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Perivascular Stem Cells Demonstrate Similar Stemness and Chondrogenic Expression Potential as Mesenchymal Stem Cells

(Sel Stem Perivaskular Menunjukkan Potensi Stem dan Ekspresi Kondrogen seperti Sel Stem Mesenkimal)

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ABSTRACT

Perivascular stem cells (PSCs), namely pericytes, are more accessible than traditional sources of mesenchymal stem cells (MSCs) such as bone marrow and serve as an excellent alternative cell source for treating articular cartilage damage. However, evidence of its multipotent or chondrogenic potential compared to MSCs appears lacking. The present study was thus conducted to (i) Isolate and characterize rat adipose tissue (AT)-derived and peripheral blood (PB)-derived PSC, as well as bone marrow (BM)-derived and PB-derived MSCs; (ii) Establish their multipotentiality; and (iii) Trilineage differentiation of their potentials *in vitro*. PSCs from AT and PB were isolated using magnetic-activated cell sorting, while MSCs were isolated from BM and PB using density gradient centrifugation. Immunophenotyping of PSCs and MSCs was analysed using flow cytometry. Trilineage differentiation of the cells was subsequently assessed using Haematoxylin–Fast Green–Safranin O staining for chondrogenesis, Alizarin Red S for osteogenesis, and Oil Red O for adipogenesis. Chondrogenesis was also analyzed by measuring the production of sulphated glycosaminoglycans. The results showed that both PSCs were similar to MSCs in expressing surface protein markers and the ability to undergo self-renewal and tri-lineage differentiation. However, PSCs expressed higher CD146 levels than MSCs. AT-PSCs exhibited the highest level of proteoglycan content, whereas the chondrogenic potential of PB-PSCs, BM-MSCs, and PB-MSCs demonstrated similar levels. Compared to MSCs, PSCs from various sources demonstrate comparable or higher chondrogenic potential, indicating that PSCs are a superior stem cell source for future cartilage injury treatment strategies.

Keywords: Chondrogenic expression; mesenchymal stem cell; pericyte; perivascular stem cell

ABSTRAK

Sel stem perivaskular (PSC), iaitu perisits, lebih mudah didapati daripada sumber tradisional sel stem mesenkimal (MSC) seperti sumsum tulang, menjadikannya sebagai sumber sel alternatif yang sangat baik untuk merawat kerosakan rawan artikular. Walau bagaimanapun, bukti potensi multipoten atau kondrogennya berbanding MSC masih kurang. Oleh itu, kajian ini dijalankan untuk (i) mengasing dan mencirikan tisu adiposa (AT) tikus dan PSC terbitan darah periferi (PB) serta MSC yang berasal daripada sumsum tulang (BM) dan PB; (ii) membuktikan pelbagai potensi mereka; dan (iii) mengkaji potensi pembezaan *trilineage* secara *in vitro*. PSC daripada AT dan PB telah diasingkan menggunakan pengisihan sel diaktifkan magnetik, manakala MSC diasingkan daripada BM dan PB menggunakan pengemparan kecerunan ketumpatan. Immunofenotip PSC dan MSC dianalisis menggunakan sitometri aliran. Pembezaan *trilineage* sel kemudian dinilai menggunakan pewarnaan dan ujian. Krondrogenesis dianalisis dengan mengukur tahap glikosaminoglikan tersulfat. Keputusan menunjukkan bahawa PSC adalah serupa dengan MSC yang ditunjukkan dengan ekspresi penanda protein permukaan dan keupayaan untuk menjalani pembaharuan diri dan pembezaan tiga keturunan. Walau bagaimanapun, PSC ekspresi tahap CD146 dalam PSC adalah lebih tinggi daripada MSC. AT-PSC menunjukkan kandungan proteoglikan tertinggi, manakala potensi kondrogen PB-PSC, BM-MSC dan PB-MSC menunjukkan tahap yang sama. Berbanding

dengan MSC, PSC daripada pelbagai sumber menunjukkan potensi kondrogen yang setanding atau lebih baik, menunjukkan bahawa PSC berpotensi menjadi sumber sel stem yang unggul untuk strategi rawatan kecederaan rawan masa hadapan.

Kata kunci: Ekspresi kondrogen; perisit; sel stem mesenkimal; sel stem perivaskular

INTRODUCTION

Injuries to articular cartilage caused by direct trauma or excessive and repetitive physical stress, are likely to result in progressive cartilage degeneration due to its limited self-repair capacity. Continual degenerative changes of the articular cartilage may progress to severe osteoarthritis (OA), which often leads to significant functional impairment in the affected individuals. To date, there is no definitive treatment that can cease or reverse the structural and functional defect of the articular cartilage, particularly in the severe stages of osteoarthritis. The current treatment paradigm for osteoarthritis is mainly focused on strategies to alleviate pain, maximize joint function, and attenuate further OA progression. Surgical interventions such as microfracture to stimulate the recruitment of bone marrow, autograft or allograft replacement of osteochondral tissues, and the implantation of *in vitro* cultured chondrocytes to facilitate the development of a new cartilage matrix have not demonstrated long-term efficacy. In recent years, there has been a resurgence of interest in exploring novel strategies, such as using mesenchymal stem cells (MSCs) to potentially treat cartilage damage before it progresses to osteoarthritis (OA). However, such efforts have been hampered by several factors, including donor site morbidity caused by extracting MSCs from bone marrow or adipose tissues. As an alternative, pluripotent stem cells have garnered attention due to their higher chondrogenic potential and accessibility, making them a promising option for cartilage repair and regeneration. An alternative source that avoids such issues is therefore preferred, one of which being suggested is perivascular stem cells (PSCs). Research conducted by Crisan et al. (2008) demonstrated that PSCs can be isolated from the peripheral circulation and subsequently expanded for applications in regenerative medicine (Cantoni et al. 2015; Jung et al. 2011).

PSCs originate from a population of cells called pericytes, which are typically embedded within the basement membrane of smaller vessels, such as arterioles, capillaries, and venules (Winkler, Bell & Zlokovic 2010). Pericytes exhibit similar morphology to fibroblasts and, at times, are indistinguishable from their progenitor stages of development. It has been shown that perivascular localisation is often necessary to distinguish these two cell types (Thomas, Cowin & Mills 2017). Fibroblasts are typically located in the interstitial connective tissue outside the vessel wall, whereas pericytes reside within the basement membrane of microvessels, in close physical association with endothelial cells (Armulik, Genove & Betsholtz 2011; Chang et al. 2002). PSCs contribute to tissue development, regeneration, homeostasis, and repair due to their ability to undergo distinctive self-renewal and differentiation, which is identical to MSCs. PSC has, therefore, been recognised as a potential source of mesenchymal stromal progenitor cells, although its application to treat diseased conditions has not been well established. One reason for this is the process of identifying PSCs, which has proven to be a challenge. For example, there is no specific marker that defines the phenotypic characteristics of pericytes, although it has been suggested that a combination of $CD146^{+}$, $CD31^{-}$, and $CD45^{-}$ may prove to be useful. Other studies have suggested that the marker profile $CD146^{+}$, $CD31^{-}$, $CD34^{-}$, and $CD45^{-}$ more closely resembles the typical expression pattern of pericytes (Bouacida et al. 2012; Crisan et al. 2008; Thomas, Cowin & Mills 2017).

Recent interest has emerged in using innovative strategies, including MSCs, for early cartilage damage treatment to prevent progression to osteoarthritis (OA). However, extracting MSCs from bone marrow presents significant challenges, including complications at the harvest site, such as pain, infection, bleeding, or delayed recovery. Although pericytes and other perivascular stem cells have shown greater bone-forming potential compared to conventional MSCs, their potential for cartilage formation remains largely unexplored. Moreover, PSCs may offer higher chondrogenic potential and easier accessibility, making them a promising alternative for cartilage repair.

Peripheral blood is particularly noted as a readily accessible source of stem cells. Therefore, this study aimed to isolate, identify, and characterize PSCs derived from peripheral blood and compared with adipose tissue. Subsequently, the research focused on inducing PSCs along the chondrogenic lineage to demonstrate their potential for isolation, expansion, and differentiation into chondrogenic cell types. The goal is to establish the feasibility of using PSCs for effective cartilage repair and regeneration. The comparison of trilineage differentiation potential, phenotypic marker expression, and sulphated glycosaminoglycan (S-GAG) quantification after chondrogenic differentiation was conducted to evaluate the similarities between PSCs and MSCs. Trilineage differentiation (osteogenic, adipogenic, and chondrogenic) is a standard criterion used to confirm the multipotency of mesenchymal stem cells. Meanwhile, S-GAG quantification serves as a key indicator of cartilage matrix production, providing functional evidence of chondrogenic potential relevant to cartilage repair applications.

MATERIALS AND METHODS

HARVESTING AT, PB, AND BM SPECIMENS FROM RATS

This study was conducted with ethic approval from the Institutional Animal Care and Use Committee at University Malaya (reference no.: 2018-210903/ORTHO/R/CPP). Fourteen 16-week-old Sprague-Dawley (SD) male rats (N=14) weighing between 400 - 500 g were obtained from the Animal Experimental Unit (AEU). The rats were anaesthetised, and the peripheral blood was collected using intracardiac puncture. The peripheral blood (PB) was aspirated slowly into spray-coated ethylenediaminetetraacetic acid (EDTA) Tubes (BD Vacutainer®, UK). Adipose tissue (AT) was obtained from the back and groin area of rats. The visible blood vessels were removed using a sharp blade. The tibia and femur of both legs were exteriorised after removing the muscular and tendinous attachments. Adipose tissue, femur, and tibia of both legs were stored separately in a 50 mL centrifuge tube (Sewon Precision Mold Limited™ (SPL), North Korea) containing PBS and 5% penicillin/streptomycin (p/s). Bone marrow (BM) was harvested using a centrifugation-based isolation method. In brief, the metaphyseal region was removed from the femur and tibia to expose the hollow interior of the long bones. The bottom of a sterile 1.5 mL microcentrifuge tube is incised, and the distal end of the long bone (knee joint end) is placed facing down into the tube, which is then nested into a 15 mL centrifuge tube and centrifuged at 4,000 rpm for 15 s. The BM that spun out is visually inspected for confirmation. Subsequently, the 1.5 mL microcentrifuge tube containing the bone is discarded, and the bone marrow is suspended in 2 mL of PBS.

ISOLATION OF PB- AND BM-DERIVED MSCS AND AT-DERIVED STROMAL CELLS

BM suspensions were mixed with 1X Phosphate-Buffered Saline (PBS) solution (Gibco, USA), resulting in a total volume of 20 mL of the solution. Peripheral blood was mixed with an equal volume of 1X PBS. Three milliliters (3 mL) of Ficoll-Paque 1.073 (GE Healthcare, USA) were placed into these 15 mL centrifuge tubes. The 4 mL diluted mixture of the specimen was layered gently on top of the Ficoll-Paque. The tubes were centrifuged at 2500 rpm for 25 min at 24 °C. The PB- or BM-derived mononuclear cells were carefully extracted and transferred into a new 15 mL centrifuge tube. This was followed by a washing step using PBS in a 1:1 dilution, and the sample was centrifuged at 1200 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended using 1 mL of fetal bovine serum (FBS) (Hyclone™, USA).

A total of 1.75 g (3.5% w/v) bovine serum albumin (BSA) (Sigma-Aldrich®, USA) and 0.05 g (1 mg/mL) Collagenase type II powder (Gibco, USA) were weighed and mixed with the 50 mL DMEM to prepare the AT digest solution. The adipose tissue (15 g) was washed with 35 mL cold PBS and minced into 3 mm pieces. Then, it was mixed with 25 mL filtered AT digest solution and shaken for 70 min at 200 rpm and 37 °C. Later, the tube was centrifuged at 1800 rpm for 10 min, and the supernatant was aspirated. The cell pellet was re-suspended in 2 mL of PBS and passed through 70 µm and 40 µm cell strainer (Falcon™, USA) separately to remove any undigested tissue. The pellet was then washed and resuspended in 2 mL of DMEM containing 10% FBS. Cell viability was measured using the trypan blue assay.

The BM, PB, and AT cell mixtures were cultured in a cell culture medium, which consisted of DMEM with 10% FBS, 1% GlutaMAX®, and 1% Penicillin/Streptomycin. Cultures were kept at 37 °C in a humidified incubator with 5% CO_2 atmospheric conditions. Suspended cells were discarded after seven days of culture, and only adherent cells were left to grow on the flask surface. The cell culture medium was then changed every three days.

ISOLATION OF AT- AND PB-DERIVED PERIVASCULAR STEM CELLS

Perivascular stem cells (PSCs) from AT or PB, defined as the population of cells with $CD146^+$, $CD31^-$, and $CD45^-$ surface markers, were isolated using magnetic-activated cell sorting. At passage zero, both cells were cultured until reaching 90% confluence and later detached using TrypLE™ Express (Gibco, USA). Following the manufacturer's recommendation, stain buffer solution was prepared and kept cold (2-8 °C). The detached cell pellet was re-suspended, stained with CD31 and CD45 primary FITC-conjugated antibodies (Miltenyi™, Germany), washed, and mixed with Anti-FITC Microbeads Ultrapure (Miltenyi, Germany). The cell suspension was then washed and applied onto the LD column, which was placed in the magnetic field of a suitable MACS Separator (Miltenyi, Germany). The flow-through containing unlabelled cells was then collected. All unwanted $CD31^+$ and $CD45^+$ cells were magnetically labelled and removed from the cell suspension. The $CD31^-$, $CD45^-$ cell pellet was resuspended in 1 mL of cell culture medium. The cell suspension was cultured in five T75 culture flasks containing 10 mL of cell culture medium to further expand the population.

The $CD31^-$ and $CD45^-$ cells from PB and AT were detached from the flask at passage one to ensure minimal *in vitro* manipulation and preservation of native phenotype. These were stained with the CD146 primary PE-conjugated antibody (Miltenyi, Germany), then mixed with Anti-PE Microbeads Ultrapure (Miltenyi, Germany) and later incubated for 15 min in the refrigerator (2-8 °C). The washed cell suspension was applied onto the LS column, and the flow-through containing unlabelled cells was subsequently collected. The column was washed, allowing the magnetically labelled cells to be flushed out into the tube. The labelled $CD146^+$, $CD31^-$, and $CD45^-$ cells were then collected for culture to expand the population.

Correspondingly, cell numbers were determined using cell counting via a standard hemocytometer. PB- and BM-derived MSCs, AT- and PB- PSCs were characterized using light microscopy to assess cell morphology and any observable abnormalities.

IMMUNOPHENOTYPING ANALYSIS BY FLOW CYTOMETRY

The cultured PB-PSCs and AT-PSCs were analysed using the CytoFlex™ flow cytometer (Beckman Coulter™, USA). Data selected for analyses represents the cell population gated at P3. Cultured BM-MSCs and PB-MSCs were used as a control. The following antibodies were used for flow cytometry analysis: CD146-PE (Miltenyi, Germany), CD31-APC (Miltenyi, Germany), and CD34-PE (Raybiotech®, USA), CD44-APC (Bio-techne®, USA), CD45-PE (BD Pharmingen™, USA), CD90-PerCP (BD Pharmingen™, USA), Purified Mouse Anti-Rat CD73 primary antibody (BD Pharmingen™, USA) and Rat Anti Mouse secondary antibody (BD Pharmingen™, USA). Cell cultures that reached 80-90% confluence was detached and placed into respective vials depending on their intended analysis, as shown in Table 1. Staining was performed in accordance with the manufacturer's instructions. Unstained cells and single-color-stained cell controls were prepared to compensate for any. Samples were analysed using CytExpert Software® version 2.3 (Beckman Coulter, USA).

CHONDROGENIC, OSTEOGENIC, AND ADIPOGENIC DIFFERENTIATION

To induce chondrogenic differentiation, PSC and MSC cell pellets (at passage 3) were cultured with the StemPro™ Chondrogenesis Differentiation Kit (Gibco, USA). PSCs were detached from the cell culture flask using TrypLE Express reagent (Gibco, USA). The cell pellets were re-suspended in 1 mL PBS (Gibco, USA). Cell number was measured using a haemocytometer (Blaubrand®, Germany). The cell concentrations were adjusted to 1.5×10^6 cells/mL. The 1 mL cell suspension was placed into the 5 mL round bottom polystyrene test tube with a cell strainer snap cap (Falcon™, USA) and centrifuged at 1200 rpm for 10 min. The supernatant was discarded whilst maintaining the cell pellet shape and replaced with 1 mL prepared StemPro Chondrogenesis Differentiation medium (Gibco, USA). The tubes were then maintained at 37 °C in a 95% air-5% CO_2 humidified incubator. The medium was changed every three days throughout the duration of the experiments. The bottom of the tube was flicked very gently on day 6 to slowly detach the adherent cells. Chondrogenic-PSCs and MSCs were harvested after four weeks of incubation. Chondrogenic cultures were processed for Haematoxylin-Fast Green-Safranin-O staining. To assess the adipogenic and osteogenic differentiation ability of the isolated PSCs and MSCs, the cells were cultured according to the manufacturer's instructions for the StemPro® adipogenesis or osteogenesis differentiation kits (Gibco, USA).

BIOCHEMICAL ASSAY TO ASSESS CHONDROGENIC-PSC DIFFERENTIATION

Chondrogenesis was analyzed by measuring the Sulphated glycosaminoglycans (S-GAG) production using the 1,9-dimethyl methylene blue (DMMB) assay. The samples were processed according to the protocol specified in the Blyscan™ Glycosaminoglycan Assay Kit provided by the manufacturer (Biocolor Ltd, Northern Ireland). The cell pellets (1.5×10^6) were cultured in a 5 mL round-bottom polystyrene test tube with 1 mL chondrogenic differentiation medium. On the third, sixth, ninth, twelfth, and fifteenth days, 100 µL of chondrogenic differentiation medium was collected from the cultures for the DMMB assay. The fresh chondrogenic differentiation medium was used as a background control.

TABLE 1. Flow cytometry cells staining design

Vial	Labelled CD marker		Incubation time
Vial A1	CD31 with APC 1 µL	CD146 with PE 1 µL	50 µL 10 min 2-8 °C dark
Vial A2	CD44 with APC 5 µL	CD34 with PE 5 µL	50 µL 30 min 2-8 °C dark
Vial A3	CD45 with PE 2.5 µL	CD90 with PerCP 10 µL	50 µL 20 min ($CD90^+$); 10 min (CD45) 2-8 °C dark
Vial AP	PE isotype control 1 µL		50 µL 10 min 2-8 °C dark
Vial AF	FITC isotype control 1 µL		50 µL 10 min 2-8 °C dark
Vial AUS	Unstained		

A comprehensive analysis was performed to assess variations in sulphated glycosaminoglycan (sGAG) concentrations among different stem cell types at various time points. The statistical evaluation involved conducting a two-way ANOVA using the Duncan test in SPSS software, version 26. Before data analysis, all variables were subjected to a normality test. The mean comparison was made using the Duncan test for each sGAG production data separately at a significance level of 0.05.

MORPHOLOGICAL ANALYSIS OF CHONDROGENIC, OSTEOGENIC, AND ADIPOGENIC PSCS AND MSCS

The chondrogenic-PSCs and chondrogenic-MSCs cell pellets were washed and fixed immediately in 10% formalin (Thermo Fisher, USA) for at least 4 h. The fixed cell aggregates were then washed and embedded in paraffin wax. Sections of 5 µm thickness were obtained using a microtome and mounted on 3-aminopropyltriethoxy-silane-coated glass slides (Slides, Belona, China), air-dried, and stored at 4 °C for histological analysis. Each specimen was stained with Haematoxylin (Leica Biosystems, Germany)-Fast Green (Sigma, USA)-Safranin O (Sigma, USA) staining. Finally, the slides were rinsed with water, dried at room temperature, and fixed using a mounting solution, 1-(2-chlorophenyl) sulfonyl-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl) urea (DPX) and observed by light microscopy for detailed morphological examination. Osteogenic cultures were processed and stained with Alizarin Red S, whereas adipogenic cultures were processed with Oil Red O stain.

RESULTS

PROLIFERATION AND MORPHOLOGICAL CHARACTERISTICS OF MSC AND PSC

From 14 rats weighing 500 grams, approximately 1 mL of bone marrow (BM) and 22 mL of peripheral blood (PB) were drawn from each rat. The BM and PB yielded approximately 7-12 million and 4-6 million mononuclear cells, respectively. Following a culture period of 20-23 days, the plastic-adherent cells in the T75 cell culture flasks reached 80% confluency. This level of confluency indicated that the cells were ready for sub-culturing, demonstrating robust proliferative capacity and viability.

In the case of BM-derived mesenchymal stem cells (BM-MSCs), it was observed that approximately 1 - 2.2 $\times 10^6$ BM-MSCs were obtained at the end of passage 0 (Day 20). On day 6, the morphological assessment showed that the majority of adhered BM-MSCs still exhibited a spherical or roughly spherical shape. Only a small population of cells began displaying a spindle-like shape. By day 14 of cell culture, fibroblastic-like MSCs started adhering to the plastic surface of the cell culture flasks (Figure 1). Subsequently, the spindle-shaped MSCs underwent proliferation and were evenly distributed across the flask's surface. The BM-MSCs grew in colonies and achieved confluence within 20 days after initial attachment onto the culture flasks (Figure 1).

For PB-MSCs, about 7.1 - 11.3 $\times 10^6$ cells were obtained at the end of passage 0 (Day 23). On day 7, the morphology of the few PB-MSCs has a spindle-like appearance. The cells subsequently expanded rapidly and formed dense cell colonies. On day 15, the PB-MSC morphology was similar to elongated fibroblasts (Figure 1). The PB-MSC cells reached confluence within 23 days after cells were attached to the cell culture flasks (Figure 1).

On day 0, the sorted AT- and PB-PSCs were seen floating in medium. On day 3, the adhered cell displayed a typical fibroblast-like and spindle-shaped morphology. Cells subsequently expanded rapidly, and fibroblastic-like cells were uniformly distributed and adhered to the surface of the culture flasks on day 7. PB-PSCs showed elongated and spindle-like morphology, whereas most AT-PSCs remained short, fibroblastic-like cells. AT-PSCs and PB-PSCs reached confluence within ten days after cells were attached to the cell culture flasks. Approximately 1.1 - 1.4 $\times 10^6$ cells were obtained per flask.

IMMUNOPHENOTYPIC CHARACTERISTICS OF CULTURED PSCs AND MSCs

Table 2 shows the results of flow cytometry analysis of surface antigens expressed as percentages in various populations. In the given cell types, the CD146 marker is highly expressed in Adipose Tissue-Derived Perivascular Stem Cells (AT-PSCs) and Peripheral Blood-Derived Perivascular Stem Cells (PB-PSCs), with approximately 88.19% and 81.28% positivity, respectively. However, it is less prevalent in Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs) and Peripheral Blood-Derived Mesenchymal Stem Cells (PB-MSCs), with approximately 25.75% and 19.38% positivity, respectively. The CD90 marker is highly expressed across all cell types. The CD44 marker is highly expressed in AT-PSCs and PB-PSCs, with over 99% positivity, but less prevalent in BM-MSCs and PB-MSCs, with approximately 79.63% and 84.66% positivity, respectively. The expression of both CD31 and CD34 markers was low across all cell types. The percentages range from approximately 1.10% to 3.78%.

In vitro OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION OF PSCs AND MSCs

After a week of AT-PSCs, PB-PSCs, BM-MSCs, and PB-MSCs incubation in osteogenic induction medium, spindle-shaped cells began to divide rapidly and fill most of the plastic surface of cell chamber slides. Small particles (calcium deposits) were also seen scattered on the cells. The calcium deposits became more extensive over a more extended culture period and covered most of the culture surface area. After two weeks of induction, Alizarin Red S

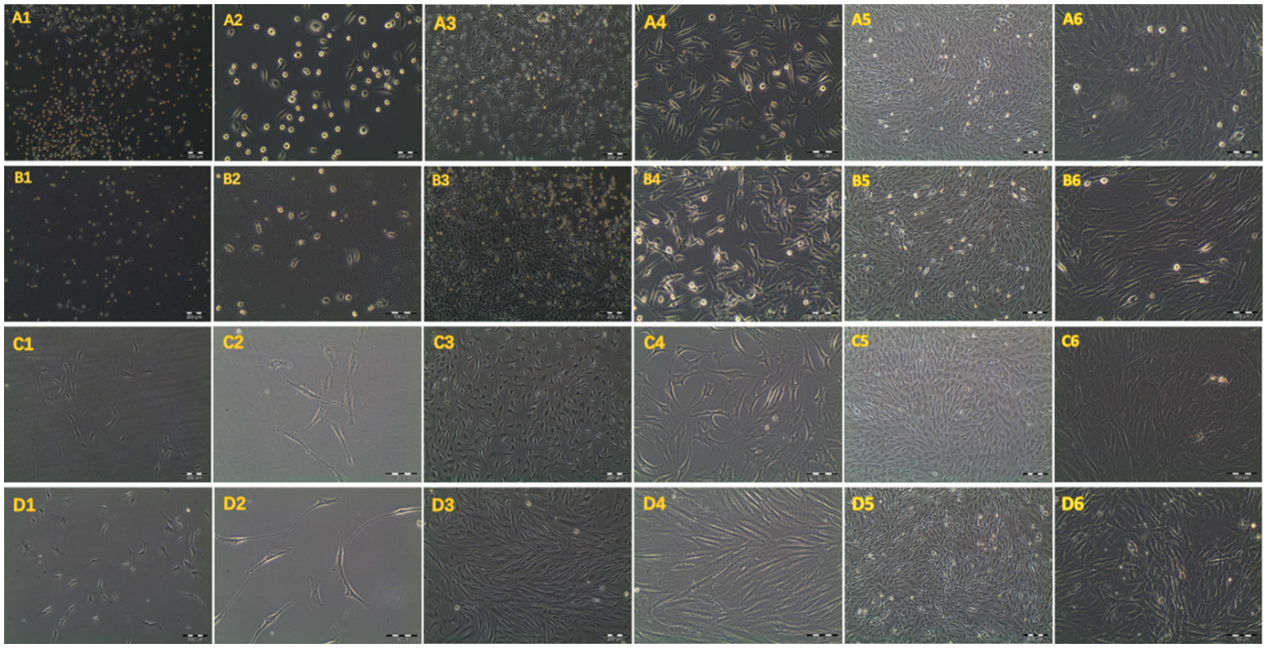

FIGURE 1. Morphology observation of MSCs and PSCs. (A1, A2): BM-MSCs at Passage 0 on day 6; (A3, A4): BM-MSCs at Passage 0 on day 13; (A5, A6): BM-MSCs at Passage 0 on day 20. (B1, B2): PB-MSCs at Passage 0 on day 7; (B3, B4): PB-MSCs at Passage 0 on day 15; (B5, B6): PB-MSCs at Passage 0 on day 23. (C1, C2): AT-PSCs at Passage 2 on day 1; (C3, C4): AT-PSCs at Passage 2 on day 3; (C5, C6): AT-PSCs at Passage 2 on day 5. (D1, D2): PB-PSCs at Passage 2 on day 2; (D3, D4): PB-PSCs at Passage 2 on day 5; (D5, D6): PB-PSCs at Passage 2 on day 7. The images were captured at 4X objective for images A1, A3, A5, B1, B3, B5, C1, C3, C5, D1, D3 and D5, while A2, A4, A6, B2, B4, B6, C2, C4, C6, D2, D4 and D6 were captured at 10X objective. The scale bar (200 μm for 4X objective; 100 μm for 10X objective) was depicted in the bottom right corner of the image

TABLE 2. Flow cytometry analysis of surface antigen expression on AT-PSCs, PB-PSCs, PB-MSCs and BM-MSCs

	AT-PSCs, %	PB-PSCs, %	BM-MSCs, %	PB-MSCs, %
$CD146^+$	88.19±4.03	81.28±2.26	25.75±2.40	19.38±3.79
$CD90^+$	97.95±0.27	98.51±0.18	94.69±3.18	86.02±6.20
$CD44^+$	99.47±0.34	99.56±0.06	79.63±12.32	84.66±8.82
$CD31^+$	1.24±0.07	2.44±0.48	1.83±0.17	1.98±0.65
$CD34^+$	1.45±0.24	2.01±0.32	1.10±0.70	3.78±3.14
$CD45^+$	16.34±2.46	21.50±2.62	12.09±9.38	14.59±3.46

staining was performed on the osteogenic-PSCs and MSCs. Calcium deposits were observed as evident by the presence of orange red stain in the osteogenic-PSCs and osteogenic-MSCs (Figure 2), especially in high cell-density areas. In contrast, cells that were not incubated in the osteogenic medium had a slower proliferation rate. Crystal particles were absent in this group, and the morphology of individual cells could be observed clearly under microscopy (Figure 2).

After a week of AT-PSCs, PB-PSCs, BM-MSCs, and PB-MSCs incubation in adipogenic induction medium, spindle-shaped cells began to turn into larger polygonal cells. Initially, some small vesicles (believed to be lipids) were observed in the cytoplasm of cells, becoming increasingly more prominent over time. In addition, such vesicles were also observed to coalesce to form larger possible lipid vacuoles gradually. After 14 days of induction, more cells contained such lipid particles in the cytoplasm. When these cells were stained with Oil Red O, the vesicular particles stained red, indicating lipid content (Figure 3). However, only a few small lipid-containing structures were detected in adipogenic PB-MSCs after 14 days of induction using the commercial adipogenic induction medium (Figure 3).

In vitro CHONDROGENIC DIFFERENTIATION OF PSCs AND MSCs

Both PSCs and MSCs formed physically stable chondrogenic pellets. The pellet size varied across different cell sources (Figure 4). Overall, the cell pellets formed using chondrogenic differentiation medium and MSCs derived from bone marrow were larger than those of other cells. Meanwhile, AT-PSC and PB-PSC demonstrated a more structured cartilage tissue organisation. Different types of stem cells, when induced with chondrogenic differentiation medium, can produce varying amounts of sGAG, an essential component of cartilage. The results, visualized using Safranin-O staining, showed that while all tested stem cell types could differentiate into chondrocytes, PB-PSC showed particularly high levels of sGAG compared to PB-MSCs. Safranin-O positive staining indicates the presence of sGAG. The control cells, which were not subjected to the differentiation medium, did not produce significant amounts of sGAG and showed a loose structure, underscoring the effectiveness of the chondrogenic differentiation medium in inducing chondrogenesis.

SULPHATED GLYCOSAMINOGLYCAN (S-GAG) CONTENT IN PSC AND MSC

Normality tests confirmed that the data were normally distributed. ANOVA analysis indicated significant differences in sGAG production among the various stem cell groups, as well as significant temporal variations throughout the experiment. In a three-dimensional cell pellet culture system with chondrogenic differentiation medium, AT-PSCs, PB-PSCs, PB-MSCs, and BM-MSCs all exhibited the ability to differentiate into cartilage cell types *in vitro*. The production of sulphated glycosaminoglycan (sGAG) was quantified using the 1,9-dimethyl methylene blue (DMMB) assay, demonstrating significant sGAG expression during chondrogenic differentiation. The sGAG concentrations produced by all differentiated cells increased progressively over time. The highest sGAG content was observed in chondrogenic differentiated AT-PSCs, followed by chondrogenic differentiated PB-MSCs, BM-MSCs, and PB-PSCs (Figure 5). These temporal changes in sGAG production were statistically significant for AT-PSCs, PB-PSCs, PB-MSCs, and BM-MSCs.

DISCUSSION

Perivascular stem cells (PSCs) from adipose tissue (AT) and peripheral blood (PB) were isolated using a magnetic-activated cell sorting (MACS) technique. The isolation targeted cells expressing $CD146^{+}$ and lacking $CD31^{-}$ and $CD45^{-}$, which enriched for the PSC population. This method was developed and optimized in this study to enable consistent PSC isolation from both tissue sources.

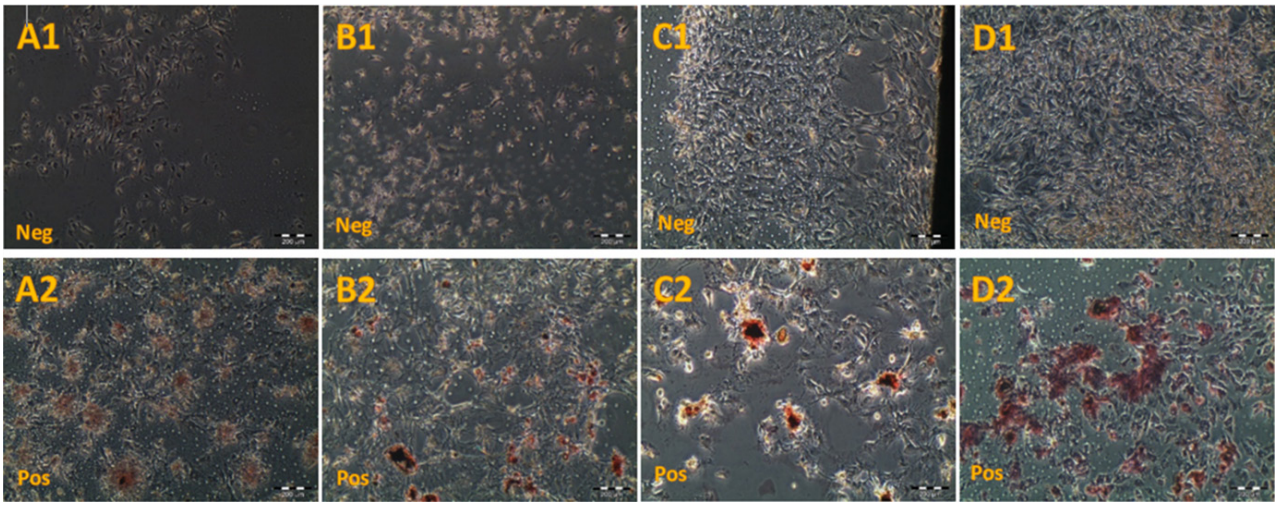

FIGURE 2. Microscopic view of Alizarin Red S stain. Alizarin Red S staining showed the deposition of calcium in cells in red. A1 (AT-PSC), B1(PB-PSC), C1(BM-MSC) and D1(PB-MSC) in normal culture medium (4X); A2 (AT-PSC), B2(PB-PSC), C2 (BM-MSC) and D2(PB-MSC) in osteogenic differentiation medium (4X) (Neg: Negative staining; Pos: Positive staining

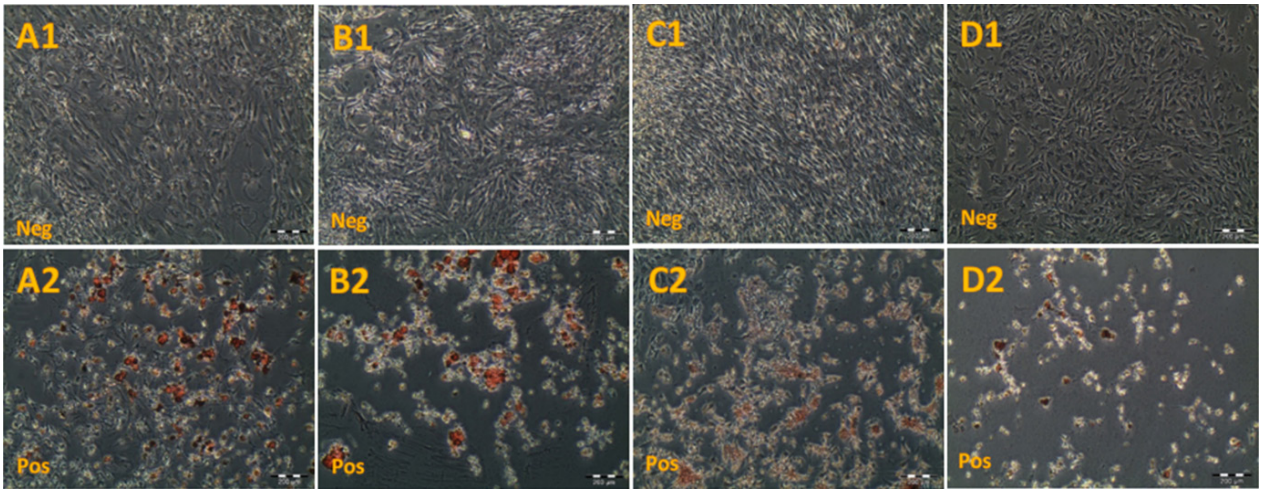

FIGURE 3. Microscopic view of Oil Red O staining. Oil Red O staining showed lipids deposits in red. A1 (AT-PSC), B1(PB-PSC), C1(BM-MSC) and D1(PB-MSC) in normal culture medium (4X); A2 (AT-PSC), B2(PB-PSC), C2(BM-MSC) and D2(PB-MSC) in adipogenic differentiation medium (4X): (Neg: Negative staining; Pos: Positive staining)

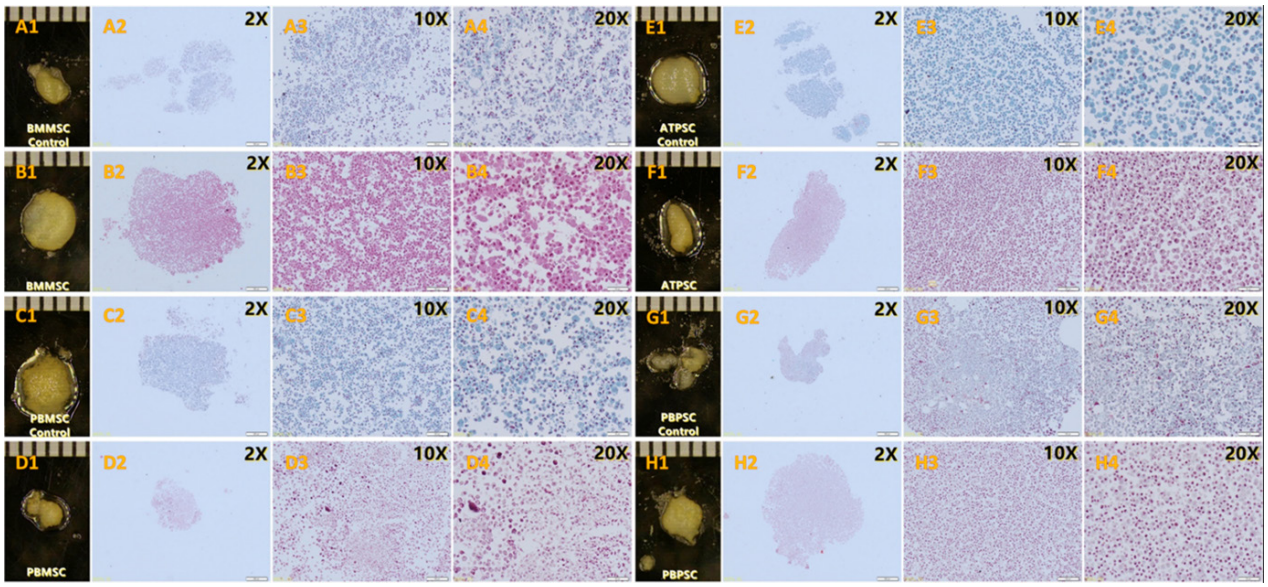

FIGURE 4. Microscopic view of Safranin O-fast green-haematoxylin staining. Safranin O staining is shown in red. A1(BM-MSC), C1(PB-MSC), E1(AT-PSC), G1(PB-PSC) cell pellet cultured in standard culture medium (Control, scale unit: mm); A2-A4(BM-MSC), C2-C4(PB-MSC), E2-E4(AT-PSC), G2-G4(PB-PSC) Haematoxylin-Fast-Green-Safranin O-stained section from pellets of cells cultured in standard culture medium (2X, 10X & 20X) (Scale bar: 500 µm, 100 µm & 50 µm); B1(BM-MSC), D1(PB-MSC), F1(AT-PSC), H1(PB-PSC) cell pellet cultured in chondrogenic differentiation medium (Control scale unit: mm); B2-B4(BM-MSC), D2-D4(PB-MSC), F2-F4(AT-PSC), H2-H4(PB-PSC) Haematoxylin-Fast-Green-Safranin O stained section from pellets of cells cultured in chondrogenic differentiation medium (2X, 10X & 20X) (Scale bar: 500 µm, 100 µm & 50 µm)

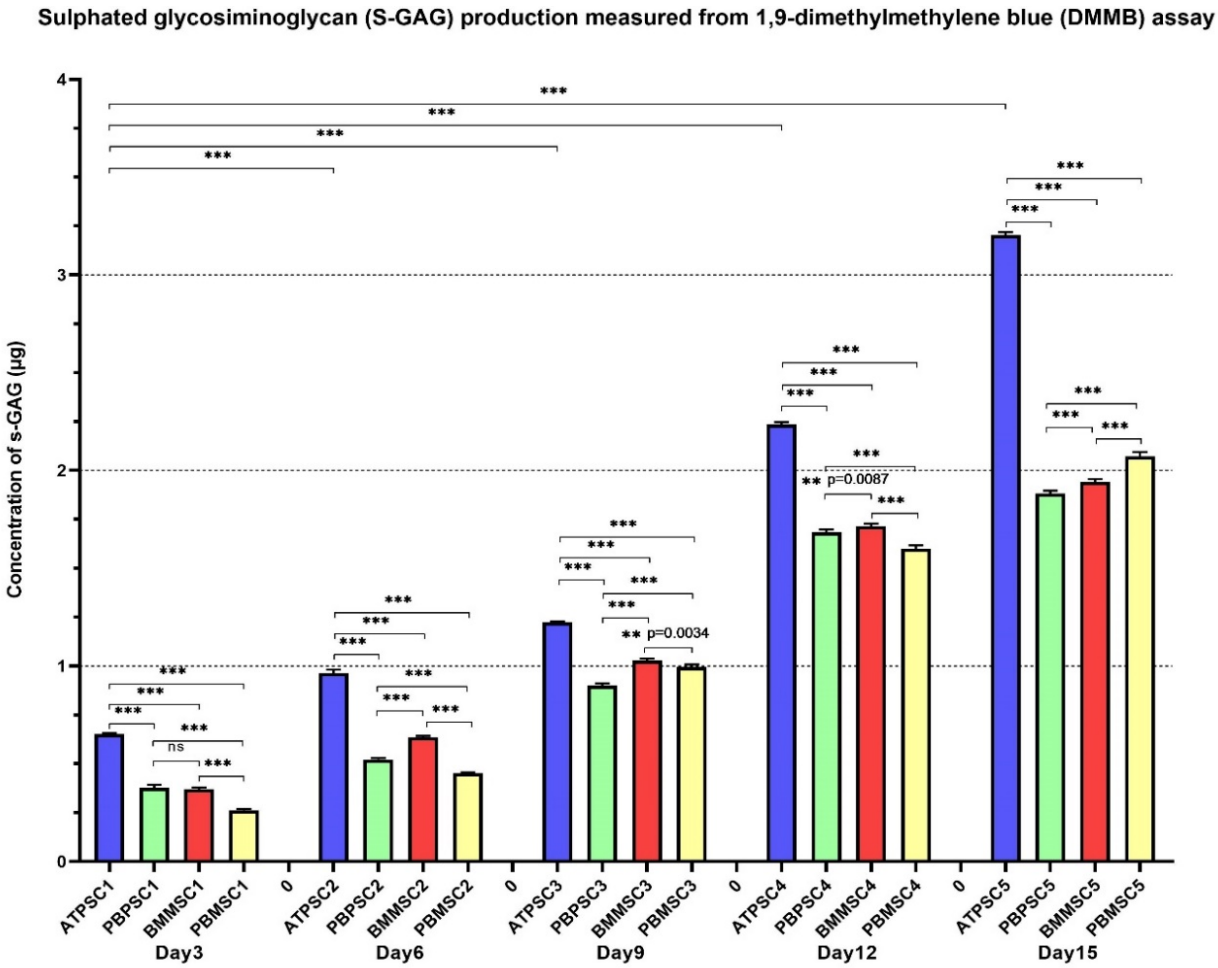

FIGURE 5. Sulphated glycosaminoglycan (S-GAG) production was measured from 1,9-dimethylmethylene blue (DMMB) assay. All differentiated cell types significantly expressed the production of s-GAG during the chondrogenic differentiation. The concentration of GAG gradually increased over time, with the highest content observed in chondrogenic differentiated AT-PSCs, followed by PB-MSCs, BM-MSCs, and PB-PSCs

The adipose tissue and peripheral blood are easily accessible and the techniques for their collection are well-established. The ability to isolate PSCs from easily accessible sources, such as adipose tissue and peripheral blood, holds tremendous potential for advancing clinical and translational medicine. It paves the way for safer, more effective, and personalized treatments, enhances research capabilities, and may reduce healthcare costs, ultimately improving patient outcomes and quality of life. The adipose-derived stromal vascular fractions (AT-SVF) are heterogeneous cells known to promote plasticity and adaptability (Boyd et al. 2013; Stratman et al. 2009; Yoshimura et al. 2006). Comparatively, the concentration of pericytes in bone marrow concentrate (BMC) is 15 times lower than that in AT-SVF (Jang et al. 2015).

Peripheral blood (PB) is also a promising source of mesenchymal stem cells (MSCs) (Caplan 2007; Hass et al. 2011). Although the isolation and culturing of MSCs from PB have been challenging (Dominici et al. 2006; Kassis et al. 2006), previous studies have demonstrated the existence of MSCs in peripheral blood (Valenti et al. 2008). Notably, Chong et al. (2012) showed that PB-derived MSCs share characteristics and differentiation capabilities with bone marrow-derived MSCs. However, our research is the first to demonstrate the isolation of peripheral blood-derived PSCs using cell sorting techniques. Some studies suggested that uncultured cells, such as adipose tissue-derived stromal vascular fraction (AT-SVF) and peripheral blood mononuclear cells (PBMC), contain biologically and clinically heterogeneous cell populations that can be sorted and applied without *in vitro* culture expansion (Jang et al. 2015). However, the present study did not use uncultured cells for cell sorting due to difficulties in obtaining sufficient numbers of PSC cells from the limited amount of adipose tissue and peripheral blood collected from the rats.

Nonetheless, the sorted population of PSCs still required passage 2 for adequate expansion, especially notable for peripheral blood PSCs, which necessitated a longer duration of culture to obtain a suitable quantity of adherent fibroblasts for cell sorting. Several factors contribute to the extended time required for obtaining adherent fibroblasts from peripheral blood Perivascular Stem Cells (PB-PSCs). Firstly, peripheral blood contains a lower concentration of PSCs than other sources, resulting in a slower proliferation rate and an increased duration needed to reach a sufficient cell population (Crisan et al. 2008; Tormin et al. 2011). Secondly, PB-PSCs may display slower attachment and expansion kinetics, contributing to the prolonged time required for cell sorting (James et al. 2012; Wanjare, Kusuma & Gerecht 2013). Thirdly, PB-PSCs may exhibit higher heterogeneity, necessitating additional time to identify and select the desired population with optimal characteristics (Corselli et al. 2012; Sacchetti et al. 2016). Lastly, the inherent variability among individuals, which is influenced by factors such as age and health status, can impact the growth and expansion rates of PB-PSCs. To expedite PB-PSC expansion, it is recommended to optimize growth factors, culture conditions, and conduct further research to understand the underlying mechanisms involved.

In the present study, PSCs derived from AT or PB with a $CD146^+$ CD31-CD45- phenotype were isolated using MACS technology. Previous studies have utilized magnetic cell sorting (MACS) to isolate various perivascular stem cells from different tissues and species. For instance, Meyers et al. (2019) induced *in vivo* bone ossification using MACS-derived PSCs and Wu et al. (2017) isolated $CD146^+$ PSCs from rat growth plates for *in vitro* studies using the MACS technique. These $CD146^+$ cells demonstrated better colony-forming ability and potential for chondrogenic differentiation compared to non-enriched cell fractions (Wu et al. 2017). MACS offer advantages such as convenience, increased yield, improved efficiency, better technical reproducibility, and lower costs compared to fluorescence-activated cell sorting (FACS) (Grützkau & Radbruch 2010; Semple et al. 1993; Ulrich et al. 2015). MACS provides a clinically viable PSC enrichment technique. However, compared to FACS, MACS-derived Perivascular Stem Cells (PSCs) yield a lower number of cells and require a longer processing time (Sutermaster & Darling 2019). Additionally, this technique may leave behind a larger number of endothelial and inflammatory contaminants (James et al. 2012; Sutermaster & Darling 2019). The potential impact of endothelial contaminants on chondrogenic differentiation *in vitro* or cartilage formation *in vivo* is still unclear. Furthermore, the absence of a definitive, exclusive marker for PSCs complicates their identification, but it does not negate the need for improved isolation strategies or diminish their demonstrated therapeutic potential. Functional properties, including multipotency, immunomodulation, and trophic support, continue to justify the exploration of PSCs as a valuable cell source for regenerative medicine (Alvino et al. 2018; Campagnolo et al. 2010; Corselli et al. 2012). Establishing a nomenclature, marker array, and functionality tests to identify subpopulations of pericytes using specific markers is thus recommended. Standardization of pericyte expansion and purification processes for clinical applications is also necessary.

In this study, we identified PSCs as purified $CD146^+$, $CD31^-$, and $CD45^-$ populations. The CD146, also known as MCAM (melanoma cell adhesion molecule) is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. It is strongly correlated with blood vessels, particularly vascular endothelium, pericytes, and smooth muscle cells (Martinez et al. 2015; Sorrentino et al. 2008). The CD146 has also been implicated as a marker for multipotency, which may explain its presence in pericytes (Cathery et al. 2018). Therefore, subsequent studies have utilized CD146 as a biomarker to isolate and characterize pericytes (Crisan et al. 2008; Sweeney, Ayyadurai & Zlokovic 2016).

Pericytes uniformly express CD146 and serve as efficient markers for separating pericytes from the heterogeneous AT-SVF (Crisan & Dzierzak 2016; Flanagan et al. 2012; Pelikánová 2016; Xu et al. 2017). CD146 expression remains stable after several passages (Crisan et al. 2008; Mitchell et al. 2006). Additionally, $CD146^+$ cells tend to suppress arthritis progression, with intra-articular injection of $CD146^+$ MSCs attenuating the progression of collagen-induced arthritis (CIA). $CD146^+$ MSCs express lower levels of interleukin-6 (IL-6) and can inhibit the activation of Th17 cells, making them more capable of protecting cartilage than CD146- cells. These findings suggest the potential therapeutic role of $CD146^+$ cells in treating inflammatory arthritis (Wu et al. 2016). Our study also supports the strong expression of CD146 in PSCs (AT-PSCs, 88%; PB-PSCs, 81%) compared to the MSC populations (BM-MSCs, 25%; PB-MSCs, 19%) we have isolated. This difference in expression profiles highlights a characteristic distinction between PSCs and MSCs. However, it should be noted that CD146 is also expressed by $CD31^+$ endothelial cells and a subset of $CD45^+$ lymphocytes (Wang & Yan 2013). To exclude $CD45^+$ hematopoietic stem cells and $CD31^+$ endothelial cells from the $CD146^+$ population, we used CD31 and CD45 cell markers to distinguish them from pericytes (Crisan et al. 2008; Zimmerlin et al. 2010). Through trilineage differentiation, our results demonstrate the multipotentiality of PSCs and their potential application in cartilage tissue regeneration.

PSCs isolated from adipose tissue and peripheral blood share similar characteristics and chondrogenic potential to classical sources of MSCs, including BM-MSCs (Caplan 2008). CD146 is highly expressed in PSCs but only modestly expressed in the MSC population. However, some studies suggest that MSCs should have

a high expression of CD146. In our study, we could not collect a large amount of adipose tissue and peripheral blood from a single rat due to the limitations of rat size as an animal model. Therefore, we performed cell sorting and cell population expansion twice until we obtained the passage 3 cell population to ensure an adequate number of cells for flow cytometry analysis. Previous studies have shown that $CD146^+$ MSCs may have a shorter doubling time and higher proliferation rate than $CD146^-$ MSCs. When cultured concurrently, $CD146^+$ MSC populations tend to outpace the growth of CD146- cells, reducing the latter's population. This could explain why MSCs in our cell culture expressed lower levels of CD146 in this study. Furthermore, these cells share similar immunophenotypic expressions, including $CD44^+$, $CD90^+$, $CD31^-$, $CD34^-$, and $CD45^-$, with BM and PB sources of mesenchymal stem cells, supporting the notion that these elusive MSCs are derived from perivascular cells (Crisan et al. 2008). PSCs isolated from adipose tissue and peripheral blood exhibit similar chondrogenic potential to BM and PB sources of MSCs. The chondrogenic PSCs produced comparable levels of extracellular matrix proteins, specifically S-GAG levels and proteoglycans, to the chondrogenic BM-MSCs. Previous studies have demonstrated the chondrogenic potential of adipose tissue-derived pericytes, showing the expression of SOX9 and type II collagen genes in differentiated pericytes (Cai et al. 2011). Hindle et al. (2016) implanted adipose-derived pericytes into hydrogels to repair damaged cartilage in sheep models. The transplanted pericytes were found embedded in the repaired articular cartilage, indicating their potential for cartilage tissue engineering *in vivo*.

CONCLUSIONS

The present study demonstrates PSCs and MSCs from different resources demonstrate comparable chondrogenic potential, suggesting their potential usefulness in future cartilage damage treatment strategies and thus averting OA. In addition, PSCs can undergo osteogenic and adipogenic differentiation, making them multipotential, which is comparable to MSCs. In terms of identifying these cells, PSCs derived from adipose tissue and peripheral blood expressed $CD146^+$, $CD31^-$, and $CD45^-$, similar to MSCs. PSCs and MSCs share the same cell morphology and express $CD44^+$, $CD90^+$, $CD31^-$, $CD34^-$ and $CD45^-$. However, only PSCs express CD146.

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