
**UNIVERSITI PUTRA MALAYSIA**

# *GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELLS DERIVED FROM HUMAN CARTILAGE*

**PRATHEEP A/L SANDRASAIGARAN**

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# GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELLS DERIVED FROM HUMAN CARTILAGE

**By**

**PRATHEEP A/L SANDRASAIGARAN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

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## DEDICATION

A Special gratitude to my father who would I like to sincerely dedicate my entire work and success of this thesis.

The glory and the splendours of a man are not solely lies on his ability; it is the gene and the karma that he inherits, so do i. My father is my first guru and his gene and good karma that enable me to accomplished my work. The success of this thesis is not the most ultimate, there are many more to come.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**GENERATION AND CHARACTERISATION OF MESENCHYMAL STEM CELLS DERIVED FROM HUMAN CARTILAGE TISSUE**

By

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**July 2014**

**Chair: Associate Professor Rajesh Ramasamy, PhD**

**Faculty: Medicine and Health Sciences**

Mesenchymal stem cells (MSCs) were initially discovered as stromal cells that possess unique characteristics as compared to other counterparts of multipotent stem cells. Besides the capability of self-renewal and differentiating into a variety of mature cells, MSCs also exert potent immuno-regulatory activities on various immune cells. This exclusive characteristic has enabled MSCs to be recognised as an ideal cell based treatment in the field of regenerative medicine, gene therapy and immunotherapy. As regeneration of cartilage tissue *in situ* is hampered by limited intrinsic growth, this study explores the feasibility of generating human MSCs from sports injured patients' cartilages and investigates the possibility to differentiate them into cartilage tissues. For this, MSCs were generated from tissues that was harvested from a non-weight bearing region of cartilage during an arthroscopy procedure and characterised based on morphology, immunophenotype and immunomodulatory properties. Furthermore, the MSCs generated from their original physiognomy (cartilage) are believed to support the cartilage regeneration much greater. The cartilage tissues in laboratory were subject to enzymatic digestions and cultured in plastic culture ware. A series of experiments were designed using the cells from passage three onwards. Initially, the cells were cultured at 200 cells/cm$^2$ and harvested at day 10 and 12 respectively to determine the cells' growth kinetics and population doubling time. Cells generated from these tissues showed spindle-shaped fibroblast morphology with a population doubling time of approximately 27 hours. Next, the cells were then stained with respective antibodies with fluorescent conjugated markers and analysed in flow cytometer. When the right cells' populations were gated, a common surface markers that are related to mesenchymal

origin however not haematopoietic were observed (CD29+, CD73+, CD90+, CD105+, HLA-ABC+, CD271-, CD14 , CD19-, CD45-, CD86-, CD80-, CD34- and HLA-DR-). Besides that, these cells were also subjected to the cell differentiation analysis. The cells were allowed to confluent before cultured with the respective differentiation media according to the manufacturer's instructions. Cells' cyto-staining assay and PCR analysis on isolated total RNA showed the cells are capable of differentiating into mesodermal lineages (chondrocytes, adipocytes and osteocytes). In term of stemness, human cartilage derived cells expressed the early embryonic markers of SOX2, REX1, OCT4, NANOG; hence indicating their inherent pluripotency. Such results has confirmed cartilage tissues hold the aptitude to generate mesenchymal stem cells and these cells were termed as human cartilage derived mesenchymal stem cells (hC-MSCs). Further experiments reveal that the hC-MSCs are able to suppress proliferation of activated T-lymphocytes, demonstrating that their immunomodulatory effects are analogous to bone marrow derived MSCs. In the presence of hC-MSCs, the proliferation of the T cells was severely inhibited in dose dependent manner but their activation profile was well preserved. They further affirm the requirement for the cell-to-cell contact during their immuno-inhibitory activity. These outcomes were confirmed in the hC-MSCs: T cells co-culture assay and further analysis of CD25 expressions by activated T cells shows no variations when they were cultured either with or without the presence of hC-MSCs. Furthermore when the activated T cells were co-cultured with hC-MSCs, the immune cells were arrested in $G_0/G_1$ phase of the cell cycles and their commitments into S phase were not permissible. Based on the acquired laboratory data, it has been shown that human cartilage sample could serve as a good source to generate mesenchymal stem cells and the functional properties of human cartilage mesenchymal stem cells in term of differentiating into mature chondrocytes plus ability to prevent the expansion of activated T cells has endeavoured as a new paradigm to treat destructive autoimmune diseases of joints such as rheumatoid arthritis. Moreover, this study has further strengthened the fundamental findings on human cartilage mesenchymal stem cells biology, thus adding value to the existing clinical therapy.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

**PENJANAAN DAN PENCIRIAN SEL INDUK MESENKIMA DARI TISU RAWAN MANUSIA (hC-MSCs)**

Oleh

**PRATHEEP A/L SANDRASAIGARAN**

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Sel induk mesenkima (MSCs) pada asalnya ditemui dalam stroma tulang, memiliki ciri-ciri yang unik berbanding dengan sel induk multipoten yang selaras dengannya. Selain daripada keupayaan pembaharuan dan membezakan dirinya kepada pelbagai sel matang, MSCs juga melaksanakan aktiviti imunomodulasi ke atas pelbagai sel imun. Ciri-ciri eksklusif ini membolehkan MSCs diiktiraf sebagai rawatan ulung berdasarkan sel dalam bidang perubatan regeneratif, terapi gen dan imunoterapi. Disebabkan pertumbuhan semula tisu rawan *in situ* terjejas oleh pertumbuhan intrinsik yang terhad, kajian ini menerokai kebolehlaksanaan menjana MSCs manusia dari pesakit yang mengalami kecederaan rawan semasa bersukan dan menyiasat kemungkinan untuk membezakan mereka kepada tisu rawan. Untuk ini, MSCs telah dijana daripada tisu rawan yang diterima dari kawasan kurang tekanan mekanikal pada tulang rawan semasa prosedur artroskopi dan dicirikan berdasarkan morfologi, ciri-ciri imun dan imunmodulasi. Tambahan lagi, MSCs dijana daripada fisiognomi asalnya (rawan) dipercayai dapat menyokong pertumbuhan semula tulang rawan dengan lebih baik. Tisu tulang rawan diproses di makmal dengan rawatan enzim dan dikultur dalam kelalang plastik. Satu siri eksperimen telah direka dengan menggunakan sel-sel daripada 'passage' tiga dan seterusnya. Pada mulanya, sel-sel ini telah dikultur pada 200 cells/cm$^2$ dan dikaji masing-masing pada hari yang ke-10 dan ke-12 untuk menentukan kinetik pertumbuhan sel dan masa untuk penggandaan populasinya. Sel dihasilkan daripada tisu ini menunjukkan morfologi fibroblas dan mempunyai masa gandaan-dua lebih kurang 27 jam. Seterusnya, sel ini kemudiannya dikultur dengan antibodi yang ditanda dengan pendarfluor dan dianalisis dengan menggunakan flow cytometer. Apabila populasi sel-sel yang betul

telah dipagar, penanda permukaan yang kebiasannya berkaitan dengan sel induk mesenkima diperhatikan, walau bagaimanapun tidak untuk haematopoietik ($CD29^+$, $CD73^+$, $CD90^+$, $CD105^+$, HLA-$ABC^+$, $CD271^-$, $CD14^-$, $CD19^-$, $CD45^-$, $CD86^-$, $CD80^-$, $CD34^-$ and HLA-$DR^-$). Selain itu, sel-sel ini juga tertakluk kepada analisis pembezaan sel. Sel-sel ini dibiarkan memenuhi ruang kelalang kultur sebelum ia dikultur dengan media pembezaan mengikut arahan pengeluar. Pencerakinan sel dan analisis PCR pada RNA menunjukkan sel-sel ini mampu membezakan diri kepada keturunan mesodermal (kondrosit, adiposit dan osteosit). Dalam kandungan sel induk awal, sel induk mesenkima yang diperolehi dari tisu rawan manusia menunjukkan penanda awal embrio SOX2, REX1, OCT4, NANOG; lantas menunjukkan kebolehan pluripotensi yang wujud. Keputusan ini mengesahkan tisu rawan mempunyai kebolehan untuk menjana sel induk mesenkima dan sel-sel ini telah diklasifikasi sebagai sel induk mesenkima diperolehi dari rawan manusia (hC-MSC). Kajian lebih mendalam mendedahkan bahawa hC-MSC dapat mengawal pertumbuhan T-limfosit yang aktif, sekaligus mendedahkan bahawa kesan immunomodulasi mereka seakan-akan sama seperti MSC diperolehi dari sum-sum tulang. Dalam kehadiran hC-MSCs, pertumbuhan sel T terbantut dan ini bergantung kepada nisbah sel induk mesenkima, namun profil pengaktifan sel T tetap dipelihara. Ini mengesahkan lagi keberkesanan hC-MSCs saling memerlukan perhubungan antara satu sama lain semasa aktiviti imunomodulasinya. Keputusan ini telah disahkan dalam kajian hC-MSC: sel T kultur bersama dan analisis selanjutnya dalam ekspresi CD25 oleh sel-sel T aktif tidak menunjukkan sebarang variasi apabila mereka dikultur sama ada dengan atau tanpa kehadiran hC-MSC. Tambahan pula apabila sel-sel T yang aktif dikultrur bersama dengan hC-MSCs, sel-sel imun ini diperangkap dalam fasa $G_0/G_1$ ketika pada kitaran sel dan komitmen mereka ke fasa S tidak dibenarkan. Berdasarkan data makmal yang diperolehi, ia telah terbukti bahawa sampel tisu rawan manusia boleh menjadi sumber yang baik untuk menjana sel induk mesenkima dan sifat-sifat sel induk mesenkima dari tisu rawan manusia boleh membezakan diri kepada kondrosit matang dan ditambah dengan keupayaan untuk mencegah perkembangan sel T aktif, telah menbina satu paradigma baru untuk merawat penyakit autoimun seperti ‘reumatoid artritis’. Selain itu, kajian ini telah mengukuhkan lagi pemahaman biologi asas terhadap sel induk mesenkima dari tisu rawan manusia, justeru menambah nilai kepada terapi klinikal yang tersedia ada.

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I certify that a Thesis Examination Committee has met on 4th JULY 2014 to conduct the final examination of Pratheep a/l Sandrasaigaran on his thesis entitled "Generation and Characterisation of Mesenchymal Stem Cells Derived from Human Cartilage" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The member of the Supervisory Committee were as follows:

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## DECLARATION

**Declaration by graduate student**

I hereby confirm that:

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| | |
|---|---|
| µ | Micro |
| $^{3}$H-TdR | Tritium incorporated thymidine |
| a2Col6 | α chain 2 of type 6 collagen |
| ACI | Autologous chondrocyte implantation |
| Ag | Aggrecan |
| AGM | Aorto-gonado-mesonephros |
| ALP | Alkaline phosphatase |
| AML | Acute myeloid leukaemia |
| APC | Antigen presenting cells |
| APC | Allophyceoerythrin |
| B7-1 | CD80 |
| B7-2 | CD86 |
| bFGF | Basic Fibroblast Growth Factor |
| BM | Bone marrow |
| BMP-2 | Bone morphogenetic protein-2 |
| bp | Base pair |
| BSA | Bovine serum albumin |
| BSP | Bone sialoprotein |
| c/EBP | CCAAT-enhancer-binding protein |
| CD | Cluster of differentiation |
| cDNA | Complementary deoxyribonucleic acid |
| CII | Type II collagen |
| $CO_2$ | Carbon dioyxide |

| | |
|---|---|
| Col | Collagen type II |
| col1a1 | Collagen type I alpha-1 |
| cpm | Count per Minute |
| CSCs | Cancer stem cells |
| CTL | Cytotoxic T cell |
| DC | Dendritic cells |
| DEPC | Diethylpyrocarbonate |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DN | Double negative |
| DNA | Deoxyribonucleic acid |
| DNAse I | Deoxyribonuclease I |
| DP | Double positive |
| ECM | Extra cellular matrix |
| EGF | Epidermal growth factor |
| ESC | Embryonic stem cells |
| F-CFU | Fibroblast-colony-forming unit |
| FACS | Fluorescence-activated cell sorting |
| FBS | Foetal bovine serum |
| FGF | Fibroblast growth factors |
| FITC | Fliorescein isothiocynate |
| Flt-3 | Fms-like tyrosine kinase 3 |
| FoxP3 | Forkhead box P3 |
| g | Gram |
| $G_0$ | Quiescence phase |

| | |
|---|---|
| $G_1$ | Gap phase |
| GvHD | Graft versus Host Disease |
| GVT | Graft-versus-tumour |
| HAM F-12 | Nutrient mixture F-12 Ham's |
| hC | Human cartilage |
| hC-MSCs | Human cartilage derived mesenchymal stem cells |
| HCT | Hematopoietic cell transplantation |
| HGF | Hepatocyte growth factor |
| HLA | Human leukocyte antigen |
| HLA-G | Human leukocyte antigen-G |
| HO-1 | Heme oxygenase 1 |
| HSC | Hematopoietic stem cells |
| HSCT | Hematopoietic stem-cell transplantation |
| IBMX | 3-isobutyl-1-methylxanthine |
| ICM | Inner cell mass |
| IDO | indoleamine 2, 3-dioxygenase |
| IFN-γ | Interferon-γ |
| Ig | Immunoglobulin |
| IGF | Insulin-like growth factors |
| IL | Interleukins |
| iNOS | Inducible nitric-oxide synthase |
| iPSC | Induced pluripotent stem cells |
| ISCT | International Society for Cellular Therapy |
| JOCD | Juvenile osteochondritis dissecans |
| l | Litre |

| | |
|---|---|
| LIF | Leukemia Inhibitory Factor |
| LNGFR | Affinity nerve growth factor receptor |
| LPL | Lipoprotein lipase |
| M | Mitotic phase |
| MAb | Monoclonal antibody |
| MHC | Major Histocompatibility Complex |
| MLR | Mixed leukocyte reaction |
| mRNA | Messenger ribonucleic acid |
| MSCs | Mesenchymal stem cells |
| NCBI | National Center for Biotechnology Information |
| NIH | National Health Institutes |
| NK | Natural killer cell |
| OA | Osteoarthritis |
| OC | Osteocalcin |
| OCD | Osteochondritis dissecans |
| OPN | Osteopontin |
| OSM | Oncostatin M |
| PALS | Periarterial lymphatic sheath |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffer solution |
| PCR | Polymerase chain reaction |
| PD | Programmed death |
| PDT | Population doubling time |
| PDGF | Platelet-derived growth factor |
| PE | Phycoerythrin |

| | |
|---|---|
| PE-CY5 | Phycoerythrin Cy5 |
| PerCP | Peridinin chlophyll protein |
| PGE2 | Prostaglandin E2 |
| PHA-L | Phytohaemagglutinin-Leukocytes. |
| PI | Propidium Iodide |
| PPARγ2 | Peroxisome proliferation-activated receptor γ2 |
| PTH | Parathyroid Hormone |
| RA | Rheumatoid arthritis |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| Scf | Stem cell factor |
| SD | Standard deviation |
| SCID | Severe combined immunodeficient |
| SDF-1 | Stromal cell derived factor 1 |
| SLE | Systemic lupus erythematosis |
| SN | Supernatant solution |
| SP | Single positive |
| stTRAIL | Secretable trimeric form of tumour necrosis factor-relate apoptosis-inducing ligand |
| TCR | T cell receptor |
| TECs | Thymic epithelial cells |
| TGF-β | Transforming growth factor-beta |
| TGF-β1 | Transforming growth factor-beta 1 |
| Th 1 | T helper 1 |
| Th-2 | T helper 2 |
| TNF-α | Tumour necrosis factor α |

| | |
|---|---|
| Tregs | T regulatory cells |
| VEGF | Vascular endothelial growth factor |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| δ | Delta |

# CHAPTER 1

## INTRODUCTION

Mesenchymal Stem Cells (MSCs) are a rare population of cells that was discovered in the late 1960s by a Russian scientist, Friedenstein. These are stem cells that constitute a non-haematopoietic population in the adult bone marrow (BM) which have the capability to regulate immune cell responses (Friedenstein, Chailakhjan *et al.* 1970; Gotherstrom 2007). The cells are classified as multipotent progenitors due to their ability for self-renew and differentiate into tissues of mesodermal origin such as adipocytes, osteoblasts, tendon and chondrocytes (Pittenger, Mackay *et al*. 1999). On-going animal studies and one-off clinical observations in recent days has emerged to support the therapeutic ability of MSCs to enhance the engraftment of haematopoietic stem cells, reduce graft versus host disease (GvHD) at post bone marrow transplantation and suppression of immune responses (Le Blanc, Rasmusson *et al*. 2004; Le Blanc, Samuelsson *et al*. 2007; Ramasamy, Tong *et al*. 2008; Tong, Seow *et al*. 2008). Thus, owing to such unique characteristics, MSCs has been established as a novel cell-based therapy in treating many bone and cartilage degenerative diseases.

Cartilages are structurally a soft bone with the main constitution of chondrocytes; a building block that supports the mechanical pressure exerted in our body. The intrinsic nature of the cartilage is lack of nutrients and oxygen supply, hence, any damages or deformities sustained by cartilage is hard to heal and requires complicated surgical methods (Detterline, Goldberg *et al*. 2005). Moreover, the conventional treatments such as intra articular injections, visco-supplementation and physiotherapy are only able to reduce the symptoms but none of them promotes cartilage repair (Detterline, Goldberg et al. 2005). This limited capacity of cartilage regeneration at synovium has necessitated an exploration for alternate therapies to overcome a non-conducive microenvironment that failed to trigger repopulation of tissue resident stem cells or recruitment of bone marrow derived stem cells. In line with this, MSCs have emerged as a novel cell-based therapy in treating cartilage degenerative diseases.

In recent years, human cartilage has been shown to contain multipotent stem cells, potentially MSCs, which preserving the ability to differentiate into mesodermal lineages (Alsalameh, Amin *et al*. 2004; Csaki, Schneider *et al*. 2008). Although, MSCs which were generated from other anatomical parts of human body are able to

differentiate into mature chondrocytes, yet it has been widely believed that, the chondrogenic differentiation ability of cartilage origin MSCs is much greater (Dazzi, Ramasamy *et al*. 2006; Peng, Jia *et al*. 2008). Isolation and generation of these multipotent stem cells from human cartilage therefore would be able to serve as an ideal source to repair the cartilage damages. However any attempt to repair cartilage deformities must also involve the consideration for autologous or allogeneic purpose which is highly depending on their regenerative and therapeutics comprehensiveness. Therefore in this study, MSCs was generated from cartilage of sports injury patients and the physiology of the stem cells was analysed in relation to the guidelines provided by the International Society for Cellular Therapy (ISCT); MSCs are plastic adherent, able to differentiate in mesodermal lineage and expresses a panel of MSCs surface molecular markers.

Mesenchymal stem cells are lesser immunogenic, escaping the host immune recognition and also suppress the immune responses, hence made MSCs an excellent therapeutic candidate for treating immune mediated diseases in allogeneic settings (Parekkadan and Milwid 2010). Therefore it would be ideal to utilise cartilage derived MSCs to treat knee related degenerative diseases such as in rheumatoid arthritis and osteoarthritis where MSCs could contribute for restoration of damaged/corroded cartilage tissue while preventing the immune responses exerted by auto reactive immune cells towards cartilage. Hence, the second part of this study, has been focused on the immunomodulatory property of human cartilage derived MSCs, as the immunomodulation is a part of MSCs characterisation. Besides that, the mechanism used by the stem cells to induce immunosuppression on mitogen activated T cells and how does the stem cells evade the immune system were briefly covered in this section.

The hypotheses of this study are:

**1.1** Mesenchymal stem cells can be generated from human cartilage tissues.

**2.1** The generated human cartilage derived mesenchymal stem cells (hC-MSCs) exhibit similar characteristics as to standard bone marrow (BM) derived MSCs.

**3.1** Human cartilage derived MSCs may induce anti-proliferative response in mitogen stimulated T cells.

This study is a preliminary step in establishing the cartilage derived MSCs as a most potential candidate for both regenerative and therapeutics options in cartilage related defectives. Prior to such advance experiments, fundamental studies has to be carried out in order to understand the regenerative and immunological nature of the cells and hence the works presented in this study is believed to serve as a basic data to accommodate future clinical setup in cartilage defect repairs.

The objectives of this project are:

General Objective:

1 To generate and characterise mesenchymal stem cells derived from human cartilage (hC-MSCs).

Specific objective:

1 To isolate mesenchymal stem cells from human cartilage.

2 To characterise the phenotypes and differentiation potential of cartilage derived stem cells for mesenchymal stem cells as per standard guidelines.

3 To investigate the immunomodulatory property of human cartilage derived MSCs on T-lymphocytes.

4 To investigate the mechanism of hC-MSCs to induce immunomodulatory activity on T-lymphocytes.

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