

Characterization of synovium-derived mesenchymal stem cells from joints with osteochondritis dissecans

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History

- Received: Aug 13, 2020
- Accepted: Oct 15, 2020
- Published: Oct 31, 2020

DOI : 10.15419/bmrat.v7i10.645



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ABSTRACT

Introduction: Osteochondritis dissecans (OCD) is a pathologic condition that occurs in children as well as adults. OCD is often managed based on the extent of ischemia and the stage of the disease. Synovial tissue collected during an arthroscopic procedure might serve as an ideal source for autologous mesenchymal stem cells (MSCs) with a potential for regenerative medicine of cartilage. Therefore, the present *in vitro* study aimed to evaluate the potency characteristics of synovium-derived MSCs (SMSCs) from joints with OCD for prospective autologous therapy. **Methods:** Primary culture of SMSCs was established and basic cellular properties, such as morphology, growth kinetics and clonal propagation ability, were analyzed. The expression of phenotypic markers, including CD29, CD44, CD90, CD34 and CD45, was assessed by flow cytometry and immunocytochemistry. Mesodermal differentiation into osteocytes, chondrocytes and adipocytes was performed using standard protocols. Expression of chondrocyte-specific markers was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). **Results:** Isolated SMSCs displayed fibroblast-like morphology with > 95% cell viability and had high proliferative rates with a shorter doubling time. The cells showed positive expression of CD29, CD44 and CD90, but were negative for CD34 and CD45 markers. Upon induction, SMSCs were successfully differentiated into osteogenic, chondrogenic and adipogenic lineages. Chondrogenesis was more prominent in SMSCs than osteogenesis. Chondrogenesis was further confirmed by the expression of aggrecan and collagen type II α 1 markers. **Conclusion:** SMSCs showed greater proliferation and an enhanced ability for chondrogenic differentiation. Synovium can be harvested with minimal tissue damage and donor-site morbidity and might serve as an alternative autologous source of MSCs for cartilage-regeneration.

Key words: Synovial-derived MSCs, Potency, Differentiation, Osteochondritis dissecans

INTRODUCTION

Osteochondritis dissecans (OCD) is a condition due to ischemic insult to the subchondral bone, resulting in damage to hyaline cartilage of the joint and leading to separation of the bone and the overlying cartilage; it affects both children and adults^{1,2}. In OCD, the bone and cartilage break loose, causing pain and hindering joint motion. Its etiology is still unexplained. The most commonly affected joints are the knee, ankle and elbow³. The condition, if untreated, can progress to uneven joint surfaces, leading to the defects being replaced by fibrous cartilage and progressing to osteoarthritis⁴. OCD is often managed based on the extent of ischemia and the stage of separation from the underlying bone, and surgery is performed to replace the fragment and fix it with implants⁵. This procedure may not be successful in all cases. Hence, cartilage transplant and cartilage grafting could be a promising procedure. In joints, naturally existing mesenchymal stem cells (MSCs) can be found in both synovial and

solid joints as well as the ligaments of the mammalian body. Human synovial-derived MSCs (SMSCs) were first described by De Bari et al., when they successfully isolated them in 2001⁶. MSCs have also been isolated and characterized from the meniscus, ligaments, fat pad, and cartilage of the joints, suggesting that MSCs play a crucial role in the maintenance and function of these tissues⁷.

Several studies have shown that MSCs are involved in the maintenance and regeneration of connective tissues, and are known to migrate to tissues as a result of injury or inflammation, where they participate in the repair of damage⁷⁻⁹. Indeed, a recent case series report provided an option for treating juvenile OCD using human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs)¹⁰. Further, a study on rats with articular cartilage defects demonstrated that SMSCs were able to differentiate into cartilaginous cells depending on the microenvironment¹¹. Synovial tissues, as source of MSCs, may be preferable for

Cite this article : Rao S, Shetty S, Nitilapura N, Shetty V, Parashiva G K, Basavarajappa M K. **Characterization of synovium-derived mesenchymal stem cells from joints with osteochondritis dissecans.** *Biomed. Res. Ther.*; 7(10):4075-4085.

transplantation as well as cell-banking purposes, and investigations in this regard are scanty^{7,12,13}. However, studies report the consideration of SMSCs as a promising alternative; it is still critical to examine whether autologous SMSCs reside in the joints of OCD patients. Therefore, the aim of this *in vitro* study herein was to isolate SMSCs from joints with OCD, and to characterize them in terms of morphology, growth kinetics, clonal propagation ability, expression of MSC-specific positive (CD29, CD44, and CD90) and negative (CD34 and CD45) markers, and potential for osteogenic, chondrogenic and adipogenic differentiation. The findings have provided evidence to utilize the synovium as a potential autologous source of MSCs for cartilage regeneration.

MATERIALS — METHODS

Collection of synovial tissue

The study obtained prior permission from the Institutional Ethics Committee (No. NU/CEC/2017/0182) and Institutional Committee for Stem Cell Research (No. NU/ICSCR/2016-17/002A/P10) (Mangaluru, India). A total of five samples of human synovial membrane were collected, and three cell lines were selected for performing qualitative and quantitative analysis using various cell-based assays⁶. The samples were obtained from male patients undergoing knee arthroscopy surgery for OCD with cartilage damage (age range: 35 to 45 years; mean age: 39.25 years). Informed consent was obtained for all patients and was a requisite for the treatment procedure. Patients with rheumatoid arthritis were excluded. The procedure was performed under anesthesia, and the synovium was harvested from the suprapatellar pouch using arthroscopic basket forceps or a suction rotatory aspirator device.

Chemicals and media

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO), media from Thermo Fisher Scientific (Gibco-Invitrogen, Life Technologies, MA), and cell culture plastic wares from Thermo Fisher Scientific, unless otherwise specified.

Isolation of synovium-derived mesenchymal stem cells (SMSCs)

Isolation of SMSCs was performed according to the protocol described by De Bari *et al.*⁶, with minor modifications. Briefly, the collected samples were washed 3-4 times thoroughly with Dulbecco's phosphate buffered saline (DPBS) and minced into small pieces. Then, they were treated with 0.05 $\mu\text{g}/\text{mL}$ type

II collagenase enzyme in Dulbecco's modified essential medium (DMEM) at 37°C overnight. From the cell suspension, enzyme was removed by centrifugation and was plated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin until cells reached 80-90% confluence for trypsinization. The cells were cultured at 37°C with 95% humidity and 5% CO₂. Seeding density for primary culture was 8000 cells/cm², while sub-passage seeding density was maintained at approximately 3000-3500 cells/cm². Out of five synovial membranes collected, SMSCs were successfully established and cultured in four samples. For analyses, three cell lines from passage 2 (P2) to passage 5 (P5) were used, and all experiments were carried out at least in triplicate.

Partial clonal propagation of SMSCs

SMSCs (50 cells/dish) were cultured in 100-cm² cell culture dish using a partial clonal population technique as described previously¹⁴. Culture media was changed every 3 days. Once the colonies reached more than 25 cells, they were harvested and further sub-passaged for experiments.

Viability assay

Cell viability at P0 to P4 were performed by trypan blue exclusion test with a hemocytometer.

Proliferation rate and population doubling time (PDT)

SMSCs were plated at 10,000 cells/well in 12-well plates and cultured for 12 days. Cells were harvested with 0.25% trypsin-EDTA on the 3rd, 6th, 9th and 12th day after seeding, and counted using a hemocytometer. The culture medium was changed every 3 days. The experiment was performed in triplicate and the proliferation rate was calculated. Population doubling time (PDT) was calculated using the formula: $PDT = t (\log 2) / (\log N_t - \log N_0)$, where t represents culture time; N₀: initial cell number; N_t: final cell number.

Colony-forming assay

Isolated SMSCs were cultured with an initial cell density of 50 cells/cm². Fresh culture media was added twice weekly. After 14 days of culture, cells were stained with 1% Crystal violet solution and colonies were observed under phase-contrast microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis

SMSCs at 80% confluence were trypsinized and fixed with 3.7% paraformaldehyde for 30 min. Cells were resuspended in 50 μ L cell-staining buffer (Biolegend, CA, USA) and incubated with antibodies, such as CD29 (eBioscience, CA, USA, 1:100), CD44 (Biolegend, 1:100), CD90 (eBioscience, 1:100), CD34 (Biolegend, 1:100) and CD45 (eBioscience, 1:100), for 4 hrs at 4°C. Then, cells were incubated in fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (eBioscience, 1:100) secondary antibody for 1 hr at room temperature. As an isotype control, mouse immunoglobulin G (eBioscience) was used. Cell fluorescence was acquired by fluorescence-activated cell-sorting (FACS) (BD FACSCalibur, Becton Dickinson) and data was analyzed using Cell Quest software (Becton Dickinson).

Immunocytochemistry analysis

SMSCs were sub-cultured into four-chambered imaging slides (Eppendorf, Hamburg, Germany) until confluent and then fixed in 3.7% paraformaldehyde for 30 min. Nonspecific sites of the cell membrane were blocked using 1% bovine serum albumin (BSA) for 1 hr at room temperature. Cells were incubated with the aforementioned primary antibodies at 4 °C for overnight followed by secondary antibody at 37 °C for 1 hr. Then, the cells were counter-stained with propidium iodide (PI) for 5 min at room temperature. Finally, the slides were mounted for observation under a fluorescent microscope (Olympus, Tokyo, Japan).

Multi-lineage differentiation potential

The ability of the SMSCs to undergo *in vitro* differentiation into osteocytes, chondrocytes and adipocytes were evaluated after 3 weeks of culture in specific medium. To assess osteogenic differentiation, SMSCs were cultured in DMEM containing 100 μ M dexamethasone, 0.01 M β -glycerophosphate and 50 mg/mL ascorbate-2-phosphate. Following this, the cells were assayed for calcium deposition using von Kossa staining method. To assess chondrogenic differentiation, SMSCs were cultured in DMEM supplemented with 1% ITS mix (10 mg/L insulin, 5 mg/L transferrin, and 5 μ g/L selenium), 0.1 μ M dexamethasone, 1 μ M ascorbate-2-phosphate, and 5 ng/mL transforming growth factor-beta3 (TGF- β 3; Peprotech, NJ). After 3 weeks of culturing SMSCs, cells were stained with Alcian blue for the confirmation of deposition of glycosaminoglycans and proteoglycan synthesis. For adipogenic differentiation, SMSCs were cultured

in adipogenic induction medium containing 100 nM dexamethasone, 0.5 mM isobutyl-methylxanthine, 10 mM indomethacin, and 10 μ g/mL insulin. To determine the potency of the responding cells for adipogenesis, the cells were stained with Oil Red O solution.

Reverse transcription- polymerase chain reaction (RT-PCR)

The expression of chondrogenic marker genes, such as aggrecan and collagen type II α 1, was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was isolated from SMSCs with nucleospin total RNA isolation kit (Takara, Shiga, Japan) and complementary DNA (cDNA) was synthesized from 1 μ g RNA using PrimeScript 1st strand cDNA synthesis kit (Takara), following the manufacturers' instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene and the primer sequences are presented in **Table 1**. PCR reactions were performed in a thermal cycler (Eppendorf, Hamburg, Germany) with the following conditions: 30 sec at 95 °C, 30 sec at 58 °C, and 90 sec at 72 °C for 30 cycles, followed by a final 10 min extension at 72°C. The PCR products were separated in 1.5% agarose gel. Average intensity of band was analyzed with the Gel Doc™ XR1 (Bio-Rad, CA).

Statistical analysis

All data are presented as the mean \pm standard deviation. Comparison of values was carried out by one-way and two-way analysis of variance (ANOVA) with Tukey's multiple comparison test using GraphPad Prism 8.0 software (GraphPad, CA, USA). The level of significance was considered at $P < 0.05$.

RESULTS

Establishment of primary culture, morphology and clonal propagation of SMSCs

SMSCs were successfully established by plastic-adherent culture method. Initially, the cells exhibited small, spindle shape, but later they formed a monolayer by showing fibroblast-like morphology (**Figure 1A-B**). SMSCs under primary culture conditions had the ability to form clusters. Cells reached 90% confluence by day 15 and were subjected for sub-passaging after trypsinization. Representative images of clonal propagation of SMSCs are presented in **Figure 1 C-D**. A low seeding density of cells at P1 showed their proper attachment on day 3 and active proliferation by day 7 of culture. Single cell colonies were found around day 20.

Table 1: Details of chondrogenic genes and primer sequences

Sl. No.	Gene name	Sequence (5' - 3')	Size (bp)	Accession No.
1	Aggrecan	F: AGTCACACCTGAGCAGCATC R: AGTTCTCAAATTCATGGGGTGTC	147	NM_001135.3
2	Collagen II α I	F: GGATGGCTGCACGAAACATAACCGG R:CAAGAAGCAGACCGGCCCTATG	157	NM_001844.4
3	GAPDH	F: AATGGGCAGCCGTTAGGAAA R: GCGCCCAATACGACCAAATC	151	NM_001256799.2

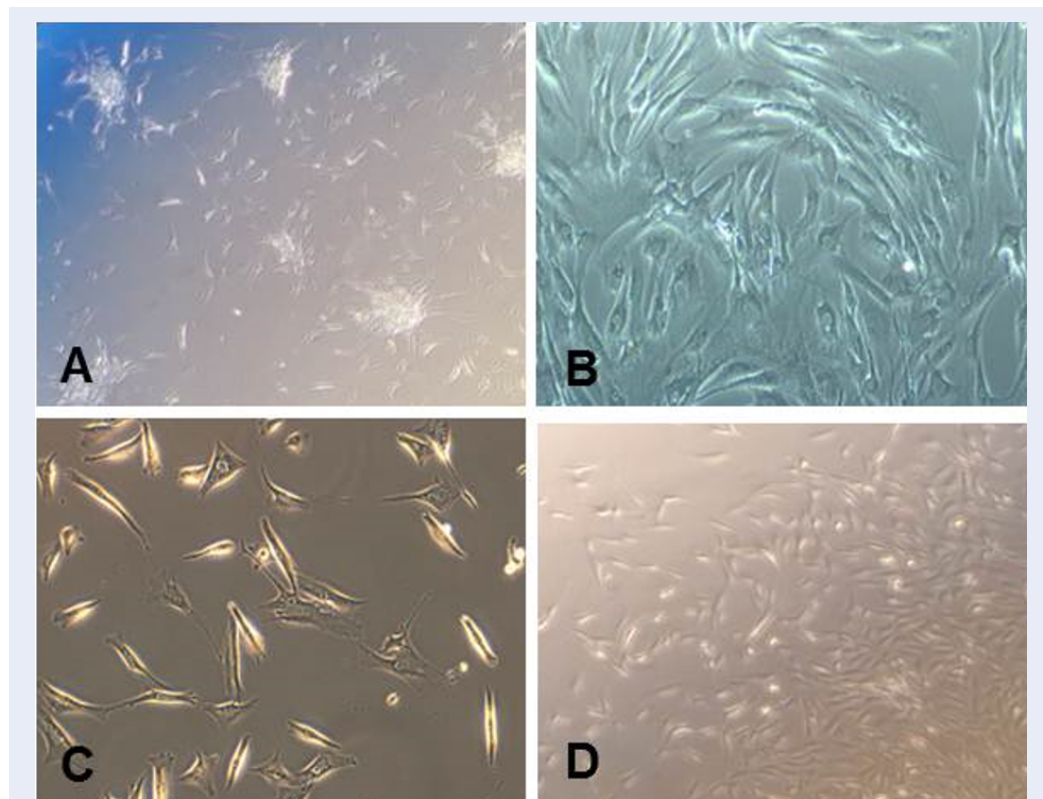


Figure 1: Morphology and clonal propagation of SMSCs. A. Plastic adherence with spindle-shape and formation of clusters by SMSCs under primary culture conditions. B. Cells showing characteristic fibroblast-like morphology at passage 3. C. SMSCs seeded at low density for clonal propagation on day 7. D. Formation of clonal clusters by SMSCs on day 20 of culture. Images A, C, D: 10x and B: 20x magnification (n = 3).

Viability of SMSCs

The results of percentage viability of SMSCs (SMSCs-1, 2 & 3) from P0 to P4 are presented in **Figure 2 A**. Viability at every passage of SMSCs was determined by trypan blue staining. At P0, the viability ranged between 81 and 88%. However, from P1 to P4, the viability of all SMSCs was found to be more than 98%. Further, there were no significant ($p > 0.05$) differences observed between the passages and cell lines, except at P0, where the values were lower and found

significantly different ($P < 0.05$) compared to later passages.

Proliferation and population doubling time (PDT) of SMSCs

Proliferation rate of SMSCs (SMSCs-1, 2, & 3) is presented graphically in **Figure 2 B**. With uniform initial seeding densities (5,000 cells per well), cell counts were obtained at every 3 days of culture up to day 12 (performed in triplicate). A significant increase

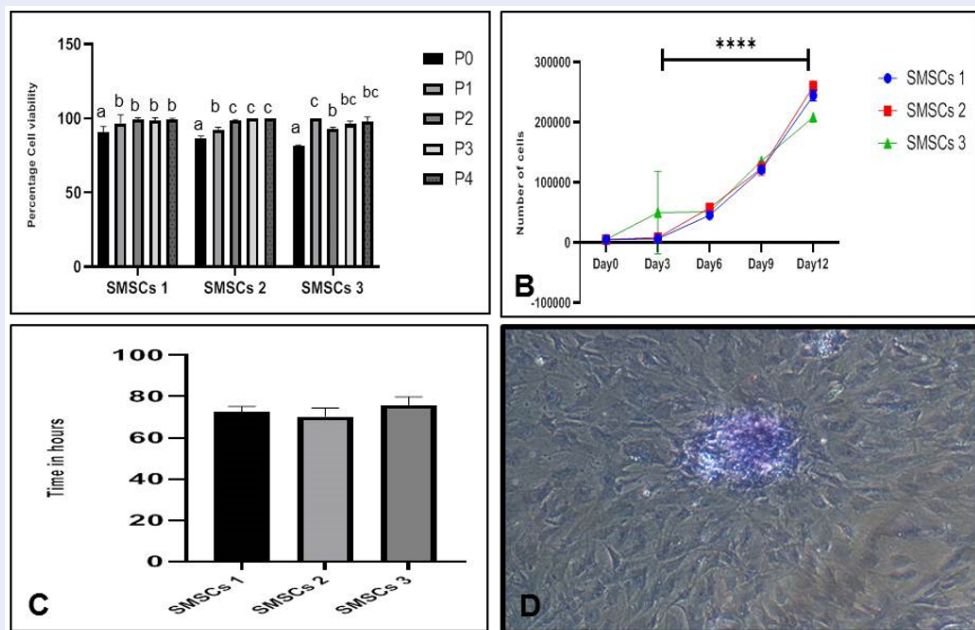


Figure 2: Basic cellular properties of SMSCs in three different cell lines. A. Cell viability of SMSCs was observed more than 98% in every passage from P0 to P4. **B.** All three cell lines were highly proliferative log phase of proliferation was observed from day 3 to day 12. **C.** 70-75 hrs Population doubling time was recorded. **D.** Representative image of colony forming ability of SMSCs stained with crystal violet. Image D: 4x magnification (n = 3).

in cell number was noticed at every stage of counting ($P < 0.05$). The mean proliferation counts were comparable across the three cell lines of SMSCs during expansion. Similar to other diploid cells, SMSCs grew at a rather consistent rate during early days of culture and then with a gradual increase in cell proliferation and number, and attained a peak as days of culture increased (Figure 2 B).

In support of the considerable proliferative abilities of SMSCs, the time for population doublings remained shorter and ranged between 70 and 75 hrs when examined at P3 (Figure 2 C). Moreover, there was no significant ($P > 0.05$) difference in PDT observed between the cell lines.

Colony-forming ability of SMSCs

The initial growth of SMSCs in primary culture on a plastic surface was documented by the formation of single-cell-derived colonies. Later at P4, SMSCs were cultured up to 14 days in a 6-well culture plate with a seeding density of 50 cells/cm², and the colonies were observed by staining with Crystal violet solution. SMSCs exhibited lesser ability to form larger colonies and decreased clonogenic potential over time. The number of colonies formed was also decreased with increased passages.

Cell surface marker analysis of SMSCs

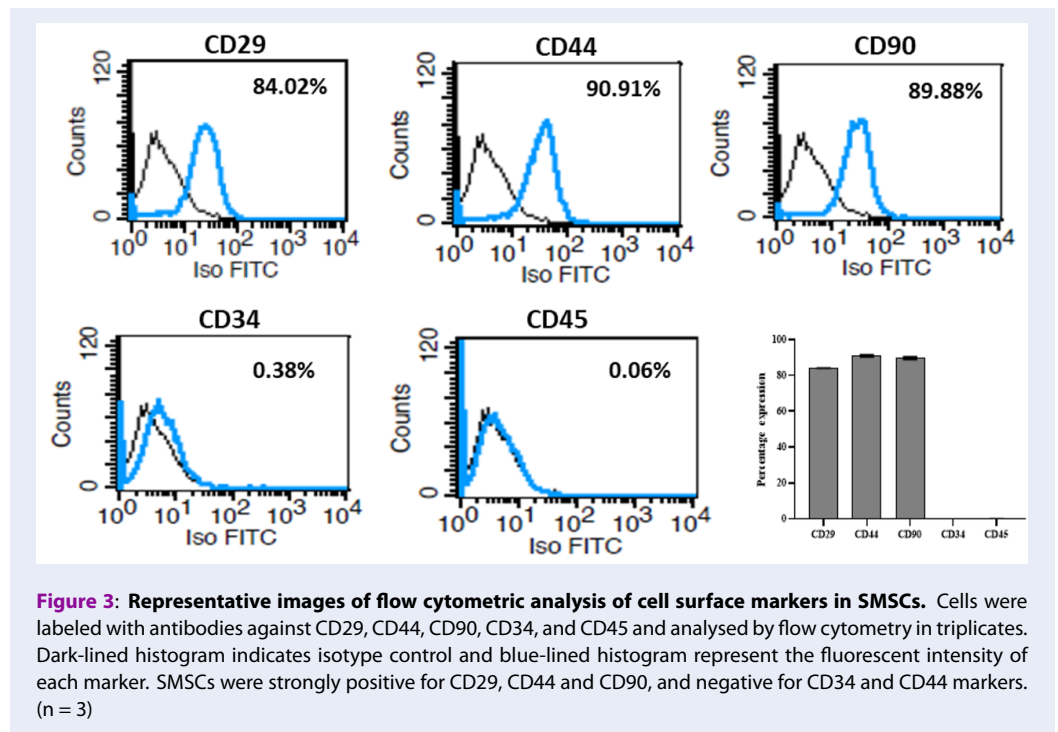
Cell surface markers in SMSCs were analyzed by both flow cytometry and immunocytochemistry, and the representative results are presented in Figure 3 and Figure 4, respectively. MSC-specific marker expression in clonally-expanded SMSCs was quantitatively and qualitatively examined. Assays clearly demonstrated the positive expression of CD29, CD44 and CD90 as MSC-specific markers. However, there was no expression of CD34 and CD45 markers (hematopoietic lineage) in the cultured SMSCs (Figure 3). These results were further supported by the immunofluorescent expression of positive markers, but no expression of hematopoietic markers.

Osteogenic differentiation of SMSCs

Following three weeks of osteogenic induction, SMSCs displayed a characteristic osteogenic phenotype, and the accumulation of calcified mineralized matrix was evidenced by von Kossa staining (Figure 5 A). No such mineralization was visible in the uninduced (control) SMSCs.

Chondrogenic differentiation of SMSCs

The results of chondrogenic differentiation ability of SMSCs are presented in Figure 5 A. SMSCs, after



three weeks of chondrogenic induction in the presence of TGF-beta, revealed the synthesis and secretion of proteoglycans and glycosaminoglycans (GAGs), as demonstrated by Alcian blue staining. In the control, SMSCs showed retention of fibroblast-like cell morphology with no chondrogenesis. In addition to cytochemical staining of chondrocytes differentiated from SMSCs, chondrogenic marker expression was assessed by RT-PCR (Figure 5 B). In control, a weaker expression of aggrecan and collagen II α 1 was detected. However, in induced SMSCs, a prominent expression of these two chondrogenic specific markers was observed, thus confirming the production of cartilage-specific proteoglycans.

Adipogenic differentiation of SMSCs

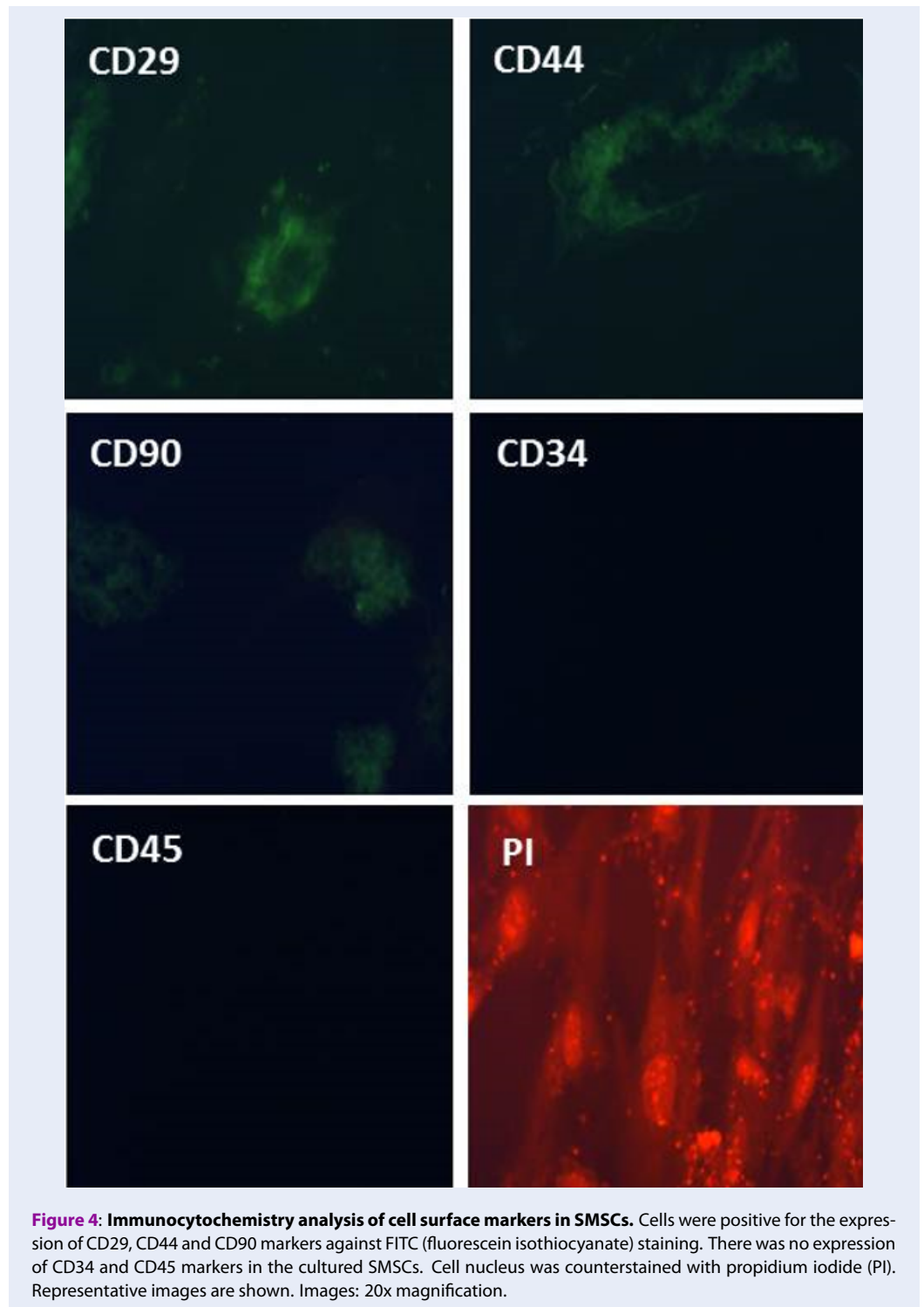
SMSCs cultured in adipogenic induction medium showed intracellular accumulation of neutral fat globules with adipocyte phenotypic features (Figure 5 A). Oil Red O staining clearly demonstrated the formation of lipid droplets prominently in cultures. However, no differentiation was observed in control SMSCs.

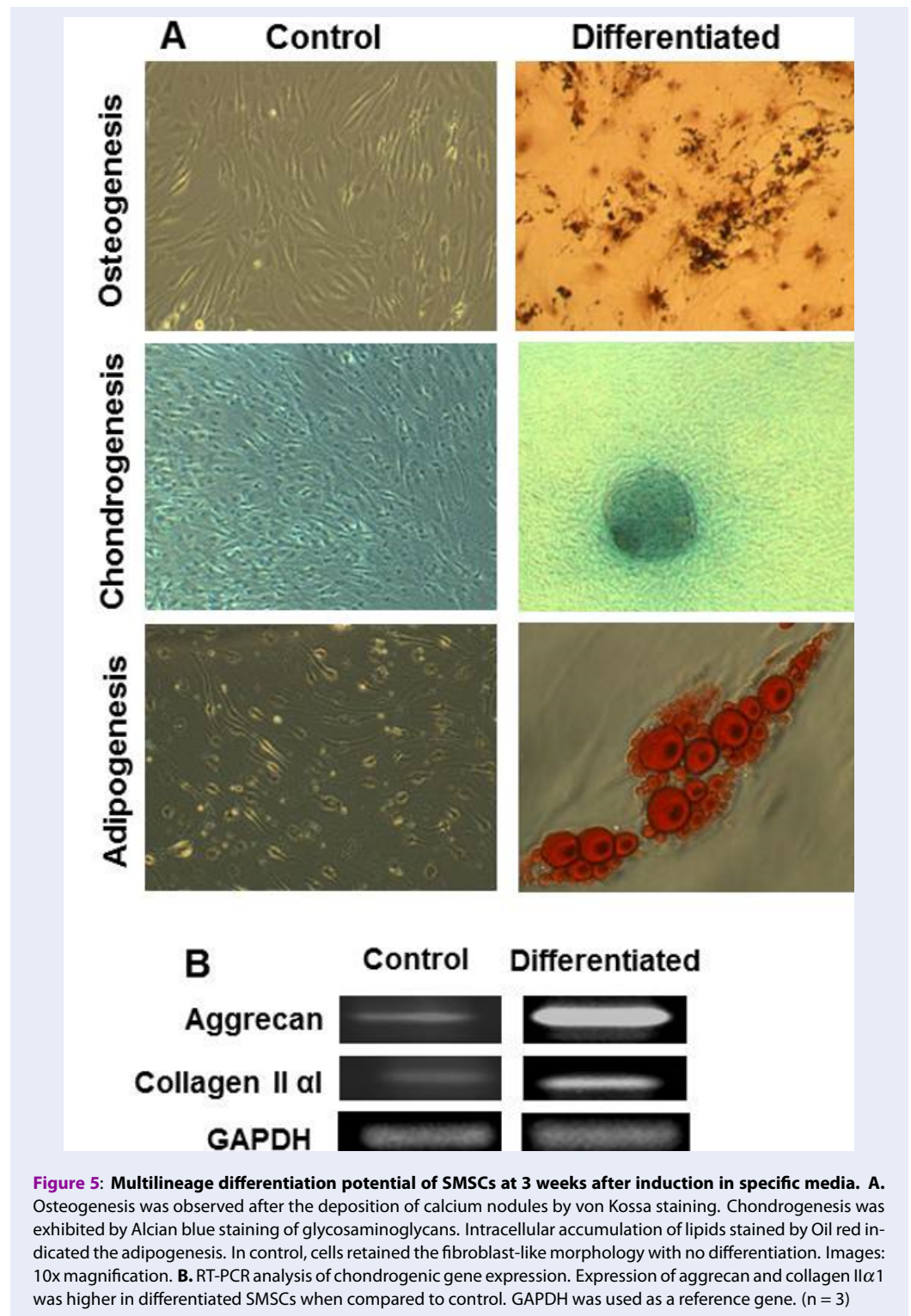
DISCUSSION

The present study successfully isolated SMSCs from joints with OCD, and characterized them based on their morphology, growth kinetics, clonal propagation ability, expression of MSC-specific positive

and negative markers, and multilineage differentiation potential. Under *in vitro* culture, these cells showed plastic adherence and fibroblast-like morphology with an ability to form colonies/clusters at lower seeding density with viability of more than 90%. These characteristics of SMSCs at the initial phase of culture expansion indicated the efficiency of the isolation process adopted for the synovial tissue biopsies. As previously opined, the quality of collected tissue determined the isolation success rather than the amount of biopsy processed¹². Further, these observations on the morphology and early growth kinetics of SMSCs are in agreement with the findings of other studies^{6,12,13}.

In this study, the colonies obtained from partial clonogenic assay showed greater proliferation capacity than that of cells grown at optimum cell density. Hence, these colonies were employed for further analyses. Lower seeding densities enhanced the proliferation of SMSCs with greater homogeneity, and this feature is attributed to a reduction in contact inhibition. It has been shown that SMSCs proliferate rapidly when passaged by plating the cells at low densities of 10-100 cells/cm²¹⁴. In addition, a greater clonogenicity and chondrogenic capacity of progenitor cell subsets that co-exist within the synovium has also been reported¹⁵. These results emphasized the need for establishing pure cell populations with the clinically de-





sired tissue formation potentials. However, it is suggested that heterogeneity with individual clonal populations in SMSCs might lead to a variable proliferation and differentiation potential.

The clinical application of SMSCs needs the availability of a large number of functionally competent cells. In this study, SMSCs exhibited a higher proliferation rate with a shorter duration for population doublings. Replicative senescence is a normal phenomenon observed in all adult diploid cells¹⁶ and these features were not found in *in vitro*-expanded SMSCs, at least until passage 5. Earlier studies revealed that synovial-derived MSCs maintained linear growth curve and possessed greater proliferation^{17,18}. It is noteworthy to mention that proliferation and population doublings are largely dependent on the age and condition of the donor, and also the yield of cells at primary culture¹². Although our results are comparable with earlier reports, a weaker clonogenic capacity at later passages of SMSCs needs to be studied further as it directly indicates the modulation of cell behavior and proliferative ability.

Due to unavailability of specific criteria that define the multipotent SMSCs, these cells are usually assessed in functional terms based on phenotypical marker expression and capacity for multilineage differentiation. In this study, phenotypic characterization of SMSCs using different MSC-specific markers, such as CD29, CD44, CD90, CD34 and CD45, showed that they were of mesenchymal origin with typical expression and did not include hematopoietic cells. The expression of membrane-associated proteins, integrins and adhesion molecules provides strong evidence for their stemness characteristics¹⁹. The observations correspond with earlier reports that describe SMSCs which are positive for CD29, CD44 and CD90, and negative for CD34 and CD45 (as hematopoietic markers)^{12,13}.

In vitro-cultured SMSCs showed more inclination towards chondrogenic differentiation capacity when compared to osteogenic ability. Moreover, the cells also possessed adipogenic differentiation capacity under specific culture conditions. Although the ability for multilineage differentiation is a defining feature of MSCs, it is observed that cells from different tissue sources show predisposition in their differentiation pattern as noticed in this study with SMSCs for chondrogenesis. Chondrocyte-specific gene expression, such as aggrecan and collagen II α 1, further emphasized that SMSCs seem to be specialized, at least in part, towards chondrogenic lineage due to their tissue source. In an earlier study, the detection of type II collagen protein by immunostaining

and of collagen types IIA, IIB, IX, and X messenger RNA (mRNA) confirmed the cartilage phenotype of the tissue generated *in vitro*⁶. In addition, MSCs isolated from various intraarticular tissues including synovium showed gene expression profiles closely resembling each other⁷. However, an impaired chondrogenesis and enhanced osteogenesis have been evident in MSCs derived from osteoarthritis patients²⁰. It has also been reported that dedifferentiated chondrocytes from articular cartilage of healthy and osteoarthritic donors have been shown to exhibit MSC characteristics. However, the reported rates of tripotent cloned cell populations arising from them varied from 10% to 30%^{21,22}. However, we did not observe any diminished chondrogenic potential of SMSCs derived from the joints of OCD patients.

CONCLUSIONS

Collectively, it is concluded that synovial tissue is an important source of MSCs because of its proximity towards articular cartilage. Given that the condition of OCD may not affect the potency of SMSCs, synovium-derived MSCs could serve as a good autologous source for cartilage tissue regeneration.

ABBREVIATIONS

- ANOVA:** Analysis of variance
- DMEM:** Dulbecco's modified essential medium
- DPBS:** Dulbecco's phosphate buffered saline
- FBS:** Fetal bovine serum
- GAGs:** Glycosaminoglycans
- GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase
- MSCs:** Mesenchymal stem cells
- OCD:** Osteochondritis dissecans
- RT-PCR:** Reverse transcription-polymerase chain reaction
- SMSCs:** Synovial-derived mesenchymal stem cells

ACKNOWLEDGMENTS

The authors gratefully acknowledge the research support from Nitte (Deemed to be University).

AUTHOR'S CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published and consent to be accountable for all aspects of the work. All authors read and approved the final manuscript.

FUNDING

This project was funded by Nitte (Deemed to be University) Faculty Research grant (NUFR2-067) from Nitte (Deemed to be University).

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study obtained prior permission from the Institutional Ethics Committee (No. NU/CEC/2017/0182) and Institutional Committee for Stem Cell Research (No. NU/ICSCR/2016-17/002A/P10).

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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