UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL CELLS FROM HUMAN BONE MARROW AND THEIR DEVELOPMENT INTO BONE CELLS

WAN NAZATUL SHIMA BT SHAHIDAN

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By

WAN NAZATUL SHIMA

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

April 2007

Dedication

Special thanks are dedicated to my loving parents, Shahidan bin Mohd and Sharipah Norida bt Syed Nordin for their moral support and prayers. Not forgetting to my dearest husband, Ahmad Amharie bin Zainal Bakri and our daughter Nur Alya Humaira for their understanding, support and enriching love during my endeavour. I love you all.

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

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Chairman: Professor Abd Manaf Ali, PhD

Faculty: Biotechnology and Biomolecular Sciences

Everyday thousands of people suffer from bone diseases that lead to destruction of bone tissue. Various types of bone allograft, xenograft and synthetic biomaterial are now used for bone replacement therapy. The future of bone reconstruction lies in the use of stem cell technology for bone development. Within the bone marrow stroma there are exists a subset of nonhematopoietic cells referred to as mesenchymal stem cells (MSCs) or mesenchymal progenitor cells (MPCs). These cells can be expanded *ex vivo* and induced, either *in vitro* or *in vivo*, to terminally differentiate into osteoblasts, chondrocytes, adypocytes, tenocytes, myotubes, neural cells, and hematopoietic supporting stroma. The multipotential of these cells, their easy collection and culture, as well as their high *ex vivo* expansive potential makes these cells an attractive therapeutic tool. The purpose of this study was to isolate, expand and characterize mesenchymal stem cell from human bone marrow in Mesenchymal Stem Cell Growth Medium (MSCGM), to compare the cell growth in the MSCGM and Dulbecco Modified Eagle's Medium (DMEM) with 10%

Fetal Bovine Serum (FBS) medium, to differentiate MSCs into osteoblast cells and to determine the osteogenic potential of the differentiated MSC by detecting the expression of osteoblast-specific genes such as collagen type 1, osteocalcin, runx 2, osteopontin and alkaline phosphatase. In this study, MSCs were isolated from human bone marrow and cultured in MSCGM medium and DMEM-10% FBS medium. Culture-expansion of MSCs was characterized by the presence of CD 105 marker using Labelled Streptavidin Biotin (LSAB) method. The MSCs were cultured in the MSCGM and DMEM-10% FBS medium within five days. The MSCs were then cultured in osteogenic medium containing DMEM medium supplemented with Fetal Bovine Serum (FBS), antibiotics, ascorbic acid, β-glycerol phosphate and dexamethasone to differentiate into osteoblasts. Their osteogenic differentiation was determined with the formation of a mineralized extracellular matrix visualized by Von Kossa staining and Alkaline Phosphatase (ALP) assay. Moreover, osteogenic differentiation was also judge by RT-PCR profiling of osteoblast gene expression. MSC first attached to the dish surface and exhibited fibroblast-like spindle shape. The cells were also identified as MSCs based on their immunophenotype of CD 105 which resulted in funchia coloured staining. The MSCs culture in MSCGM medium expanded and proliferated rapidly compared to MSCs culture in DMEM-10% FBS medium. Incubation of bone marrow-derived MSCs in the osteogenic medium for 3 weeks resulted in a dramatic increase in ALP activity and accumulation of calcium deposit, as assessed by ALP assay and Von Kossa staining, respectively. This osteogenic potential upon culture in osteogenic medium was further confirmed by the RT-PCR analysis where the expressions of osteoblast specific genes were confirmed by molecular weight produced on agarose gel. We conclude that

MSCGM is the best choice for expanding and proliferating MSCs and suggest that MSC from bone marrow have pure osteogenic potential and have the capability to differentiate into osteoblast. This potential assures that bone marrow can be a legitimate source of MSCs for production of osteoblast which can be utilized in bone replacement therapy.

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

PEMENCILAN DAN PENCIRIAN SEL MESENKIMA DARIPADA TULANG SUM-SUM MANUSIA DAN PERKEMBANGANNYA KEPADA SEL-SEL TULANG.

Oleh

WAN NAZATUL SHIMA BT SHAHIDAN

April 2007

Pengerusi: Profesor Abd Manaf Ali, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Setiap hari hampir beribu manusia mati menderita penyakit tulang disebabkan oleh kerosakan tisu tulang. Pelbagai jenis allograf, xenograf tulang dan tulang sintetik telah digunakan untuk terapi penggantian tulang. Pembinaan semula tulang bergantung kepada teknologi sel stem. Di dalam tulang sum-sum terdapat subset sel bukan hematopoitik yang dirujuk sebagai sel mesenkima atau generasi sel mesenkima. Sel-sel ini boleh dikembangbiakkan atau diaruhkan samada *in vitro* atau *in vivo* dan akhirnya bertukar kepada osteoblas, chondrosit, adiposit, tenosit, myotiub, sel neuro dan tisu penyokong hematopoitik. Oleh kerana sel ini mempunyai pelbagai potensi, mudah dikumpulkan dan dikultur, juga mempunyai daya kembang biak *ex vivo* yang tinggi, menjadikan sel ini sebagai satu alat terapeutik yang menarik. Tujuan kajian ini adalah untuk memencilkan, mengembang biakkan dan mencirikan sel mesenkima daripada tulang sum-sum di dalam media MSCGM, membandingkan perkembangan sel di dalam media MSCGM dan media

DMEM-10% FBS, menukarkan sel mesenkima kepada sel osteoblas dan menentukan kebolehan osteogenik sel mesenkima bertukar kepada sel osteoblas melalui ekspresi lima gen khusus osteoblas; collagen type 1, osteocalcin, runx 2, osteopontin dan alkaline phosphatase. Di dalam kajian ini sel stem mesenkima telah dipencilkan dan dikultur di dalam media MSCGM dan media DMEM-10% FBS. MSC kultur yang berkembang biak telah diuji sifat dan ciri-cirinya melalui kehadiran penanda permukaan sel CD 105 dengan kaedah Labelled Streptavidin Biotin (LSAB). Sel mesenkima telah dikultur di dalam media MSCGM dan juga media DMEM-10% FBS dalam lingkungan lima hari. Sel mesenkima kemudiannya dikulturkan di dalam media DMEM dengan tambahan Fetal Bovine Serum (FBS), antibiotic, askorbik asid, β-glycerol Phosphate dan dexamethasone untuk membezakan kepada sel osteoblas. Pembezaan osteogenik yang optimum telah ditentukan daripada pembentukan matrik mineral ekstraselular yang dilihat secara visual melalui tompokan Von Kossa dan pegujian Alkaline Phosphatase (ALP). Tambahan lagi pembezaan osteogenik telah diadili melalui ekspresi osteoblas gen profil RT-PCR. Sel mesenkima pada mulanya melekat pada permukaan bekas kultur dan menunjukkan bentuk seperti gelungan fibroblast. Sel ini juga telah dikenal pasti sebagai MSC berdasarkan pada immunofenotip (CD105), menyebabkan pewarnaan warna funchia. Sel mesenkima yang dikultur di dalam media MSCGM tumbuh dah berkembang biak dengan cepat berbanding dengan sel mesenkima yang dikultur di dalam media DMEM-10% FBS. Pengeraman MSC daripada sel tulang sum-sum di dalam media osteogenik selama tiga minggu menghasilkan peningkatan dalam aktiviti ALP dan pengumpulan mendapan kalsium melalui penilaian pengujian ALP dan tompokan Von Kossa. Potensi osteogenik telah disahkan lagi menggunakan analisis RT-PCR di mana ekspresi gen khusus telah

menunjukkan jaluran DNA di atas gel agarose. Kami merumuskan bahawa media MSCGM adalah pilihan terbaik untuk pertumbuhan dan kembang biak sel mesenkima dan mencadangkan bahawa sel mesenkima daripada tulang sum-sum mempunyai potensi osteogenik yang tulen dan berkeupayaan untuk bertukar kepada sel osteoblas. Potensi mesengenic ini meyakinkan bahawa sel stem daripada tulang sum-sum adalah multipotensi MSCs dan menunjukkan bahawa tulang sum-sum merupakan sumber MSC yang sah untuk menghasilkan osteoblas yang dapat digunakan dalam terapi penggantian tulang.

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I certify that an Examination Committee has met on 2nd April to conduct the final examination of Wan Nazatul Shima bt Shahidan on her Master of Science thesis entitled "Isolation and Characterization of Mesenchymal Stem Cell from Bone Marrow and Their Development Into Bone Cells." in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Dr. Nor Aripin Shamaan
Professor
Biochemistry Department
Fakulty of Bioteknology dan Biomolecule Sciences
Universiti Putra Malaysia
(Chairman)

Dr. Rozita Rosli
Associate Professor
Fakulti Perubatan dan Sains Kesihatan
Universiti Putra Malaysia
(Internal Examiner)

Dr. Md. Zuki Abu Bakar
Associate Professor
Praclinical Veterinary Science Department
Fakultiy of Medical Veterinar
Universiti Putra Malaysia
(Internal Examiner)

Dr. Ruszymah Bt. Hj. Idrus
Professor
Fisiology Department
Universiti Kebangsaan Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 JUNE 2007

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Abd. Manaf bin Ali, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Noorjahan Banu Alitheen, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Muhajir Hamid, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Abd. Rani Samsudin, PhD
Lecturer
Pusat Pengajian Sains Pergigian
Universiti Sains Malaysia.
(Member)

_____
AINI IDERIS, PhD
Professor/ Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 17 JULY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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WAN NAZATUL SHIMA BT SHAHIDAN

Date: 29 MAY 2007

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BMC	Bone marrow derived cell
BM-MSC	Bone Marrow Mesenchymal Stem Cell
CPD	Citrate Phosphate Buffer
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleotide triphosphate
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
GPDH	Glycerol-3-Phosphate-Dehydrogenase
HLA	Human Leucocyte Antigen
HSCs	Haematopoietic Stem Cells
IFNγ	Interferon Gamma
LSAB	Labelled Streptavidin Biotin
MDA	Muscular Dystrophy Association
MNC	Mononuclear Cell
MSCGM	Mesenchymal Stem Cell Growth Medium
MSCs	Mesenchymal Stem Cells
PBS	Phosphate Buffered Saline
PBSCs	Peripheral Blood Stem Cells
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid

RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Runx 2	Runt-related transcription factor-2
UCB	Umbilical Cord Blood
β-ME	β-mercaptoethanol

CHAPTER 1

INTRODUCTION

A stem cell is a cell that has the ability to divide (self-replicate) for indefinite periods often throughout the life of the organism. Under the right conditions, or given the right signals, stem cells can give rise (differentiate) to the many different cell types that make up the organism. Stem cells have the potential to develop into mature cells that have characteristic shapes and specialized functions, such as bone cells, heart cells, skin cells or nerve cells (Chandross *et al*., 2001).

Many of the terms used to define stem cells depend on the behavior of the cells in the intact organism (*in vivo),* under specific laboratory conditions (*in vitro),* or after transplantation *in vivo*, often to a tissue that is different from the one from which the stem cells were derived. For example, the fertilized egg is said to be totipotent from the latin word *totus,* meaning entire because it has the potential to generate all the cells and tissues that make up an embryo and that support its development *in utero*. The fertilized egg divides and differentiates until it produces a mature organism. The term pluripotent used to describe stem cells that can give rise to cells derived from all three embryonic germ layers; mesoderm, endoderm, and ectoderm. These three germ layers are the embryonic source of all cells of the body. All of the many different kinds of specialized cells that make up the body are derived from one of these germ layers. “Pluri” derived from the latin word *plures* that means several or many. Thus, pluripotent cells have the potential to

give rise to several type of cell, a property observed in the natural course of embryonic development and under certain laboratory conditions. Unipotent stem cell, a term usually applied to a cell in adult organisms, means that the cells in question are capable of differentiating along only one lineage. “Uni” is derived from the latin word *unus*, which means one. Also, it may be that the adult stem cells in many differentiated, undamaged tissues are typically unipotent and give rise to just one cell type under normal conditions. This process would allow for a steady state of self-renewal for the tissue. However, if the tissue becomes damaged and the replacement of multiple cell types is required, pluripotent stem cells may become activated to repair the damage.

Embryonic stem cell and adult stem cell are two types of stem cell. The embryonic stem cell is defined by its origin that is from one of the earliest stages of the development of the embryo, called blastocyst. Specifically, embryonic stem cells are derived from the inner cell mass of the blastocyst at a stage before it would implant in the uterine wall. It can give rise to cells derived from all three germ layers. The adult stem cell is an undifferentiated (unspecialized) cell that is found in a differentiated (specialized) tissue; it can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated. Adult stem cells have been found in the bone marrow, blood stream, cornea and retina of the eye, the dental pulp of the tooth, liver, skin, gastrointestinal tract, and pancreas (Slack *et al*., 2000). Unlike embryonic stem cells, at this point in time, there are no isolated adult stem cells that are capable of forming all cells of the body.

Adult stem cell compartment consists of two sub-populations which are hematopoietic stem cells (HMCs) and mesenchymal stem cells (MSCs). A hematopoietic stem cell is a cell that form blood and immune cells. They are responsible for the constant renewal of blood and the production of new blood cells each day. The cells are isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death, called apoptosis; a process by which cells that are detrimental or unneeded undergo self-destruct. Adult mesenchymal stem cells are adult human pluripotent progenitor cells found in bone marrow, peripheral blood, cord blood, adipose tissue and liver. They have self-renewal capacity without differentiation in long term culture. Under certain conditions, MSC could be differentiated into adipocytes, chondrocytes, astrocytes, tenocytes, cardiomyocytes, hepatocytes, neurons, muscle cells, endothelial and endodermal cells (Sanchez-Ramos *et al*., 2000). MSCs have generated a great deal of excitement and promise as a potential source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiate into functional cell types that constitute the tissue in which they exist. MSCs are considered a readily accepted source of stem cells because such cells have already demonstrated efficacy in multiple types of cellular therapeutic strategies, including applications in treating children with *osteogenesis imperfecta* (Horwitz *et al*., 2002), haematopoietic recovery (Koc *et al*., 2000), and bone tissue regeneration strategies (Petite *et al*., 2000).

Despite the great interest in MSC, there is still no established protocol for isolation and expansion of the cells in culture. In most experiments isolated MSC from bone marrow mononuclear cells were determined based on their tight adherence to tissue culture plastic. The isolated cells were initially heterogenous, and were difficult to be distinguished from other adherent cells. Several methods have been developed to prepare more homogenous populations but none of protocols have earned wide acceptance (Hung *et al*., 2002). However, today there are many studies have isolated MSCs and controlled, *in vitro*, its differentiation into cartilaginous tissue and bone using new technology for repairing injured tissues of mesenchymal origin (Renata *et al*., 2006)

In this study, we have tried a method according to Friedenstein (1982) who was the first to identify a fibroblast-like cell in the bone marrow that can be cultured undifferentiated *in vitro* (Bruder *et al*., 1994) with modification in terms of media use and have succesfully differentiated it into bone cells. Bone formation in the embryo, and during adult fracture repair and remodeling, involves the progeny of cells called mesenchymal stem cells. These cells continuously replicate themselves, while a portion become commited to mesenchymal cell lineages such as bone, cartilage, tendon, ligament, and muscle. The differentiation of these cells, within each lineage, is a complex multistep pathway involving discrete cellular transitions much like that, which occurs during hematopoiesis. Progression from one stage to the next depends on the presence of specific bioactive factors, nutrient and other environmental cues whose exquisitely controlled contributions orchestrate the entire differentiation phenomenon (Price *et al*., 1993).