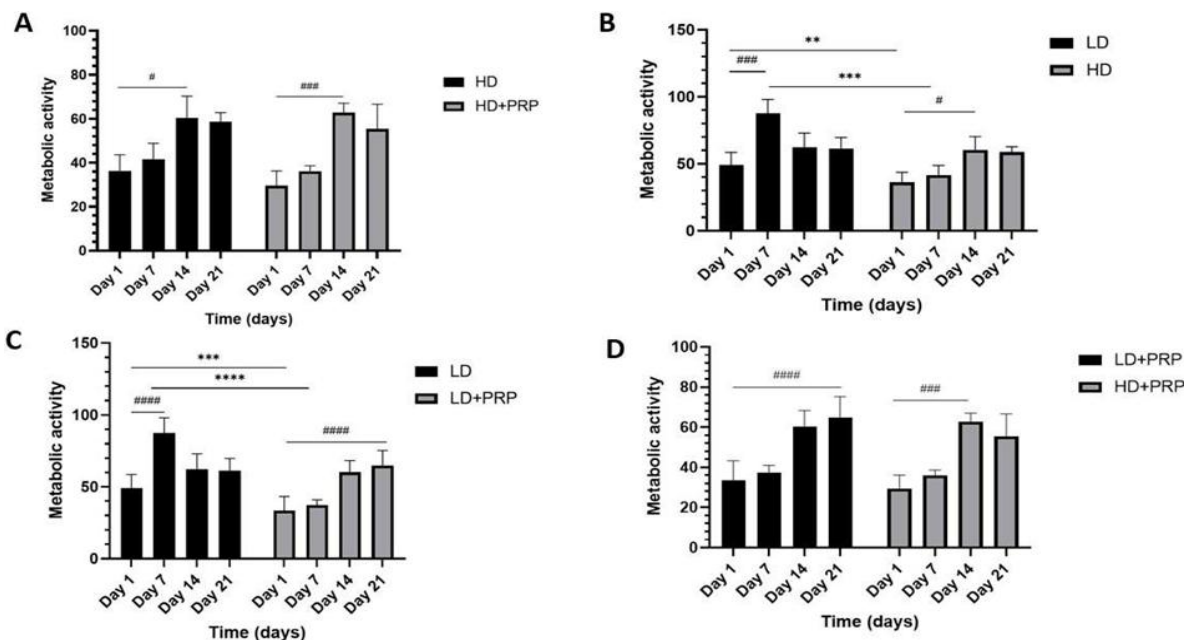
|                                                                                              | Low Density (LD) |                 | High Density (HD) |                 |
|----------------------------------------------------------------------------------------------|------------------|-----------------|-------------------|-----------------|
| Overall Density ( $\text{g}/\text{cm}^3$ )                                                   | 0.15 $\pm$ 0.01  |                 | 0.19 $\pm$ 0.01   |                 |
| Porosity (%)                                                                                 | 88.2 $\pm$ 0.2   |                 | 86.2 $\pm$ 0.9    |                 |
|                                                                                              | Inner            | Outer           | Inner             | Outer           |
| Partial Density ( $\text{g}/\text{cm}^3$ )                                                   | 0.19 $\pm$ 0.01  | 0.13 $\pm$ 0.01 | 0.26 $\pm$ 0.01   | 0.16 $\pm$ 0.01 |
| Pore size ( $\mu\text{m}$ )                                                                  | 65.5 $\pm$ 16.4  | 61.7 $\pm$ 15   | 63.1 $\pm$ 31     | 62.2 $\pm$ 20.8 |
| Data are presented as Mean $\pm$ 1 SD. All tests had (n=3) and were repeated at least twice. |                  |                 |                   |                 |

**Table 3:** Summary of density, porosity and pore size.

### In-vitro Cellular Proliferation of hADSCs on 3D LD and HD Scaffolds

hADSCs seeded on each scaffold showed increases in cellular proliferation with and without PRP treatment. A similar trend in cellular proliferation was observed for hADSCs seeded on HD scaffolds with and without PRP stimulation. Proliferation was highest in both groups at Day 14 (61%  $\pm$ 4 for HD with PRP and 63%  $\pm$ 10 for HD without PRP) with statistically significant

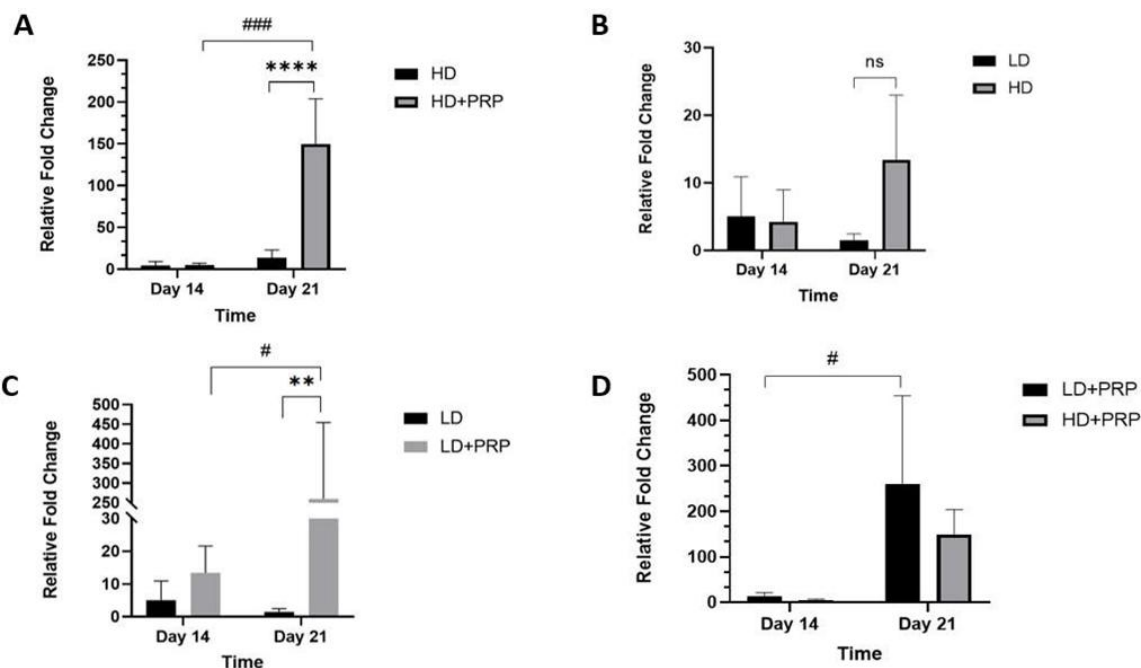
increases ( $p=0.0134$ ,  $0.0005$ , respectively) relative to Day 1 ( $29\%\pm 17$ ,  $30\%\pm 7$  for HD with and without PRP respectively) (Fig. 2). Comparative analysis of the cellular proliferation of hADSCs seeded on LD and HD scaffolds revealed a significant increase for LD group on Day 1 ( $p=0.0074$ ) and Day 7 ( $p<0.0001$ ) (Fig. 2). When examining the trend in cellular proliferation of hADSCs seeded on LD scaffolds over time, a transient increase was observed for LD group without PRP stimulation. This increase was highest at Day 7 ( $88\%\pm 11$ ). This contrasts with a gradual increase over time seen in the LD group stimulated with PRP. Initial cellular proliferation of hADSCs seeded on LD scaffolds without PRP on Day 1 ( $49\%\pm 10$ ) and Day 7 ( $88\%\pm 11$ ) was observed to be higher when compared to LD samples with PRP stimulation ( $34\%\pm 10$  and  $38\%\pm 4$ ,  $p=0.0003$ ,  $<0.0001$  respectively) (Fig. 2). hADSCs in the LD scaffold group exhibited the highest cellular proliferation on Day 21 with PRP treatment ( $65\%\pm 10$ ) ( $p<0.0001$ ) (Fig. 2). There was no significant difference in cellular proliferation between LD and HD scaffolds in the presence of PRP.



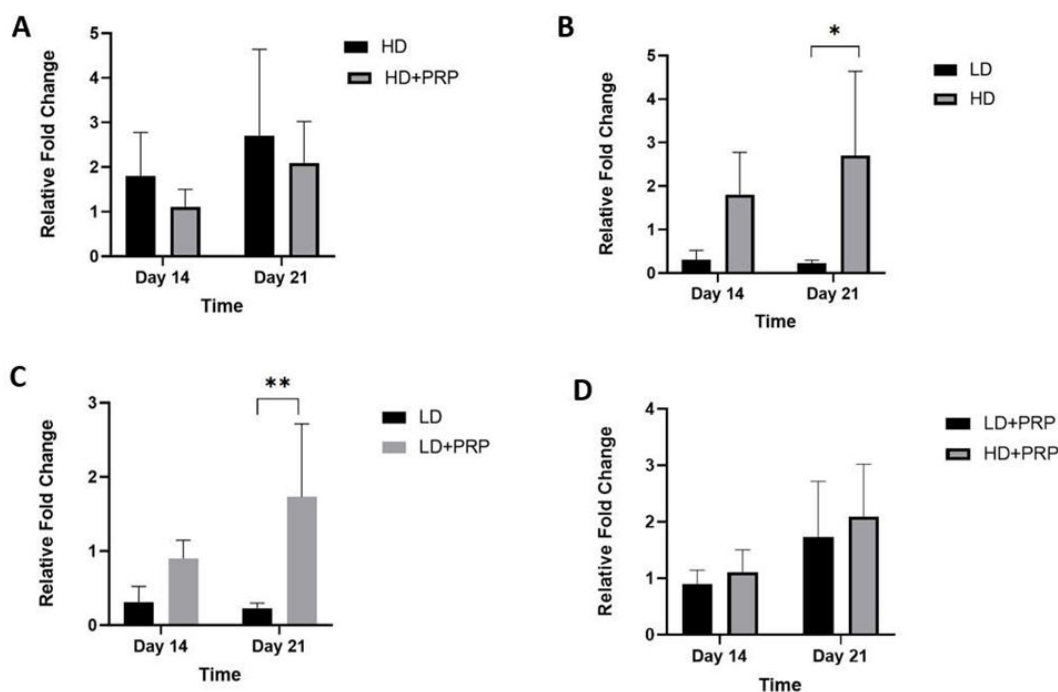
**Figure 2:** Cell proliferation ( $n=5$ ): Quantification of cellular activity using absorbance values detected after Alamar Blue Reduction at the different time points (Days 1, 7, 14, 21). Note: All values are represented as means and standard deviations. Statistical significance: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ . #Significant difference between groups; # Significant difference across time.

#### *Fibrochondrogenic Potential of hADSCs Cultured on 3D Scaffolds for Meniscus Tissue Repair*

To examine the fibrochondrogenic differentiation potential of hADSCs cultured on LD and HD scaffolds for meniscal tissue repair, relative gene expression changes for Aggrecan (ACAN) and Collagen I (COL I) were quantified for Day 14 and Day 21. No difference was found in ACAN gene expression level between all groups on Day 14 (Fig. 3). In absence of PRP, hADSCs seeded on HD scaffold had greater level of ACAN expression compared to LD ( $p=0.0352$ ) (Fig. 3). In the presence of PRP, hADSCs seeded on both scaffolds demonstrated higher gene expression for ACAN compared to untreated groups at Day 21 (Fig. 3). This increase was found to be more statistically significant for HD scaffolds ( $p<0.0001$ ) than LD scaffolds ( $p=0.0055$ ). When analyzing the effect of PRP treatment over time within groups, the increase of ACAN expression in groups treated with PRP was statistically more significant for LD than HD scaffolds ( $p=0.0298$ ) (Fig. 3). However, no significant comparative difference was found in ACAN gene expression between hADSCs seeded on LD and HD scaffolds with PRP at Day 14 and Day 21 (Fig. 3). Quantification of gene expression level for COL I for HD groups revealed an increase without PRP stimulation on Days 14 and 21 (Fig. 4). However, this increase was not found to be statistically significant. The opposite trend was observed in the LD groups, treated and untreated with PRP. A significant increase in COL I gene expression was found in hADSCs seeded on LD scaffolds with PRP stimulation at Day 21 ( $p=0.0030$ ) (Fig. 4). Comparison of COL I gene expression in hADSCs unstimulated with PRP and seeded on LD and HD scaffolds showed higher levels of expression in HD groups at Day 14 and 21 (Fig. 4). This increase was only found to be statistically significant on Day 21 ( $p=0.0146$ ). The effect of PRP treatment on COL I expression over time was not found to be significant for all groups. In addition, there was no significant difference found between HD and LD scaffolds in the presence of PRP (Fig. 3).



**Figure 3:** Gene expression analysis (n=5) Relative fold change in aggrecan (ACAN) gene expression, a chondrogenic ECM marker found in meniscal tissue and articular cartilage. Note: All values are represented as means and standard deviations. Statistical significance: \*,#p ≤ 0.05, \*\*,##p ≤ 0.01, \*\*\*,###p ≤ 0.001, \*\*\*\*,####p ≤ 0.0001. \*Significant difference between groups; #Significant difference across time.

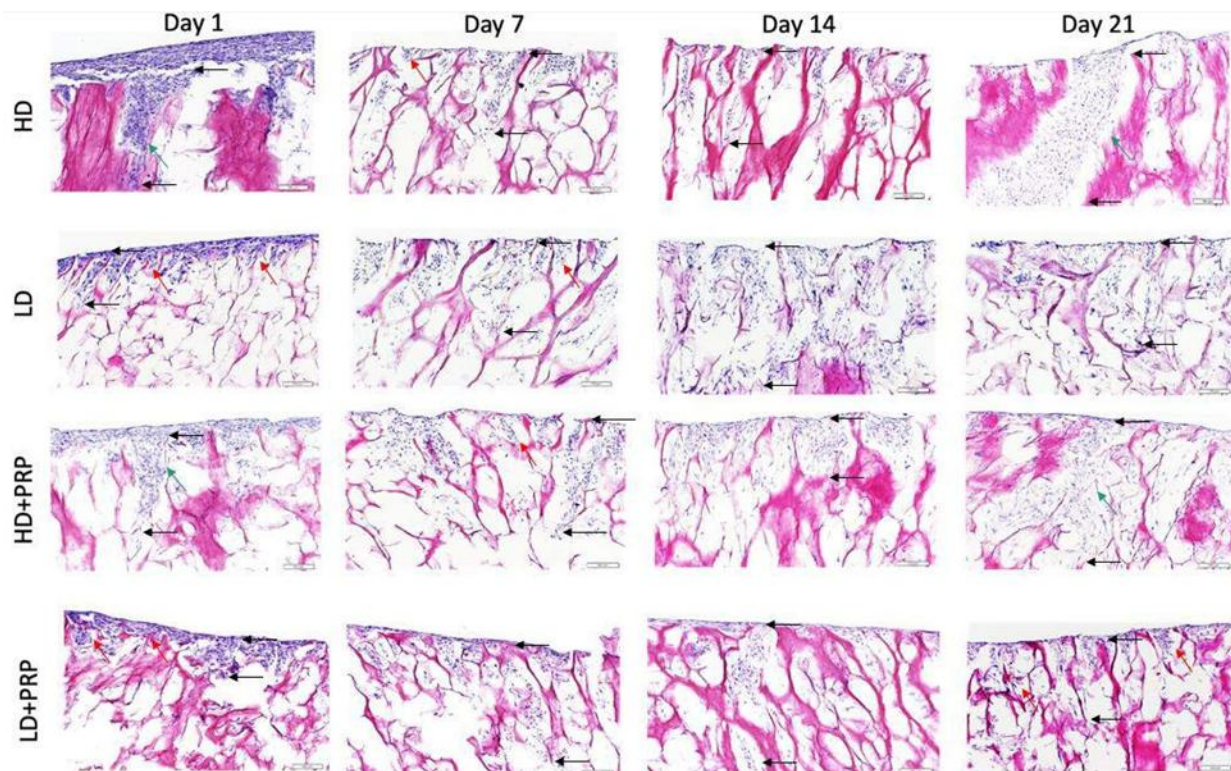


**Figure 4:** Gene expression analysis (n=5) Relative fold change in type I Collagen (COL I) gene expression, the most abundant collagen in the meniscus. Note: All values are represented as means and standard deviations. Statistical significance: \*,#p ≤ 0.05, \*\*,##p ≤ 0.01, \*\*\*,###p ≤ 0.001, \*\*\*\*,####p ≤ 0.0001. \*Significant difference between groups; # Significant difference across time.

#### *In-vitro Cell Infiltration of hADSCs Seeded on 3D LD and HD Collagen Scaffolds*

To visualize the cellular infiltration and morphology of the hADSCs on LD and HD scaffolds, micrographs of samples were taken after hematoxylin and eosin staining. From Day 1 to Day 21, a progressive cellular infiltration was observed for all groups with

and without PRP treatment. hADSCs seeded on HD scaffolds with and without PRP treatment showed a very thick and dense layer of cells on the surface after 24 hours of cell seeding; in contrast to LD scaffolds with and without PRP treatment, which demonstrated numerous cell clusters migrating through the pores of the scaffold (Fig. 5). Cell penetration became uniform at Day 7 across all groups. However, on Day 21, HD groups with and without PRP treatment exhibited cellular infiltration deep within the scaffold compared to LD scaffolds with and without PRP treatment.



**Figure 5:** Histology (n=2): Micrographs of HandE-stained samples of biodegradable collagen type I scaffolds Low (LD) and High (HD) densities seeded with hADSCs at Days 1, 7, 14, 21 in an in-vitro culture system with and without PRP stimulation. Dark purple (hematoxylin) stains represent cell nuclear components and pink (eosin) stains display ECM features including collagen scaffold. Scale bar: 100 µm. Two parallel black arrows point from surface to extent of cellular migration within the scaffold display distance migrated by the cells. Red arrows indicate small cell clusters. Green arrows indicate large cell clusters.

## Discussion

This report is a continuation of our effort to develop novel tissue engineering strategies to address the clinical unmet need for a torn meniscus repair. Tissue engineering involves three key components, scaffold, cells and bioactive molecules. The strategy provides the essential needs for the treatment to regenerate the injured tissues. The scaffolds serve the most important role because they serve as a temporary meniscus substitute, provide mechanical resistance and facilitate the meniscus regeneration during the post-surgery rehab program. In our previous preliminary studies (2020 and 2022 ORS conference abstracts), we have optimized the meniscus scaffolds which were designed to closely bio-mimic the native meniscus. Thus, we wanted to further explore their effect on cellular behavior for meniscus tissue regeneration. In this *in-vitro* proof of concept study, LD and HD scaffolds were used to evaluate how cell-scaffold interaction and the presence of PRP could affect stem cells fate and drive their fibrochondrogenic differentiation potential.

After initial seeding, we observed that LD and HD scaffolds allowed for cellular infiltration and aggregation within the scaffold. LD scaffolds resulted in a uniform cell distribution over time. HD scaffolds allowed for deeper cellular invasion but an uneven spatial distribution of cells. This is based on the observed depth of penetration of the infiltrated cells after histological analysis shown in Fig. 4. Micrographs of cell-seeded scaffolds at Day 1 and Day 21 particularly highlighted this difference (Fig. 5). SEM images of both scaffolds revealed similar pore size (Fig. 1). Pore interconnectivity was distinguishable due to the presence of thicker collagen fibers in the HD scaffolds. This may explain the unequal spatial distribution of cells in HD scaffolds. Our finding

suggests that cell infiltration and cellular spatial distribution can be controlled by the internal microarchitecture of the scaffold. This is consistent with previous findings [24-26]. In the present study, cellular proliferation of hADSCs was observed to be significantly higher in LD scaffolds than in HD scaffolds on Days 1 and 7 (Fig. 2). LD scaffolds showed smaller cell clusters within the pores than HD scaffolds, which may allow for more surface area for cells to grow (Fig. 5). These findings corroborate previous knowledge that there may be an optimal pore microarchitecture that is suitable for cell growth and penetration [27,28].

When assessing the fibrochondrogenic potential of hADSCs in both scaffold groups, our results indicated that, in the absence of PRP, the HD scaffolds were more effective in driving fibrochondrogenic differentiation than the LD scaffolds. This finding highlights that scaffold density can induce changes in gene expression. PRP treatment upregulated ACAN gene expression of hADSCs regardless of scaffold density (Figure 3). This suggests that there are bioactive factors in PRP that can enhance chondrogenic potential of hADSCs on collagen scaffold. In the presence of PRP, only LD scaffolds showed a significant increase in COL I gene expression (Fig. 4). As a result, LD scaffolds displayed similar fibrochondrogenic potential with HD and HD+PRP. The increase of both COL I and ACAN expression on LD scaffolds suggests that PRP can provide a source of messengers that stimulate fibrochondrogenic differentiation of hADSCs on collagen scaffold (Fig. 3,4). PRP has been shown to harbor a pool of chondrogenic growth factors such as PDGF, TGF- $\beta$ , FGF and IGF-1 [29-31]. In addition, recent microarray profiling has also revealed the abundance of a diversity of microRNAs (miRNA) in human platelets with a repertoire of target genes involved in numerous biological functions [32,33]. Osman, et al., has predicted five functional groups that are regulated by the 20 most abundantly found miRNAs in human platelets, the mechanistic Target of Rapamycin (mTOR) signaling was identified as one of them [34]. This pathway has been associated with cytoskeletal reorganization and lineage determination of MSCs via  $\beta$ 1-integrin and protein kinase B (AKT) [35-37]. However, the precise mechanism of action of PRP remains to be fully elucidated. Overall, the present study demonstrates proof-of-concept evidence of these cell-seeded and adjuvant treated tissue engineered scaffold as a template for directing cellular differentiation of hADSCs towards a fibrochondrogenic lineage *in-vitro*.

Other researchers have pointed to the importance of scaffold density in promoting cellular infiltration, attachment and then driving the desired phenotype of endogenous or seeded cells for meniscus regeneration [38,39]. In their *in-vitro* study, Ruprecht, et al., investigated the migration potential of endogenous meniscus cells or exogenous human bone marrow derived MSCs on a meniscus derived matrix scaffold [40]. They demonstrated that scaffolds seeded with native meniscus cells led to accelerated cellular infiltration and tissue growth and promoted meniscus healing. In alignment with our study, high-density scaffolds provided deeper cellular invasion compared to low-density. However, their study did not mention a difference in spatial distribution of infiltrated cells. Our study showed evidence of a variation in cell distribution between low- density and high-density collagen scaffolds through histology (Fig. 5).

Other studies have also investigated the ability of Type I collagen scaffolds and other biodegradable scaffolds in combination with PRP to enhance meniscus tissue regeneration and have found that these scaffolds enhance healing in animal models compared to untreated controls [41-49]. However, the current work is the first study to our knowledge that combines a focus on comparing between Type I collagen scaffold densities and comparing between untreated and PRP treated scaffolds. In fact, this is the first *in-vitro* study to investigate the ability of PRP to stimulate fibrochondrogenic differentiation of hADSCs seeded on Type I collagen scaffold.

#### Future Research

Our findings showed that these scaffolds are conducive to cellular attachment, proliferation and migration. Further study might be needed to test for the potential of PRP to induce fibrochondrogenic differentiation of primary cells. The next goal is to test its potential to accelerate the regeneration of meniscus tissue when fixed to a meniscus lesion in an animal model. Ultimately, the clinical application of this scaffold coupled with the regenerative potential of PRP is to provide a custom-designed implant to a meniscal defect that is suitable for meniscal tissue repair and regeneration after partial or subtotal meniscectomy. Perhaps the greatest challenge to translating preclinical research in meniscus repair such as this study, is the inherent complexity that comes with the many permutations of scaffold composition and density, cell-scaffold pairing and biologic adjuvant. The effects of each of these elements are poorly understood let alone in combination with each other. Future research should also focus on comparisons across these different combinations to elucidate which combinations holds the most promise. Scaffold degradation time also needs to be evaluated to design the ideal micro-architecture to promote the regenerative potential of seeded cells.

### Limitations of the Study

The static seeding method used in this study may not yield a uniform infiltration and distribution of cells within a scaffold. Cells are directly pipetted at a high concentration onto the surface of the scaffold introducing local variations in cell density and distribution. Other methods such as needle injection, vacuum seeding or rotational seeding could be better suited for enhancing uniformity and reproducibility. In addition, cell differentiation was assessed using gene expression changes in a static culture. It is well known that the chondrogenic and osteogenic potential of MSCs is affected by mechanical stimuli [48]. The application of compression combined with fluid flow, may create a microenvironment more physiologically relevant to the meniscus. Furthermore, a low dose of PRP was used in our study. We have not explored if higher doses induce a higher regenerative potential in hADSCs seeded on these scaffolds [49].

### Conclusion

Ultimately, our findings reveal a regenerative potential of hADSCs that is influenced by the 3D scaffold microarchitecture and PRP. The present study adds to the growing body of preclinical evidence suggesting that Type I collagen scaffolds in combination with PRP could drive the fibrochondrogenic differentiation of hADSCs *in-vitro*. We believe our approach can serve as a potential clinical application in the future for one-stage meniscal repair to enhance the regeneration of damaged meniscal tissue.

### Conflict of Interests

The author has no conflict of interest to declare.

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