Characterisation and immunosuppressive activity of human cartilage-derived mesenchymal stem cells

Pratheep Sandrasaigaran · Satar Jabbar Rahi Algraittee · Azfar Rizal Ahmad ·
Sharmili Vidyadaran · Rajesh Ramasamy

Received: 21 September 2017 / Accepted: 7 December 2017 / Published online: 1 March 2018
© Springer Science+Business Media B.V., part of Springer Nature 2018

Abstract Mesenchymal stem cells (MSCs) exert potent immuno-regulatory activities on various immune cells and also differentiate into various mesodermal lineages besides retaining a distinct self-renewal ability. Such exclusive characteristics had enabled MSCs to be recognised as an ideal source for cell-based treatment in regenerative medicine and immunotherapy. Thus, considering MSCs for treating degenerative disease of organs with limited regenerative potential such as cartilage would serve as an ideal therapy. This study explored the feasibility of generating human cartilage-derived MSCs (hC-MSCs) from sports injured patients and characterised

based on multipotent differentiation and immunosuppressive activities. Cartilage tissues harvested from a non-weight bearing region during an arthroscopy procedure were used to generate MSCs. Despite the classic morphology of fibroblast-like cells and a defined immunophenotyping, MSCs expressed early embryonic transcriptional markers (SOX2, REX1, OCT4 and NANOG) and differentiated into chondrocytes, adipocytes and osteocytes when induced accordingly. Upon co-culture with PHA-L activated T-cells, hC-MSCs suppressed the proliferation of the T-cells in a dose-dependent manner. Although, hC-MSCs did not alter the activation profile of T cells significantly, yet prevented the entering of activated T cells into S phase of the cell cycle by cell cycle arrest. The present study has strengthened the evidence of tissue-resident mesenchymal stem cells in human cartilage tissue. The endogenous MSCs could be an excellent tool in treating dysregulated immune response that associated with cartilage since hC-MSCs exerted both immunosuppressive and regenerative capabilities.

Keywords Mesenchymal · Cartilage · Sports injuries · Cell differentiation · Cell cycle

Abbreviations

MSCs Mesenchymal stem cells

P. Sandrasaigaran · S. J. R. Algraittee ·
S. Vidyadaran · R. Ramasamy (✉)
Stem Cell and Immunity Research Group, Immunology
Laboratory, Department of Pathology, Faculty of
Medicine and Health Sciences, Universiti Putra Malaysia
(UPM), 43400 Serdang, Selangor, Malaysia
e-mail: rajesh@upm.edu.my

P. Sandrasaigaran
School of Science and Engineering, Manipal International
University, No 1, MIU Boulevard, 71800 Putra Nilai,
Negeri Sembilan, Malaysia

S. J. R. Algraittee
Department of Medical Microbiology, College of
Medicine, Kerbala University, Kerbala 46001, Iraq

A. R. Ahmad
Columbia Asia Hospital Cheras, Lot 33107, Jalan
Suakasih, 43200 Cheras, Selangor, Malaysia

Introduction

Albeit serving as a cushion for shock absorption during mechanical movement of lower extremities, cartilage is well implicated in many disease conditions that affect significant young and old populations in terms of sports injuries and degenerative diseases. The intrinsic nature of the cartilage that lacks nutrients and oxygen supply impede the tissue repair. Hence, any damages or deformities of cartilage are often difficult to heal and require surgical interventions (Detterline et al. 2005). Although treatments to alleviate symptoms and replenishment are available through intra-articular injections, viscosupplementation and physiotherapy, yet it is unable to elicit or promote the regeneration of damaged cartilage (Detterline et al. 2005). This limited capacity for cartilage regeneration at the synovium has necessitated an exploration for alternative therapies, and mesenchymal stem cells have emerged as a novel cell-based therapy in treating degenerative diseases that are associated with cartilage.

Mesenchymal stem cells are multipotent cells with the ability for self renewal and differentiate into tissues of mesodermal origin such as osteoblasts, tendon and chondrocytes (Pittenger et al. 1999). In the human body, MSCs serve as a continuous source of reparative cells with no tissue-specific characteristic but can differentiate into different cells of connective tissue lineages. This differentiation occurs in response to signals triggered by tissue damage such as fracture, necrosis, tumours and most importantly, inflammation (Palermo et al. 2005). Although MSCs can be differentiated into various mature cells, yet such broad differentiation ability can be affected by the local microenvironment where it leads to tissue-specific or guided differentiation (Otto and Rao 2004). Besides serving as a pool of the mesenchymal and parenchymal cells, MSCs dispose of attributes for the several therapeutic properties. Cotransplantation of MSCs enhanced the engraftment of haematopoietic stem cells and reduced graft versus host disease (GVHD) at post allogeneic bone marrow transplantation (Le Blanc et al. 2004, 2007; Ramasamy et al. 2008). Hence, such unique characteristics rendered MSCs as a novel cell-based therapy in treating many orthopaedic related degenerative cartilage diseases.

Recent studies and findings have highlighted that cartilage may host the multipotent stem cells within a

specific niche as a reservoir of tissue resident stem cells and being able to produce mature chondrocytes upon the demand of homeostasis (Alsalameh et al. 2004; Csaki et al. 2008). Although not directly harvested from the cartilage tissue, the first isolation of multipotent MSCs from joint tissue was reported in 2001. MSCs were isolated from a digested human synovial membrane and showed a typical mesodermal differentiation (De Bari et al. 2001). Since then many have reported cells with similar morphology, phenotype and differentiation capacity that adhere to the standard minimal definition for MSCs, however, some termed these cells as cartilage derived stromal cells (Su et al. 2006).

Although the ability to differentiate into chondrocytes is a hallmark for MSCs' characterisation regardless the tissues' origins and ontogenesis, however, the quality and inclination of chondrogenesis differ significantly based on the source of MSCs. It has been widely believed that the chondrogenic differentiation ability of MSCs derived from cartilage is much greater as compared to other sources (Dazzi et al. 2006; Peng et al. 2008). Based on cartilage matrix formation, Peng and coworkers demonstrated that cartilage MSCs possess the highest ability for chondrogenesis when compared to bone marrow and adipose tissue-derived MSCs (Peng et al. 2008). MSCs derived from the synovium appeared to show a higher chondrogenic potential when compared with cells derived from the BM (Lee et al. 2009; Sakaguchi et al. 2005). Similarly, MSCs from the synovial fluid as well possess a predominant chondrogenic differentiation capacity when these cells were compared with bone marrow MSCs (Jones et al. 2008).

Therefore, in this study, we investigated the presence of MSCs from the cartilage of patients undergoing knee surgery due to sports injuries. The biological features shown by cartilage-derived adherent cells, such as plastic adherence, ability to differentiate into the mesodermal lineage and expression of a panel of common MSCs surface molecular markers, further confirmed the existence of MSCs within the cartilage tissue. The current study does not only provide an additional strength for the existence of tissue-resident mesenchymal stem cells in the cartilage vicinity, but shed light for future exploitation of regenerative and immunosuppressive properties of MSCs towards ameliorating cartilage-based degenerative diseases.

Materials and methods

Sample collection and transportation

Human cartilage of 20–30 g was collected from patients undergoing knee surgery or arthroscopy due to sports injury, and the tissue was harvested from a non-weight-bearing region of cartilage. The samples were collected from Hospital Columbia Asia (Cheras, Kuala Lumpur, Malaysia) and were processed within 3 h of the collection. The ethical approval was sought from the Ethics and Research Committee (Faculty of Medicine and Health Sciences, Universiti Putra, Malaysia). All samples were collected with written and informed consent. The samples were collected in sterile 50 mL tube containing RPMI 1460 medium supplemented with 1% penicillin and streptomycin (Gibco BRL, Invitrogen, USA) with 0.5% fungizone (Gibco BRL, Invitrogen, CA, USA), 0.1% gentamicin (Gibco BRL, Invitrogen), and transported in ice box to maintain the temperature below metabolic rate (4 °C).

Sample processing

The samples were finely minced to approximately 1 mm³ followed by enzymatic digestion; incubation in 20 ml of 0.4% collagenase type II (Gibco BRL, Invitrogen) and 0.01% DNase I (Sigma Aldrich, St. Louis, MO, USA) for 50–60 min gentle agitation. After incubation, the supernatant containing single cell suspension was collected and passed through 70 and 40 µm cell strainers (Becton–Dickinson, San Jose, CA, USA) and centrifuged for 10 min at 1800 rpm. Supernatant was discarded and the collected cells were centrifuged again with 1× PBS solution before plated in T25 plastic culture ware (Becton–Dickinson) with DMEM/F12 medium supplemented with 1% penicillin and streptomycin (Gibco BRL, Invitrogen) with 0.5% fungizone (Gibco BRL, Invitrogen), 0.1% gentamicin (Gibco BRL, Invitrogen) and 40 ng/ml of recombinant human basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ, USA). Cell culture medium was changed twice weekly until the adherent cells were confluent. The cultures were maintained till passage three (P3), and further downstream analysis was done using cells from P3 to P7.

Immunophenotyping of hC-MSC

The cell surface protein expression was assessed and analysed using LSRFortessa II (Becton–Dickinson) flow cytometer. Approximately $0.2\text{--}1 \times 10^6$ cells were stained with a specific monoclonal antibody conjugated with a fluorescent marker. Cells at 80–90% confluence were harvested and stained with Anti-huCD29-APC, Anti-huCD73-PE, Anti-huCD90-FITC, Anti-huCD105-PE, Anti-huCD271-PE, Anti-huCD45-PerCP, Anti-huCD34-FITC, Anti-huCD80-FITC, Anti-huCD86-FITC, HLA-A-B-C-PECy5, HLA-DR, DP, DQ-FITC, Anti-huCD3-PE, STRO-1, Anti-huCD19-PE and isotype controls. All monoclonal antibodies were purchased from the Becton-Dickinson, except Anti-huCD105 and STRO-1 were purchased from R&D System (ME, USA) and Thermo Fisher Scientific (San Diego, CA, USA), respectively. A minimum of 10,000 events (cells) was acquired from the target population excluding the death cells. Flow cytometry data were analysed with BD FACS-Diva software.

Mesodermal lineage differentiation

Adipogenic and osteogenic differentiation assays were conducted in 24 well plates for cyto-staining assay and in T25 culture flasks for gene expression analysis. Confluent hC-MSCs were cultured with STEMPRO Adipogenesis Differentiation Kit (Gibco, Invitrogen) and STEMPRO Osteogenesis Differentiation Kit (Gibco, Invitrogen) medium to induce cell differentiation. The induction medium was replaced every 3 days until day 14 for adipogenesis and until 21 days for osteogenesis. The cells were stained with Oil Red O and Alizarin Red S stain, respectively. On the other hand, for the gene expression studies, the cells in the T25 flasks were harvested with the Trizol reagent. Using reverse transcriptase and polymerase chain reaction (PCR), adipocyte marker gene (lipoprotein lipase (LPL)) was assessed. For osteogenesis, three genes (osteopontin, osteocalcin and parathyroid hormone (PTH)) were assessed using the same method. Chondrogenic differentiation assay required a special 2D micro-mass culture method. The cells were prepared before the experiments as per STEMPRO Chondrogenesis Differentiation kit (Gibco, Invitrogen) in Ø60 mm petri dishes. Cells were maintained in culture for 17 days with media changed every 3 days.

Table 1 Primers used for transcription factors of early embryonic genes

Primer	5'–3'	Sequence	Amplicon size	Anneling temperature (°C)
4-Oct	Forward	CGACCATCTGCCGCTTTGAG	535	58
	Reverse	CCCCCTGTCCCCATTCCTA		
Sox2	Forward	ATGCACCGCTACCACGTGA	434	56
	Reverse	CTTTGCACCCCTCCCATTT		
Rex1	Forward	CAGATCCTAAACAGCTCGCAGAAT	306	64
	Reverse	GCGTACGCAAATTAAAGTCCAGA		
NANOG	Forward	AGTCCCAAAGGCAAACAACCCACTTC	164	56
	Reversed	ATCTGCTGGAGGCTGAGGTATTTCTGTCTC		

Table 2 Primers used for transcription factors of mesodermal lineage genes

Primer	5'–3'	Sequence	Amplicon size	Anneling temperature (°C)
Osteocalcin	Forward	ATCAGAGCCCTCACACTCCT	377	48
	Reverse	CAAGGGGAAGAGGAAAGAAG		
PTH	Forward	AACTACTACTGGATTCTGGTGG	541	56
	Reverse	CTCCAAGATTTCTTGATCTCAG		
Osteopontin	Forward	GAAGGACAGTTATGAAACGAGT	499	60
	Reverse	AACATAGACATAACCCTGAAGC		
Lipoprotein lipase	Forward	CTTCTGTTCTAGGGAGAAAGTG	577	48
	Reverse	TGCTGTGTAGATGAGTCTGATT		
Aggrecan	Forward	GCCTTGAGCAGTTTCACCT	397	55
	Reverse	CTCTTCTACGGGGACAGC		
Collagen type II	Forward	CCAGGACCAAAGGGACAGAAAG	399	48
	Reverse	TTCACCAGGTTTACCAGGATTG		
GAPDH	Forward	TTGCAACTGTTTTAGGACTTT	567	56
	Reverse	AGCATTGGGAAATGTTCAAGG		

once. Alcian blue dye was used to stain the differentiated cells. Aggrecan and Collagen type II genes were assessed for chondrocyte differentiation. Total RNA was extracted from hC-MSCs, hC-MSCs differentiated to adipocytes, osteocytes and chondrocytes using TRIzol[®] Reagent (Invitrogen). Reverse transcription (RT-PCR) was performed using the GoScript[™] Reverse Transcription System (Promega, Madison, WI, USA) and cDNA and Taq DNA Polymerase kit (Qiagen, Germantown, MD, USA). Gene of interest was assessed using primers synthesised from EURO-GENTEC AIT Pte Ltd (Singapore) as shown in Tables 1 and 2.

T cell proliferation assay

Briefly, 5×10^4 peripheral mononuclear cells (PBMC) were co-cultured with hC-MSCs at various

ratios; 1:1, 1:5, 1:10, 1:50 and 1:100 in 96 well plate. To induce proliferation of T-cells, 5 µg/ml of phytohemagglutinin (PHA-L) (Roche, Mannheim, Germany) were added to the co-cultured cells and they were incubated for 72 h. Before analysis, tritiated thymidine (³H-TdR) (0.037 MBq/well [0.5 µCi/well] (Perkin Elmer, Richmond, CA, USA) was added to the cells at the final 18 h and cells were analysed in Microbeta Trilux beta counter (Perkin Elmer). For transwell assay, hC-MSCs (1×10^5 cells) were plated in the upper chamber of the 12 well-transwell system (1 µm sized pores) and incubated overnight for cell adherence. Non-adherent cells were discarded by changing the medium on the next day, and subsequently, PBMC (1×10^6 cells) were seeded in the lower chamber and activated with 5 µg/ml of PHA-L. Apart from that, the conditioned medium, supernatant from hC-MSCs, was also tested for its anti-

Fig. 1 Morphology and early embryonic markers expression of cartilage-derived adherent cells. Cartilage samples were treated with an enzyme for disassociation into single cell suspension. The cells were left in mesenchymal stem cell culture medium. **a**, **b** adherent cells noticed from day four onwards in passage zero (P0). **b** The cells' morphology was not well defined with contaminants suspended in the medium (P0). **c** Short and thick adherent cells were noticed which started to form from day eight

at P0. **d** Cells were observed with well-defined spindle-shaped fibroblast-like morphology from P2 onwards. Cells were observed under phase contrast microscope at 100× magnification. **e** The analysis of mRNA transcripts of these cells showed early embryonic markers; NANOG, OCT 4, REX 1 and SOX 2. The cells were positive for pluripotent genes, and GAPDH served as a reference gene (housekeeping gene). Results are representative of 3 independent experiments

proliferative action. Peripheral blood mononuclear cells alone and hC-MSCs alone with PHA-L stimulation were used as the positive and the internal control of the experiments, respectively.

Cell cycle assay

Human cartilage derived MSCs were co-cultured along with resting or activated T-cells at 1:10 ratio for 72 h. The cells were later harvested and fixed with 70% ethanol at -20°C overnight. After 24 h, the cells were washed with $1\times$ PBS and incubated with propidium iodide (PI) (Molecular Probe, Invitrogen) at a concentration of 100 $\mu\text{g}/\text{ml}$ and RNase (Sigma) at a concentration of 20 ng/ml for 30 min. 5 μl of 20,000 ng/mL FITC dye was also incorporated during the 30 min' incubation to distinguish the G_0/G_1 stage of the cell cycle. The results were acquired via a

LSRFortessa II flow cytometer and analysed using the Modfit software. Resting or activated T-cells without hC-MSCs co-culture were used as the negative and positive controls, respectively.

T-cells activation assay and analysis

The hC-MSCs were co-cultured at the ratio of 1:10 with resting or stimulated T-cells for 72 h in a 24 well plate. At the end of the culture, T-cells were harvested and washed with 0.1% BSA in $1\times$ PBS. The cells were then labelled with Anti-huCD3-PE, Anti-huCD4-FITC, Anti-huCD8-PerCP and Anti-huCD25-APC (Becton–Dickinson) for 30 min before acquired via a LSRFortessa II flow cytometer and gated using FACS Diva software. Resting T-cells and activated T-cells alone served as negative and positive controls, respectively.

Statistical analysis

The data are expressed as mean ($\pm$ SD). The Student *t*-test was performed to compare the values of two means. The significance level was determined as $p < 0.05$.

Result

Morphology and early embryonic markers expression of cartilage-derived adherent cells

Adherent cells were observed from cartilage samples from day four onwards (Fig. 1a). Besides, colonies of cells with altered morphologies and impurities were observed in early days of primary culture (Fig. 1b). However, the cells' morphology started to be defined as fibroblast-like and spindle shaped after 8 days in culture. The impurities also started to decrease along with media changes, and after passage two, more homogenous cells with unanimous spindle-shaped fibroblast-like morphology were noticed (Fig. 1c, d). When total RNA was isolated from cells after passage three (P3), the cells were identified to express early undifferentiated embryonic pluripotent markers; SOX 2, REX 1, OCT 4 and NANOG (Fig. 1e). This was assessed by reversed transcription PCR method. However, the gene expression was not quantified.

Flow cytometry analysis of cells' surface antigens

The cells stained with FITC, PE, APC and PE-Cy5 conjugated monoclonal antibodies were analysed using FACS Diva software, and the result showed positive expression for integrin (CD29), endoglin (CD105), ecto-5'-nucleotidase (CD73), Thy-1 (CD90) and HLA-A-B-C. On the other hand, cells were either negative or showing minimal expression ($< 2\%$) for common leukocyte antigen (CD34 and CD45), B cell (CD19), monocytes (CD14), CD271, HLA-DR, STRO-1 and the T cells' co-stimulatory marker CD86 and CD80 (Fig. 2) which is confirming the standard immunophenotyping profile of MSCs.

Differentiation of spindle-shaped fibroblast-like cells into mesodermal lineages

Cartilage derived spindled shaped fibroblast-like adherent cells were found to be able to differentiate into

Fig. 2 Flow cytometry analysis of cells' surface antigens. The cells were stained with monoclonal antibodies conjugated with FITC, PE, APC and PE-Cy5 were analysed for surface antigen expressions using flow cytometer. The cells (10,000 events) were gated based on the cell size [forward scatter (FSC)] and granularity [side scatter (SSC)] while excluding dead cells and debris. Isotype controls antibodies conjugated with APC, PE, FITC and PE-Cy5 fluorochromes were used to set the base line expressions while the cells were stained with antibodies with respective predominant reactivity; anti-huCD29-APC (monocytes & lymphocyte), anti-huCD73-PE (T cells, B cells, dendritic cell, endothelium & stem cell), anti-huCD90-FITC (Thy-1 cells), anti-huCD105-PE (Endoglin), HLA-A-B-C-PECy5 (MHC-I expressing cells), anti-huCD271-PE (neuronal axons, Schwann cells, perineural cells of peripheral nerves, epithelial, mesenchymal and lymphoid tissues), anti-STRO-1 (stromal elements in human bone marrow), anti-huCD14-FITC (monocytes), anti-huCD19 (B-lymphocyte), anti-huCD45-PerCP (leucocytes), anti-huCD86-FITC (activated B cells & monocytes), anti-huCD80-FITC (activated B cells & dendritic cell), anti-huCD34-FITC (hematopoietic stem cells) and HLA-DR, DP, DQ-FITC (MHC-II expression cells). Single parameter histograms were plotted and cells expressing positive cell surface markers were compared and calculated based on the isotype controls. Results showed cartilage derived adherent cells were positive for CD29, CD73, CD90, CD105 and HLA-ABC while negative for CD271, STRO-I, CD14, CD19, CD45, CD86, CD80, CD34 and HLA-DR. Results are representative of 5 individual experiments

all three mesodermal lineages. The results were confirmed with cytochemical staining assay as well as the reverse transcription PCR in which genes that expressed in differentiated cells were amplified and measured (Fig. 3). The cells differentiated into adipocyte were able to be distinguished with lipid vacuoles formation which is stained red with Oil-Red-O stain. Osteogenic differentiation was noted by Alizarin Red stain that leads to a brick red colour. On the other hand, the cells differentiated into chondrocytes resulted in the formation of proteoglycans that is recognised via blue staining by Alcian Blue. Images were captured using phase contrast microscope. Gene analysis further supported that the cells were able to differentiate into the mesodermal lineage. Cells expressing RNA transcript genes for adipocytes (Lipoprotein lipase), osteocytes (Osteopontin, Osteocalcin and Parathyroid Hormone) as well as chondrocytes (Aggrecan and Collagen type II) (Fig. 3) were noticed.

hC-MSCs inhibit activation of PBMC via cell to cell contact

The role of hC-MSCs in harnessing immune response was examined via tritiated thymidine (^3H -TdR)

proliferation assay. The result shows that hC-MSCs require a strong cell-to-cell contact to impair the T-cells proliferation. The noted immunosuppression seems to be dose-dependent of hC-MSCs numbers as the higher the ratio of hC-MSCs to T-cells was the

Fig. 3 Differentiation of spindle-shaped fibroblast-like cells into mesodermal lineages. Spindle-shaped fibroblast-like cells derived from cartilage samples were cultured using the respective differentiation media (for adipogenesis, osteogenesis and chondrogenesis) and incubated as per manufacturer's instructions. (a2) The cell morphologies were noticed to change from spindle-shaped fibroblast-like to round and stained positive for Oil Red O stain (Oil Red O stained adipocytes indicated with arrow); (b2) to change to cuboidal shape and osteogenic differentiation was confirmed with Alizarin Red S staining; (c2) meanwhile micro mass chondrocytes were stained with Alcian blue stain (Alcian blue stained chondrocytes indicated with arrow). (a1, b1, c1) Control cells neither positive for

morphology changes nor cytochemical staining. RNA was isolated from both control and differentiated cells and reversely transcribed into cDNA. (a3) Lipoprotein Lipase (LPL) gene expression was noticed for the cells cultured in adipogenesis differentiation medium. (b3) Osteopontin, Osteocalcin and Parathyroid Hormone were detected in the cells grown in osteogenesis differentiation medium. (c3) On the other hand, the cells grown in chondrogenesis differentiation medium were detected to express Aggrecan and Collagen type II genes. The results were analysed via an alpha imager after PCR amplification. GAPDH gene (house keeping gene) was used as reference gene. Results are representative of 3 experiments

better the immunosuppression was (Fig. 4a). The ratio of 1:10 of hC-MSCs to T-cells has significantly reduced proliferation of activated T cells. On the other hand, when 1:10 of hC-MSCs to T-cells were tested in a transwell assay, no suppression of T-cells was observed. hC-MSCs' culture conditioned medium also did not yield a significant suppression, however, a slight reduction of T-cells proliferation was noticed (Fig. 4b).

hC-MSCs induce T-cells arrest at G₀/G₁ of cell cycle without intervening in the activation process

When the T-cells were labelled with a PI dye that binds to the DNA and the FITC dye that stains proteins, flow cytometry analysis was able to correspondingly measure the DNA and protein synthesis in the cells. Hence, T-cells were stimulated with PHA-L, the cells proliferated extensively, and this has been supported by high protein synthesis (17.2%) and DNA (27.3%) content (Fig. 5a). The cell cycle analysis of the PHA-L activated T-cells also supported the notion that the majority of the cells entering S-phase (32.0%) and some progressing to G₂/M

Fig. 4 hC-MSCs inhibit activation of PBMC via cell to cell contact. Human cartilage derived mesenchymal stem cells (hC-MSCs) were co-cultured with T-cells either with or without PHA-L stimulants at different hC-MSCs—T-cells ratios. (a) The hC-MSCs inhibit proliferation of T-cells in a dose-dependent manner. PHA-L stimulated T-cells and non-stimulated T cells were used as the positive and negative controls, respectively. On the other hand, hC-MSCs cultured with resting T-cells at 1:5 and 1:10 significantly increased the T-cells proliferation. The data shown are the mean percentage of cells $\pm$ SD of 3 independent experiments. * $p < 0.05$ was compared to positive control (+ PHA). ^ $p < 0.05$ was compared to negative control (– PHA). (b) hC-MSCs fail to induce immunosuppression in a transwell culture system and when using solely SN medium. The data shown are the mean percentage of cells $\pm$ SD of 6 independent experiments. * $p < 0.05$ were compared to positive control (T cell + PHA)

(1.5%) (Fig. 5b). The resting T-cells were not undergoing proliferation process as the S and G₂/M phases were completely abrogated. Furthermore, supplementation of hC-MSCs (1:10 for hC-MSCs: T-cells) into resting T-cells co-culture did not induce proliferation

of T cells. On the other hand, the inclusion of hC-MSCs in culture with activated T-cells (T-cells + PHA-L) showed reduction in cell proliferation where decrease in protein (9.8%) and DNA (15.6%) contents were noted. This is corresponding to the cell cycle reports which show hC-MSCs prevent activated T-cells entering the S-phase with a drastic reduction of the cells in the S-phase (9.0%). However, no significant changes (1.6%) were observed for all cells in the G₂/M phase. All results are shown in Fig. 5. Figure 5c revealed that there was no significant difference in CD25 expression in both T-helper cells (CD4) and cytotoxic T-cells (CD8) when co-cultured with or without hC-MSCs. The resting T-cells activation profile also showed no significant differences when compared with the counterpart of resting T-cells + hC-MSCs co-culture. These outcomes indicate that hC-MSCs suppressed both the activation and proliferation of PHA-L stimulated T-cells.

Discussion

Unlike other stem cells which only or mostly reside in specific tissues or organs, mesenchymal stem cells or mesenchymal stromal cells (MSCs) are found in most of the solid organs. Since most solid organs require scaffolds and stromal compartment to consolidate and accommodate the parenchymal cells, the existence of MSCs in those organs are very much anticipated. However, the role or physiology of these tissue-resident MSCs is still unclear whether these stem cells contribute to the parenchymal regeneration or maintains the integrity of the organs. Similarly, cartilage is also reported to contain MSCs but the direct contribution of MSCs to the regeneration of cartilage is still lacking although MSCs are known as precursor for cartilage. The present study had successfully generated MSCs from human cartilage tissues, thus, further, strengthens the notion of the existence of hC-MSCs using universally accepted criteria. Moreover, we have also reported the intrinsic immunosuppressive nature of hC-MSCs and the possible mechanism of such activity in contact with immune cells.

The in vitro culture generated adherent cells of human cartilage tissue showed a typical MSCs morphology after the first 8 days which is not different from other sources of MSCs such as bone marrow,

Fig. 5 hC-MSCs induce T-cells arrest at G₀/G₁ of cell cycle without interfering in the activation process. Peripheral blood mononuclear cells (1.0×10^6) with or without hC-MSCs (1.0×10^5) were stimulated with 5 μ g/ml of PHA-L. After 72 h of incubation, T-cells were collected to be fixed and permeabilized in 70% ethanol overnight. Protein and DNA content of T-cells were analysed by staining with FITC and PI dye respectively. **(a)** The result shows that when hC-MSCs were co-cultured with PHA-L activated T-cells, hC-MSCs induced T-cell arrest by blocking synthesis of protein and DNA. **(b)** The FACS histogram shows the distribution of the cells in G₁, S and

G₂/M phases, respectively as analysed in Modfit software. Results were representative of 3 independent experiments. **(c)** PHA-activated T cells' activation profile was compared to the activated T cells to which with hC-MSCs were added (activated T cells vs. activated T cells + hC-MSCs). Resting T cells alone and resting T cells + hC-MSCs co-culture was used as the internal controls. Cells were stained with anti-huCD4, anti-huCD8, anti-huCD25 and anti-huCD3 monoclonal antibodies and results were acquired in LSRFortessa II using FACSDiva software

umbilical cord, placenta and adipose tissues (Javazon et al. 2004; Wu et al. 2013). These cells are adherent, having a well-defined short spindle-shaped fibroblast-like morphology after passage two onward. When the spindle-shaped fibroblast-like adherent cells were tested for mesodermal lineage differentiation, these cells differentiated into all three mesodermal lineages namely osteocytes, adipocytes and chondrocytes, the 'gold standard' to characterise MSCs based on common practice. The gene expression analysis further confirmed the osteogenic differentiation by gauging the presence of osteocalcin, osteopontin and parathyroid hormone in the differentiated cells but not in the control. During the adipogenic differentiation assay, the cells were transformed and developed intracellular lipid vacuoles which are distinguished with Oil Red O stain. Furthermore, the cells also undergo morphological changes from a spindle-shaped fibroblast-like cells to spherical fat cells. The expression of lipoprotein lipase (LPL), an early marker for

adipocytes was shown using reverse transcription PCR method.

Chondrogenic differentiation of hC-MSCs was enabled with 2D micro-mass culture (Tyndall et al. 2007). The 2D micro-mass culture or pellet culture is a prerequisite as chondrogenic differentiation is only achievable when there is close contact between the cells, leaving limited space, oxygen and nutrients which mimic a similar cartilage microenvironment. When hC-MSCs differentiate into chondrocytes, a highly-organised network of collagen type II with mass aggregation of proteoglycan will tend to predominate the structural makeup. Hence, differentiated cells were easily distinguished with Alcian Blue that stains the proteoglycan layer. Aggrecan is an early chondrogenic marker, and collagen type II marker was also shown positive in differentiated cells but not in the non-induced control.

In agreement with the International Society for Cellular Therapy's (ISCT) which recommends a minimal requirement for MSCs, the adherent cells

were expressing CD105, CD73 and CD90 and negative for the haematopoietic markers (Dominici et al. 2006). Besides these established markers, we have also tested the expression of CD271 and STRO-1, however, the expression of both markers was at a very negligible level. The low-affinity nerve growth factor receptor (LNGFR), CD271 is mainly reported in bone marrow (BM) derived MSCs (Quirici et al. 2002). The absence of CD271 might be due to differences in the source of MSCs. Furthermore, the expression of CD271 is not a mandatory requirement in determining the immunophenotype profile of MSCs; however, it could be useful in distinguishing the origin of MSCs, primarily bone marrow-derived MSCs. Besides, it has been noticed that the activity of LNGFR is reduced with cells' passaging (Jones et al. 2002). Additionally, the supplementation of growth factor, bFGF which was used to optimise the hC-MSCs culture was reported to rapidly reduce and hamper the expression of CD271 in *in vitro* culture (Quirici et al. 2002). On the other hand, STRO-1 is only a prominent marker in MSCs derived from human bone marrow (BM), and loss of STRO-1 was also shown in BM-MSCs at later passages (Gronthos et al. 2003). Besides, the precise function of STRO-1 either in MSCs or other cells is yet to be defined. Thus, the down-regulation of such surface markers might be necessary affect the function or physiology of hC-MSCs that maybe correspond to the release of relevant cytokines and chemokines (Kolf et al. 2007).

There are several genes such as SOX-2, REX-1, OCT-4 and NANOG which are expressed in early embryonic stem cells (ESCs). These genes serve as markers for the pluripotency of ESCs through translation of relevant proteins that mediate important signalling pathways in self-renewal (Chambers et al. 2003; Kuroda et al. 2005; Pesce and Scholer 2001; Rogers et al. 1991). The present study showed expression of similar early ESCs genes in hC-MSCs leaving an open question regarding hC-MSCs' multipotency. However, one should bear in mind that the expression of such 'primitive' genes does not promise the execution or mediation of their respective functions as we are unable to show the protein translation of particular genes. In the meantime, the expression of early ESCs markers could reaffirm the multipotency of hC-MSCs and may also hold a key for pluripotency as suggested for MSCs derived from other sources (Cho

et al. 2005; Javazon et al. 2004; Tokcaer-Keskin et al. 2009).

The KLF4 is a member of Kruppel-like factor (KLF) family which is highly expressed in mammals and often regulates cell physiological functions such as proliferation, differentiation, development, maintenance of normal tissue homeostasis and apoptosis. In the past years, the key roles of KLF4 in maintaining the self renewal ability of embryonic stem (ES) cells has been reviewed extensively. In 2006 Takahashi and coworkers had shown the ability of KLF4 to reprogram the somatic cells into pluripotent stem cells with the combination of three other transcription factors: Oct4, Sox2 and c-Myc; also known as Yamanaka factors. Although the highest efficiency of induced pluripotency is typically achieved with the Yamanaka factors, on the other hand, c-myc and KLF4 have also been shown to be unessential and dispensable for somatic cell reprogramming for pluripotency (Huangfu et al. 2008a, b). These studies indicate that OCT4 and SOX2 are critical factors required for maintenance of self renewal and pluripotency in mouse and human stem cells. Furthermore, the Thomson Factors: OCT4, SOX2, NANOG, and LIN28 were also reported to induce pluripotency of human somatic cell types. This again reinstates the surplus of KLF4 in maintaining the pluripotency state in ES cell (Yu et al. 2007). OCT4 and SOX2 have also been shown as the fundamentals for iPS by Yamanaka (2008). With regards to this, we have decided to scrutinise the early embryonic characteristics of hC-MSCs to be restricted to NANOG, OCT 4, REX 1 and SOX 2 transcriptional factors only without the inclusion of KLF4.

The immunosuppressive activity is one of the criteria that are often used to further characterise MSCs besides all three major criteria outlined by ISCT. Similar to the BM-MSCs and other common sources MSCs, hC-MSCs as well exert a profound immunosuppressive activity against activated T cells. The observed immunosuppressive activity is mediated by an active cell-to-cell contact mode and in a dose-dependent manner. In the context of the immunomodulatory function of MSCs, two properties of MSCs are worth to be discussed here, either being immune-privileged/hypoimmunogenic cells that escape the immune recognition by host immune cells or evade the host immune system by suppressing the responding immune cells. Human cartilage derived MSCs did not

express the MHC class II and the co-stimulatory molecules such as CD80 and CD86. Lacking such attribution could enable hC-MSCs to escape from being recognised by CD4 helper T cells. However, hC-MSCs exhibit MHC class I molecules and yet escapes from the immune attack of CD8 cytotoxic T-cells. Previous studies had highlighted that MSCs failed to trigger cytotoxic mechanism in CD8 T-cells and thus escapes from being lysed by the CD8-T cells (Rasmusson et al. 2007).

Mesenchymal stem cells have been previously shown to induce anergy and prevent T-cells from entering into S-phase of the cell cycle, thus inducing arrest in the G₀/G₁ phase of various immune cells (Corcione et al. 2006; Ramasamy et al. 2007a, b; Tabera et al. 2008; Vellasamy et al. 2016). Hence, when hC-MSCs were co-cultured with PHA-L activated T-cells, they prevent the lymphocytes from entering into S-phase. The protein and DNA content of activated T-cells were also reduced in the presence of hC-MSCs which is a reflection of the cell cycle arrest in T-cells. Nevertheless, the cell cycle machinery was disturbed significantly by hC-MSCs, but the existence of hC-MSCs along with T cells and mitogen did not affect the activation of T cells. It was found that the expression of CD25, an alpha component of IL-2 receptors remain increased in T cells co-cultured with hC-MSCs as compared to the stimulated T cells alone. This finding supports the notion that hC-MSCs do not influence the priming event of the T cells which is in agreement with previous studies (Ramasamy et al. 2008).

The regenerative property of hC-MSCs differentiate into chondrocytes, and the inherent immunosuppressive activity are two beneficial aspects of hC-MSCs that could be potentially exploited towards cartilage related immune and degenerative diseases. The therapeutic use of MSCs in certain diseases can ameliorate the pathological conditions especially, rheumatoid arthritis where the disease is characterised by degradation of cartilage tissue due to detrimental autoimmune response. However, it may raise an additional question of the physical existence of MSCs in the diseased cartilage tissue on the inability of endogenous MSCs to respond towards disease in order to exert a reparative action. Although not much evidence is available in this regard, yet it could be possible that the tissue microenvironment mitigates such reparative activities to take part due to the non-

conductive microenvironment. Besides, the distorted equilibrium where the magnitude of tissue injury that exceeds the capability of local stem cells to recuperate the damage may sustain the progress of the disease. However, these conundrums could be addressed, if there is a way to extract stem cells from the damaged cartilage and propagate than in vitro, so the unknown or limiting microenvironment influences can be overcome.

In conclusion, the study has indicated that the adherent cells generated from cartilage are mesenchymal stem cells. These cells exhibited similar mesenchymal stem cell's morphology, surface markers and differentiated into mesodermal lineages. Additionally, the in vitro cultures generated hC-MSCs also exerted a potent immunosuppression against PHA-L activated T cells in a dose-dependent manner. The noted immunosuppression required an initial cell-to-cell contact and was executed through an arrest in the G₀/G₁ phase of the cell cycle of T-cells. Thus, the regenerative potential and immunosuppressive activity of hC-MSCs could be potentially exploited to treat cartilage associated immune disorders.

Acknowledgements This project supported by Research University Grant Scheme (RUGS) 04-02-12-1757RU and 04/02/10/0920RU by Universiti Putra internal grant scheme. The postgraduate student, Pratheep Sandrasaigaran partially supported by special research graduate scheme by Universiti Putra Malaysia.

References

- Alsalamah S, Amin R, Gemba T, Lotz M (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis Rheumatol* 50:1522–1532. <https://doi.org/10.1002/art.20269>
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113:643–655
- Cho KJ, Trzaska KA, Greco SJ, McArdle J, Wang FS, Ye JH, Rameshwar P (2005) Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1 alpha. *Stem Cells* 23:383–391. <https://doi.org/10.1634/stemcells.2004-0251>
- Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Uccelli A (2006) Human mesenchymal stem cells modulate B-cell functions. *Blood* 107:367–372. <https://doi.org/10.1182/blood-2005-07-2657>
- Csaki C, Schneider PR, Shakibaei M (2008) Mesenchymal stem cells as a potential pool for cartilage tissue engineering.

- Ann Anat 190:395–412. <https://doi.org/10.1016/j.aanat.2008.07.007>
- Dazzi F, Ramasamy R, Glennie S, Jones SP, Roberts I (2006) The role of mesenchymal stem cells in haemopoiesis. *Blood Rev* 20:161–171. <https://doi.org/10.1016/j.blr.2005.11.002>
- De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP (2001) Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheumatol* 44:1928–1942
- Detterline AJ, Goldberg S, Bach BR Jr, Cole BJ (2005) Treatment options for articular cartilage defects of the knee. *Orthop Nurs* 24:361–366 (**quiz 367–368**)
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8:315–317
- Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortessidis A, Simmons PJ (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 116:1827–1835
- Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, Melton DA (2008a) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol* 26:795–797. <https://doi.org/10.1038/nbt1418>
- Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Melton DA (2008b) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotechnol* 26:1269–1275. <https://doi.org/10.1038/nbt.1502>
- Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* 32:414–425. <https://doi.org/10.1016/j.exphem.2004.02.004>
- Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, McGonagle D (2002) Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheumatol* 46:3349–3360. <https://doi.org/10.1002/art.10696>
- Jones EA, Crawford A, English A, Henshaw K, Mundy J, Corscadden D, McGonagle D (2008) Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. *Arthritis Rheumatol* 58:1731–1740
- Kolf CM, Cho E, Tuan RS (2007) Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthr Res Ther* 9:204. <https://doi.org/10.1186/ar2116>
- Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Tada T (2005) Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol Cell Biol* 25:2475–2485. <https://doi.org/10.1128/MCB.25.6.2475-2485.2005>
- Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 363:1439–1441. [https://doi.org/10.1016/S0140-6736\(04\)16104-7](https://doi.org/10.1016/S0140-6736(04)16104-7)
- Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, Ringden O (2007) Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia* 21:1733–1738. <https://doi.org/10.1038/sj.leu.2404777>
- Lee SY, Nakagawa T, Reddi AH (2009) Mesenchymal progenitor cells derived from synovium and infrapatellar fat pad as a source for superficial zone cartilage tissue engineering: analysis of superficial zone protein/lubricin expression. *Tissue Eng Part A* 16:317–325
- Otto W, Rao J (2004) Tomorrow's skeleton staff: mesenchymal stem cells and the repair of bone and cartilage. *Cell Prolif* 37:97–110
- Palermo AT, LaBarge MA, Doyonnas R, Pomerantz J, Blau HM (2005) Bone marrow contribution to skeletal muscle: a physiological response to stress. *Dev Biol* 279:336–344
- Peng L, Jia Z, Yin X, Zhang X, Liu Y, Chen P, Zhou C (2008) Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. *Stem cells Dev* 17:761–774. <https://doi.org/10.1089/scd.2007.0217>
- Pesce M, Scholer HR (2001) Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells* 19:271–278. <https://doi.org/10.1634/stemcells.19-4-271>
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147
- Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliers GL (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 30:783–791
- Ramasamy R, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F (2007a) Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation* 83:71–76. <https://doi.org/10.1097/01.tp.0000244572.24780.54>
- Ramasamy R, Lam EW, Soeiro I, Tisato V, Bonnet D, Dazzi F (2007b) Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth. *Leukemia* 21:304–310. <https://doi.org/10.1038/sj.leu.2404489>
- Ramasamy R, Tong CK, Seow HF, Vidyadaran S, Dazzi F (2008) The immunosuppressive effects of human bone marrow-derived mesenchymal stem cells target T cell proliferation but not its effector function. *Cell Immunol* 251:131–136. <https://doi.org/10.1016/j.cellimm.2008.04.009>
- Rasmusson I, Uhlin M, Le Blanc K, Levitsky V (2007) Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. *J Leukoc Biol* 82:887–893. <https://doi.org/10.1189/jlb.0307140>
- Rogers MB, Hosler BA, Gudas LJ (1991) Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development* 113:815–824
- Sakaguchi Y, Sekiya I, Yagishita K, Muneta T (2005) Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheumatol* 52:2521–2529
- Su W, Zhang H, Jia Z, Zhou C, Wei Y, Hu S (2006) Cartilage-derived stromal cells: is it a novel cell resource for cell therapy to regenerate infarcted myocardium? *Stem Cells* 24:349–356

- Tabera S, Perez-Simon JA, Diez-Campelo M, Sanchez-Abarca LI, Blanco B, Lopez A, San Miguel JF (2008) The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. *Haematologica* 93:1301–1309. <https://doi.org/10.3324/haematol.12857>
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676. <https://doi.org/10.1016/j.cell.2006.07.024>
- Tokcaer-Keskin Z, Akar AR, Ayaloglu-Butun F, Terzioglu-Kara E, Durdu S, Ozyurda U, Akcali KC (2009) Timing of induction of cardiomyocyte differentiation for in vitro cultured mesenchymal stem cells: a perspective for emergencies. *Can J Physiol Pharmacol* 87:143–150. <https://doi.org/10.1139/Y08-111>
- Tyndall A, Walker UA, Cope A, Dazzi F, De Bari C, Fibbe W, Feldmann M (2007) Immunomodulatory properties of mesenchymal stem cells: a review based on an interdisciplinary meeting held at the Kennedy Institute of Rheumatology Division, London, UK, 31 October 2005. *Arthr Res Ther* 9:301. <https://doi.org/10.1186/ar2103>
- Vellasamy S, Tong CK, Azhar NA, Kodiappan R, Chan SC, Veerakumarasivam A, Ramasamy R (2016) Human mesenchymal stromal cells modulate T-cell immune response via transcriptomic regulation. *Cytotherapy* 18:1270–1283
- Wu R, Gu B, Zhao X, Tan Z, Chen L, Zhu J, Zhang M (2013) Derivation of multipotent nestin(+)/CD271 (-)/STRO-1 (-) mesenchymal-like precursors from human embryonic stem cells in chemically defined conditions. *Hum Cell* 26:19–27. <https://doi.org/10.1007/s13577-011-0022-3>
- Yamanaka S (2008) Induction of pluripotent stem cells from mouse fibroblasts by four transcription factors. *Cell Prolif* 41:51–56. <https://doi.org/10.1111/j.1365-2184.2008.00493.x>
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917–1920. <https://doi.org/10.1126/science.1151526>