ISOLATION, CULTURE, *IN-VITRO* DIFFERENTIATION AND CHARACTERIZATION OF CANINE ADULT MESENCHYMAL STEM CELLS

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

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ANIMAL BIOTECHNOLOGY
(Minor Subject: Veterinary Microbiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, "**ISOLATION, CULTURE, IN-VITRO DIFFERENTIATION AND CHARACTERIZATION OF CANINE ADULT MESENCHYMAL STEM CELLS"** submitted for the degree of **Master of Science** in the subject of **Animal Biotechnology** (Minor subject: Veterinary Microbiology) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Sarabjot Singh (L-2010-ABT-10-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells capable of differentiating into various cell types, including osteocytes, chondrocytes, adipocytes, myocytes, and tenocytes, and are being used in tissue engineering and regenerative studies. This study was carried out to isolate, identify and examine the differentiation potential of canine MSCs. Bone marrow aspirates were collected from the wing of ilium from the fracture cases of dogs and processed by centrifuging over Histopaque-1077. The putative MSCs were cultured in DMEM-LG medium supplemented with 10% FCS. Initially very few cells adhered to the flask surface which started to grow and divide gradually and were subcultured at 60-70% confluency. At 3^{rd} to 5^{th} passage, when these cells were examined for their stemness properties by immunostaining, positive expression of surface markers CD44 and CD105 and negative expression of hematopoeitic markers CD34 and CD45 was detected indicating intact stemness property. These MSCs were then subjected to differentiation studies by growing in osteogenic, chondrogenic and adipogenic differentiation media for 21 days. The differentiated cMSCs were further, characterized by RT-PCR detection of the cell specific marker gene expressions, cytochemistry and immunocytochemistry. RT-PCR could detect osteopontin, SOX9 and Lpl gene expression in the differentiated osteocytes, chondrocytes and adipocytes with product sizes of 114bp, 132bp and 101bp, respectively. On cytochemistry, osteogenesis was confirmed by Von Kossa stain showing accumulation of calcium phosphate stained black in the osteocytes, chondrogenesis by blue staining of glycosaminoglycan enriched matrix of the chondrocytes by Alcian Blue staining and adipogenesis by Oil Red O stain that showed accumulation of lipid rich vacuoles in the adipocytes. In immunocytochemistry, the anti-Collagen type I, anti-SOX9 and PPAR-γ antibodies could detect the osteogenic, chondrogenic and adipogenic differentiation of the cMSC, respectively. In conclusion, canine mesenchymal stem cells (cMSC) were succesfully isolated and cultured in the laboratory and showed osteogenic, chondrogenic & adipogenic differentiation potential as revealed by cytochemistry, immunocytochemistry and RT-PCR assays

Key words: Bone marrow, mesenchymal stem cells, cell differentiation

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Signature of Major Advisor **Signature of the student**

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(SARABJOT)

ABBREVIATIONS

%	Per cent
µl	Microliter
°C	Degree Celsius
cMSC	Canine Mesenchymal Stem Cells
bp	Base pair
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
et al.	*et alii*
etc	et cet·er·a
M	Molar
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimolar
ng	Nanograms
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
Sec	Seconds
TBE	Tris borate EDTA buffer
V	Volts
IBMX	Isobutyl methyl xanthine
GAPDH	Glyceraldehyde-3- phosphate dehydrogenase
LpL	Lipoprotein Lipase
DAPI	2,4-diamidino-6-phenylindole
TGF-β1	Transforming growth factor- beta 1
TDW	Triple Distilled Water
PPAR- γ	Peroxisome Proliferator-activated receptor-γ
ODM	Osteogenic Differentiation Medium
CDM	Chondrogenic Differentiation Medium
ADM	Adipogenic Differentiation Medium
cDNA	complimentary Deoxy ribonucleic acid
FCS	Fetal Calf Serum

CHAPTER I

INTRODUCTION

Stem cells are defined as undifferentiated somatic cells having the ability to produce varieties of fully-differentiated progenies in response to the appropriate environment either *in vitro* or *in vivo*. Two broad types of mammalian stem cells are: embryonic stem cells that are isolated from the inner cell mass of blastocysts, and adult stem cells that are found in adult tissues. In a developing embryo, these stem cells can differentiate into all of the specialized embryonic tissues. Adult stem cells are undifferentiated cells, found throughout the body that multiply by cell division to replenish dying cells and regenerate damaged tissues. Unlike embryonic stem cells, the use of adult stem cells in research and therapy is not considered to be controversial as they are derived from adult tissue samples rather than destroyed animal/ human embryos. Adult stem cells in particular, represent a promising model for regenerative medicine and tissue engineering because the use of embryonic and fetal stem cells is limited by ethical considerations. Found in most adult tissues, adult stem cells act as a repair system for the body, replenishing specialized cells and maintaining the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. Different types of adult stem cells include hematopoietic, mesenchymal, mammary, endothelial, neural and testicular stem cells etc.

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into a variety of cells including fat (adipocytes), cartilage (chondrocytes), bone (osteocytes), tendon (tenocytes) and ligament, and muscle (myocytes). Mesenchymal stem cells are characterized morphologically by a small cell body with a few cell processes that are long and thin. (Netter and Frank 1987).

One of the defining characteristics of mesenchymal stem cells is their self renewal potential, the ability to generate identical copies of themselves through mitotic division over extended periods. Mesenchymal stem cells are derived from the animal's own tissue and once isolated, can be injected to provide a large concentration of the cells to the area of injury without any risk of rejection or reaction.

MSCs have been isolated from placenta, adipose tissue, lung, bone marrow and blood, Wharton's jelly from the umbilical cord, and teeth (perivascular niche of dental pulp and periodontal ligament).

Bone marrow derived mesenchymal stem cells (BM-MSCs) are multipotent cells, which are considered as a cell source for repair and regeneration of various tissues. Another property of mesenchymal stem cells is that they can be cultured, banked and frozen for future use as they maintain their functionality when thawed.

Isolation and phenotypic characterization of MSCs has been demonstrated in several vertebrate species, including human, murine, lapine, canine, ovine, avian, porcine, equine, and bovines (Adel and Mao 2004).

Recently, stem cell treatment is being conducted on horses, dogs, and cats to target a wide range of injuries and diseases such as myocardial infarction, stroke, tendon and ligament damage, osteoarthritis, osteochondrosis and muscular dystrophy in large animals (Chen *et al* 2001; Assmus *et al* 2002). Adult-derived mesenchymal stem cells are being used as a means of tissue regeneration to treat animals with injuries or defects affecting bone, cartilage, ligaments and/or tendons (Young *et al* 1998).

Canine is a promising model for the stem cell research. Given that many advances achieved in dogs are directly transferable to humans since it constitutes a large animal model, it

could be of great value for the development of new therapies. Isolating SCs in the dog model will provide the opportunity to evaluate the efficacy and safety of gene therapy and cell transplantation (Hiroaki *et al* 2006; Mahesh *et al* 2006). Canine Mesenchymal stem cells (cMSCs) have cell renewal capacity and can differentiate into the mesodermal lineages. cMSCs have been characterized on the basis of their surface markers and differentiation potential. MSCs can be used to heal fractures in canines and osteoarthritis cases in regenerative therapy.

Keeping these points in view, the present study was planned with the following objectives:

i) To isolate, culture and differentiate *in vitro* canine adult mesenchymal stem cells (cMSC).

ii) To characterize the *in vitro* differentiated canine adult mesenchymal stem cells by Cytochemistry, Immunocytochemistry and RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction).

CHAPTER II

REVIEW OF LITERATURE

2.1 Stem Cells

Stem cells are biological cells found in all multicellular organisms that can divide (through mitosis) and differentiate into diverse specialized cell types and can self-renew to produce more stem cells. In mammals, there are two broad types of stem cells; embryonic stem cells, which are isolated from the inner cell mass of blastocysts and adult stem cells, which are found in various tissues.

The first work in stem cell field was started by Alexander A. Maximow, in 1924 who used extensive histological findings to identify a singular type of precursor cell within mesenchyme that develops into different types of blood cells. Research in the stem cell field then grew out of findings by Ernest A. McCulloch and James E. Till at the University of Toronto in the 1960s. The first definitive discovery of mesenchymal stem cells was reported by Friedenstein (1974) who isolated the cells by their plastic adherent property and described them as a non-hematopoietic, clonogenic and fibroblastic cells having the capacity of producing osteoblastic, chondrocytic and adipocytic cell lineages.Stem cells can now be artificially grown and transformed (differentiated) into specialized cell types with characteristics consistent with cells of various tissues such as muscles or nerves through cell culture.

2.2 Types of Stem Cells

2.2.1 Embryonic Stem Cells

Embryonic stem (ES) cell lines are cultures of cells derived from the epiblast of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryos. A blastocyst is an early stage embryo—approximately four to five days old in humans and consisting of 50–150 cells. ES

cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm.

In 1981, embryonic stem cells (ES cells) were independently first derived from mouse embryos by two groups. Martin Evans and Matthew Kaufman from the Department of Genetics, University of Cambridge published first in July, revealing a new technique for culturing the mouse embryos in the uterus to allow for an increase in cell number, allowing for the derivation of ES cells from these embryos. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. They do not contribute to the extra-embryonic membranes or the placenta. The endoderm is composed of the entire gut tube and the lungs, the ectoderm gives rise to the nervous system and skin, and the mesoderm gives rise to muscle, bone, blood—in essence, everything else that connects the endoderm to the ectoderm. Additionally, under defined conditions, embryonic stem cells are capable of propagating themselves indefinitely. This allows embryonic stem cells to be employed as useful tools for both research and regenerative medicine, because they can produce limitless numbers of themselves for continued research or clinical use.

2.2.2 Adult Stem Cells

Adult stem cells are undifferentiated cells, found throughout the body after development, that multiply by cell division to replenish dying cells and regenerate damaged tissues. Also known as somatic stem cells, they can be found in juvenile as well as adult animals and human bodies. Pluripotent adult stem cells are rare and generally small in number but can be found in a number of tissues including umbilical cord blood (Ratajczak *et al* 2007). Most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin

(mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, dental pulp stem cell, etc.). To ensure self-renewal, stem cells undergo two types of cell division. Symmetric division gives rise to two identical daughter cells, both endowed with stem cell properties, whereas asymmetric division produces only one stem cell and a progenitor cell with limited self-renewal potential. Progenitors can go through several rounds of cell division before finally differentiating into a mature cell. It is believed that the molecular distinction between symmetric and asymmetric divisions lies in differential segregation of cell membrane proteins (such as receptors) between the daughter cells (Beckmann *et al* 2007). Adult stem cells express transporters of the ATP-binding cassette family that actively pump a diversity of organic molecules out of the cell.

2.2.3 Amniotic Stem Cells

Multipotent stem cells are also found in amniotic fluid. These stem cells are very active, expand extensively without feeders and are not tumorigenic. Amniotic stem cells are multipotent and can differentiate in cells of adipogenic, osteogenic, myogenic, endothelial, hepatic and also neuronal lines (De Coppi et al 2007). It is possible to collect amniotic stem cells for donors or for autologuous use: the first US amniotic stem cells bank was opened in 2009 in Medford, MA, by Biocell Center Corporation and collaborates with various hospitals and universities all over the world.

2.2.4 Induced Pluripotent Stem Cells

Induced pluripotent stem cells, commonly abbreviated as iPS cells or iPSCs are a type of **pluripotent stem cell** artificially derived from a non-**pluripotent** cell- typically an adult **somatic cell** - by inducing a "forced" expression of specific **genes** (Baker et al 2007). Induced pluripotent stem cells are similar to natural pluripotent stem cells, such as **embryonic stem (ES) cells**, in many aspects, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, potency and differentiability, but the full extent to their relation to natural pluripotent stem cells is still being assessed (Takahashi *et al* 2006)

iPSCs were first produced in 2006 from mouse cells and in 2007 from human cells in a series of experiments by **Shinya Yamanaka**'s team at **Kyoto University**, Japan. For this discovery he was awarded the **Wolf Prize in Medicine**.

iPSCs are an important advance in stem cell research, as they may allow researchers to obtain pluripotent stem cells, which are important in research and potentially have therapeutic uses, without the **controversial** use of embryos. Because iPSCs are developed from a patient's own somatic cells, it was believed that treatment of iPSCs would avoid any immunogenic responses; however, Zhao et al. have challenged this assumption (Zhao *et al* 2011).

2.3 Adult Stem Cells

2.3.1 Types of Adult Stem Cells

Hematopoietic stem cells

Hematopoietic stem cells are multipotent stem cells that give rise to all the blood cell types from the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/ platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells and NK-cells). The hematopoietic tissue contains cells with long-term and short-term regeneration capacities and commited multipotent, oligopotent, and unipotent progenitors. HSCs are found in the bone marrow of adults; within femur, pelvis, ribs, sternum, and other bones. Cells can usually be obtained directly from the iliac crest part of the pelvic bone, using a special needle and a syringe.

Mammary stem cells

Mammary stem cells provide the source of cells for growth of the mammary gland during puberty and gestation and play an important role in carcinogenesis of the breast. Mammary stem cells have been isolated from human and mouse tissue as well as from cell lines derived from the mammary gland. Single such cells can give rise to both the luminal and myoepithelial cell types of the gland, and have been shown to have the ability to regenerate the entire organ in mice (Liu *et al* 2005).

Intestinal stem cells

Intestinal stem cells divide continuously throughout life and use a complex genetic program to produce the cells lining the surface of the small and large intestines. Intestinal stem cells reside near the base of the stem cell niche, called the crypts of Lieberkuhn. Intestinal stem cells are probably the source of most cancers of the small intestine and colon (Barker *et al* 2008).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are of stromal origin and may differentiate into a variety of tissues. MSCs have been isolated from placenta, adipose tissue, lung, bone marrow and blood,Wharton's jelly from the umbilical cord, and teeth (perivascular niche of dental pulp and periodontal ligament). MSCs are attractive for clinical therapy due to their ability to differentiate, provide trophic support, and modulate innate immune response (Phinney and prockorp 2007).

Endothelial stem cells

Endothelial stem cells (ESCs) are one of three types of **stem cells** found in **bone marrow**. They are **multipotent**, which describes the ability to give rise to many cell types, whereas a **pluripotent** stem cell can give rise to all types. ESCs have the characteristic properties of a stem cell: self-renewal and differentiation. These parent stem cells, ESCs, give rise to **progenitor** cells, which are intermediate stem cells that lose potency. ECs were first thought to arise from extraembryonic tissues because blood vessels were observed in the avian and mammalian embryos. However, after histological analysis, it was seen that ECs were in the embryo. This meant that blood vessels come from an intraembryonic source, the mesoderm (Ferguson *et al* 2005).

Neural stem cells

The existence of stem cells in the adult brain has been postulated following the discovery that the process of neurogenesis, the birth of new neurons, continues into adulthood in rats. The presence of stem cells in the mature primate brain was first reported in 1967 (Lewis 1968). It has since been shown that new neurons are generated in adult mice, songbirds and primates, including humans. Normally, adult neurogenesis is restricted to two areas of the brain – the subventricular zone, which lines the lateral ventricles, and the dentate gyrus of the hippocampal formation (Alvarez-Buylla *et al* 2002). Although the generation of new neurons in the hippocampus is well established, the presence of true self-renewing stem cells there has been debated (Bull *et al* 2005).

Neural stem cells are commonly cultured *in vitro* as so called neurospheres – floating heterogeneous aggregates of cells, containing a large proportion of stem cells (Reynolds *et al* 1992). They can be propagated for extended periods of time and differentiated into both neuronal and glia cells, and therefore behave as stem cells. However, some recent studies suggest that this behaviour is induced by the culture conditions in progenitor cells, the progeny of stem cell division that normally undergo a strictly limited number of replication cycles *in vivo* (*Doetsch et al* 2002).

Neural stem cells share many properties with haematopoietic stem cells (HSCs). Remarkably, when injected into the blood, neurosphere-derived cells differentiate into various cell types of the immune system.

Olfactory adult stem cells

Olfactory adult stem cells have been successfully harvested from the human olfactory mucosa cells, which are found in the lining of the nose and are involved in the sense of smell. If they are

given the right chemical environment these cells have the same ability as embryonic stem cells to develop into many different cell types. Olfactory stem cells hold the potential for therapeutic applications and, in contrast to neural stem cells, can be harvested with ease without harm to the patient. This means they can be easily obtained from all individuals, including older patients who might be most in need of stem cell therapies (Murrel *et al* 2005).

Neural crest stem cells

Hair follicles contain two types of stem cells, one of which appears to represent a remnant of the stem cells of the embryonic neural crest. Similar cells have been found in the gastrointestinal tract, sciatic nerve, cardiac outflow tract and spinal and sympathetic ganglia. These cells can generate neurons, Schwann cells, myofibroblast, chondrocytes and melanocytes (Kruger *et al* 2002).

Testicular cells

Multipotent stem cells with a claimed equivalency to embryonic stem cells have been derived from spermatogonial progenitor cells found in the testicles of laboratory mice by scientists in Germany and the United States, and, a year later, researchers from Germany and the United Kingdom confirmed the same capability using cells from the testicles of humans. The extracted stem cells are known as human adult germline stem cells (GSCs) (Miller 2007).

2.4 Culture of Adult Stem Cells

Stem cells are grown in a maintained temperature of 37º C in incubators in culture dishes under high humidity. Different culture medium is used to grow and maintain different types of stem cells. It is necessary to grow stem cells in an undifferentiated state, and to make the cells

differentiate into specialized cells, when required. Human embryonic stem cells can be grown as small colonies on layers of skin cells in the presence of serum. The skin cells are known as "feeder cells" and together with the serum, provide unknown factors that nourish and support the embryonic stem cells. The cells are divided into small colonies or single cells and transferred into new culture dish when the large colonies of embryonic stem cells are grown. "Passaging" or transferring is done to grow these cells continuously. Hematopoietic stem cells are obtained from bone marrow, placenta or umbilical cord blood. As the hematopoietic stem cells differentiate very quickly it is difficult to make them grow in culture medium. Human bone marrow stem cells are isolated from the bone marrow and grown in culture media supplemented with serum from the blood. Bone marrow cells attach to the bottom of the culture dishes and can grow for several weeks before they differentiate. Human neural stem cells can grow from fetal or adult brain tissue in culture media. They grow in suspension and they do not need serum in blood (http://m.medindia.net/patientinfo/stemcells_growing.html).

Venugopal *et al* (2011) isolated and expanded Whartons Jelly derived Mesenchymal stem cells (WJ-MSCs) in 5% pooled, allogeneic human serum (HS) supplemented with 2 ng/mL of basic fibroblast growth factor. They determined their growth kinetics, *in vitro* differentiation potential, surface marker expression, and colony-forming unit potential and compared them against standard WJ-MSC cultures expanded in 10% FBS. Thus, established an efficient, complete xeno-free protocol for propagation of human WJ-MSCs.

2.4.1 Growth Media

Adult stem cells can be cultured using different available medium. The commercially available medium include the Dulbecco's modified Eagle's medium (DMEM) – low glucose (LG), α- MEM, Hams F-10 medium.

Chen *et al* (2009) compared the effect of two culture media (DMEM and alpha-MEM), two culture flasks (75 or 25 cm^2) and two different mononuclear cell seeding densities (1×10^4 or 5×10^4 MNC/cm2) on the isolation of hMSCs from bone marrow samples and analyzed if the isolation conditions affected the expansion of these cells in the first two passages. They showed that alpha-MEM is the optimal culture medium for both, isolation and expansion of mesenchymal stem cells. Moreover, the cell seeding density of 50,000 MNC/cm2 in 25 cm^2 culture flasks seems to be the best condition for the isolation step.

Azouna *et al* (2012) compared the effect of various media combining autologous HPL with or without FBS on phenotypic, proliferative and functional (differentiation, cytokine secretion profile) characteristics of human BM-derived MSCs. They showed that MSCs can be expanded in media supplemented with HPL that can totally replace FBS. HPL-supplemented media not only preserves their phenotype as well as their differentiation capacity, but also shortens culture time by increasing their growth rate.

2.4.2 Growth Factors

Growth factors are naturally occurring regulatory molecules, which bind to receptors on the cell surface. They stimulate cell and tissue function through influencing cell differentiation by changing their biochemical activity and cellular growth, and regulating their rate of proliferation. Numerous families of growth factors have already been identified and remarkable advancements have been made in understanding the pathways growth factors use to activate cellular proliferation and differentiation. Bone morphogenic protein (BMPs), epidermal growth factor (EGF), insulin – like growth factor (IGF), Fibroblast Growth Factor (FGF), cytokines,

Interleukin – γ, platelet derived growth factor (PDGF), Vascular Endothelial Growth Factor (VEGF) etc. are the growth factors involved in stem cell growth and proliferation.

NG *et al* (2008) compared the transcriptomes of marrow-derived mesenchymal stem cells (MSCs) with differentiated adipocytes, osteocytes, and chondrocytes derived from these MSCs. They also identified pathways that MSCs use to differentiate into adipogenic, chondrogenic, and osteogenic lineages. They even identified actin-mediated transforming growth factor (TGF)–β signaling, platelet-derived growth factor (PDGF) signaling and fibroblast growth factor (FGF) signaling as the key pathways involved in MSC differentiation.

2.4.3 Growth Conditions

Stem cells are grown in a maintained temperature of 37º C in incubators in culture dishes under high humidity. Different culture medium is used to grow and maintain different types of stem cells. It is necessary to grow stem cells in an undifferentiated state, and to make the cells differentiate into specialized cells, when required. Human embryonic stem cells can be grown as small colonies on layers of skin cells in the presence of serum. The skin cells are known as "feeder cells" and together with the serum, provide unknown factors that nourish and support the embryonic stem cells. The cells are divided into small colonies or single cells and transferred into new culture dish when the large colonies of embryonic stem cells are grown. "Passaging" or transferring is done to grow these cells continuously. Hematopoietic stem cells are obtained from bone marrow, placenta or umbilical cord blood. As the hematopoietic stem cells differentiate very quickly it is difficult to make them grow in culture medium.Human bone marrow stem cells are isolated from the bone marrow and grown in culture media supplemented with serum from the blood. Bone marrow cells attach to the bottom of the culture dishes and can grow for several

weeks before they differentiate. Human neural stem cells can grow from fetal or adult brain tissue in culture media. They grow in suspension and they do not need serum from blood.

Toyoda *et al* (2007) discussed how adequate numbers of MSC can be maintained in culture. They focused on identification of the cell culture conditions needed to maintain general, nonspecific potential as a stem cell over time and through replication. It would be extremely advantageous to be able to maintain MSC populations in a completely undifferentiated state and to determine and switch on specific differentiation as and when required.

2.5 *In- Vitro* Differentiation of stem cells

Differentiation is the process by which a less specialized cell becomes a more specialized cell type. Differentiation occurs numerous times during the development of a multicellular organism as the organism changes from a simple zygote to a complex system of tissues and cell types. Differentiation is a common process in adults as well: adult stem cells divide and create fully differentiated daughter cells during tissue repair and during normal cell turnover. A cell that is able to differentiate into all cell types of the adult organism is known as *pluripotent*. A cell that is able to differentiate into all cell types, including the placental tissue, is known as *totipotent*. A cell that is able to differentiate into the cells of the mesodermal lineage is known as *multipotent*.

MSCs differentiation is a complex process with a rather unknown molecular mechanism. It is believed that various parameters may involve in commitment of MSCs to differentiate into a particular cell lineages. These factors include the molecular composition of the serum, various treatments, the type of the plastic used in

culture plate and the cell interactions (Wang *et al* 2004 ; Kotto *et al* 2001). Providing the appropriate conditions, MSCs are able to generate variety of cells primarily including bone, cartilage and fat.

2.5.1 Osteogenic Differentaition

The main function of bone in skeletal system is to provide structural support for the body as well as its vital organs. The bone is a main source of minerals and plays a main role during the muscle contraction. The ability of MSCs in differentiating into osteoblastic lineages is appealing promise for cell-based treatment of bone defects especially those with large tissue loss.

For bone differentiation of MSCs, the same procedure is followed in different labs. Usually, after isolation and purifying of MSCs, passageed-2-3 cells are grown into confluency using proliferation medium containing 10-15% fetal bovine serum. Then, the proliferation medium replaces with an osteogenic medium, containing 50µg/ml ascorbic acid 2-phosphate, 10nM dexamethasone and 10mM ß-glycerol phosphate. The cultures subsequently are placed in an incubator at 37^{o}C and 5% CO_2 for 21 days, with media changes of 3 times a week (Zucconi *et al* 2010; Viera *et al* 2010).

Arpornmaeklong *et al* (2009) determined (1) the temporal pattern of osteoblastic differentiation of human embryonic stem cell–derived mesenchymal stem cells (hESC-MSCs), (2) the influence of a three-dimensional matrix on the osteogenic differentiation of hESC-MSCs in long-term culture, and (3) the bone forming capacity of osteoblast-like cells derived from hESCMSCs in calvarial defects. This current cell culture model and osteogenic cell enrichment method could provide large numbers of osteoprogenitor cells for analysis of differentiation patterns and cell transplantation to regenerate skeletal defects.

Aveline *et al* (2011) reviewed highlights, current status and progresses in the differentiation MSCs along the osteoblastic/osteocytic pathways and the ionic channel expression and evolution during this differentiation. MSCs regulate both osteogenesis and hematopoiesis, and they are responsible in part for the regenerative capacity of bone tissue.

Gao *et al* (2011) isolated and cultured human MSCs and osteo-differentiated MSCs from four individual donors. miRNA expression in MSCs and osteo-differentiated MSCs was investigated using miRNA microarrays. miRNAs that were commonly expressed in all three MSC preparations and miRNAs that were differentially expressed between MSCs and osteo-differentiated MSCs were identified. The results of this study provided an experimental basis for further research on miRNA functions during osteogenic differentiation of human MSCs.

2.5.2 Chondrogenic Differentiation

Cartilage differentiation of MSCs is usually conducted in a micromass culture system (Mackay *et al* 1998; Johnstone 2002). Approximately 200,000 cells (passage 2-3) are pelleted by centrifugation at 300g for 4 minutes, followed by incubation at 37^{o}C and 5% CO_2 in a 0.5 ml chondrogenic medium, composed of 10ng/ml transforming growth factor ß3, 500ng/ml bone morphogenetic protein-6, 100nM dexamethasone, 50µg/ml ascorbic 2-phosphate, 50 µg/ml ITS and 1.25 mg/ml bovine serum albumin. The cultures are maintained for 3 weeks with medium change of 3-day intervals. At the end of this period, the pellets are usually analyzed for differentiation using toluidine blue staining for methachromatic matrix and RT-PCR analysis for cartilage specific markers including collagen II, aggreacan, and collagen X.

Boeuf and Richter (2010) provided an overview of studies featuring comparative analysis of the chondrogenic differentiation of MSCs from different sources. It will examine the influence

of the cells' origin on the requirements for the induction of chondrogenesis and on the phenotype achieved by the cells after diff erentiation.

Mardones *et al* (2010) aimed to obtain, culture and differentiate rabbit bone Marrow derived Mesenchymal Stem Cells *in vitro* to chondral lineage. Differentiation culture had an 80% efficiency and optimal differentiation quality. Rabbit Bone Marrow derived Mesenchymal Stem Cells culture is a reproducible technique and by the use of an adequate methodology chondrogenic cells can be obtained *in vitro*. This model permits the study of chondral differentiation process and could have direct clinical application.

2.5.3 Adipogenic Differentiation

Adipogenic differentiation can be carried out in passageed-2-3 cells. The cells are grown into confluency using proliferation medium containing 10-15% fetal bovine serum. Then, the proliferation medium replaces with an adipogenic medium, containing 1µM dexamethasone, 10 mM sodium pyruvate, 500 µM 3-isobutyl-1-methyl xanthine (IBMX), 60µM indomethacin and 5µg/ml insulin. The cultures subsequently are placed in an incubator at 37^{o}C and 5% CO_2 for 21 days, with media changes of 3 times a week (Vieira *et al* 2010; Tharasanit *et al* 2011).

Vieira *et al* (2010) obtained canine adipose tissue and processed to obtain a fibroblast-like population of cells similar to human adipose-derived stem cells (hASCs). Immunoflourescence and flow cytometry showed that majority of cASCs were of mesodermal or mesenchymal origin. cASCs were able to differentiate *in vitro* into adipogenic, chondrogenic, myogenic and osteogenic cells. In conclusion, canine adipose tissue contained multipotent cells that represent an important stem cell source both for veterinary cell therapy as well as preclinical studies.

Liu *et al* (2007) compared the transcriptome profile of hAMCS and hBMSCs during directed differentiation into bone, cartilage, and fat. Data revealed considerable similarities between bone marrow-derived MSCs (BMSCs) and adipose tissue-derived MSCs (AMSCs). They also suggested that although a set of common genes may be needed for early differentiation into all three lineages, a different set of signature genes is associated with maturation into fully differentiated cells. This study not only generated a rich database for continuing molecular characterization of various MSCs but also provided a rational basis for assessing qualities of MSCs from different sources for the purpose of cell-based therapy and tissue engineering.

Csaki *et al* (2007) studied bone marrow derived canine mesenchymal stem cells (cMSCs) from three canine patients and isolated, expanded cMSCs in monolayer culture and characterized with respect to their ability for osteogenic, adipogenic and chondrogenic differentiation capacities. cMSCs treated with the osteogenic induction medium differentiated into osteoblasts, produced typical bone matrix components, β1-integrins and Cbfa-1. Canine MSCs treated with the adipogenic induction medium showed typical adipocyte morphology, produced adiponectin, collagen type I and β1-integrins, and upregulated the adipogenic specific transcription factor PPAR-gamma. cMSCs treated with the chondrogenic induction medium produced a cartilage-specific extracellular matrix, β1-integrins and upregulated the chondrogenic specific transcription factor Sox9.

Danišovič *et al* (2007) studied to verify the chondrogenic differentiation potential of human bone marrowmesenchymal stem cells (BMSCs) and adipose tissue-derived mesenchymal stem cells (AMSCs) *in vitro* in the presence or absence of transforming growth factor beta (TGF-β1). The presence of TGF-β1 led to a decrease in the levels of collagen type I mRNA and to increased levels of collagen type II mRNA only in the BMSC pellet culture. Results demonstrate

that although both mesenchymal cell types can be used in cartilage tissue engineering, the chondrogenic potential of human BMSCs is higher than that of AMSCs.

Djouad *et al* (2007) hypothesized that the induction of MSC differentiation towards chondrocytes might be induced and/or influenced by molecules from the microenvironment. Assessed the differential expression of genes associated with the microenvironment using a large-scale real-time PCR assay, according to the simultaneous detection of up to 384 mRNAs in one sample.The data suggested that crosstalk between ECM components of the microenvironment and MSCs within the cartilage is responsible for the differentiation of MSCs into chondrocytes.

Frith and Genever (2008) discussed the control of MSC differentiation at the transcriptional level. They specifically focused on the key factors that contribute to the regulation of osteogenesis, adipogenesis and chondrogenesis.

Ji *et al* (2009) aimed to make a primary comparison of the mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPCs), in an effort to understand the similarities and dissimilarities in their properties and proliferation potentials. They found that MSCs gradually lost its multiple differentiation potential and phenotype with the increase of passages, whereas MAPCs well retained pluripotency. Therefore, MAPCs may hold a greater promise in the future clinical application than MSCs

Yameen *et al* (2009) investigated the multilineage stem cell potential of bone and cartilage explant cultures in comparison with bone marrow derived mesenchymal stem cells (BM-MSCs). The results showed that the surface antigen expression of tissue-derived cells was consistent with that of mesenchymal stem cells. The tissue-derived cells were able to differentiate into osteoblast, chondrocyte and adipocyte lineage pathways when stimulated in the

appropriate differentiating conditions. However, compared with BMSCs, tissue-derived cells showed less capacity for multilineage differentiation when the level of differentiation was assessed in monolayer culture by analysing the expression of tissue specific genes by reverse transcription polymerase chain reaction (RT-PCR) and histology. Thus, cells derived from tissue explant cultures reserved certain degree of differentiation properties of MSCs *in vitro*.

Vieira *et al* (2010) obtained canine adipose tissue by biopsy from subcutaneous adipose tissue or by suction-assisted lipectomy (i.e. liposuction). Adipose tissue was processed to obtain a fibroblast-like population of cells. Immunoflourescence and flow cytometry showed that the majority of cASCs were of mesodermal or mesenchymal origin. Canine adipose stem cells are able to differentiate in vitro into adipogenic, chondrogenic, myogenic and osteogenic cells in the presence of lineage-specific induction factors.

Augello and De Bari (2010) addressed the current knowledge of the molecular basis of differentiation of cultured MSCs, with a particular focus on chondrogenesis and osteogenesis. Building on the information coming from developmental biology studies of embryonic skeletogenesis, several signaling pathways and transcription factors have been investigated and shown to play critical roles in MSC differentiation.

Teven *et al* (2011) reviewed the effect of epigenetic modifications on MSC multipotency and differentiation, with a particular focus on osteogenic and adipogenic differentiation. We also highlight potential clinical applications of MSC epigenetics and nuclear reprogramming.

2.6 Characterization of Adult stem cells

The identification of specific cell surface proteins is one of the major challenges in characterization of a cell type that defines the kind of heterotypic/homotypic interactions

between neighboring cells. Stem cells express CD44, CD73, CD90 and CD105 receptors while lacking hematopoietic stem cell markers such as CD14, CD31, CD33, CD34 and CD45. Stem cells show expression of adhesion related antigens, such as integrin αvβ3 and αvβ5, integrin subunits α4, α5 and β1, intercellular adhesion molecule-1 (ICAM-1) and CD44H, enabling their adherence to extracellular matrix molecules (Conget and Minguell 1999). MSC produce cytokines, chemokines and growth factors, such as interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, leukemia inhibitory factor, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor, macrophage colony-stimulating factor (M-CSF) and fms- like tyrosine kinase-3 ligand (flk-3L), implicated mainly in hematopoiesis (Kim *et al* 2005; Haynesworth *et al* 1996). They also express cytokine receptors IL-1R, IL-3R, IL-4R, IL-6R and IL-7R. The antibody STRO-1 has, so far, been the most useful antibody for identifying and positively selecting for MSCs in bone marrow.

Nauta and fibbe (2007) studied the imunogenicity and immunomodulatory properties of MSCs, both *in vitro* and *in vivo*, the possible underlying mechanisms, the potential clinical use of MSCs as modulators of immune responses *in vivo*, and to indicate clinical safety concerns and recommendations for future research.

Yourek *et al* (2007) studied the actin cytoskeleton and nanomechanobiology of human mesenchymal stem cells (hMSCs) using fluorescence microscopy and atomic force microscopy (AFM). Human MSCs were differentiated into chondrocytes and osteoblasts as per previous approaches. Cytochalasin D (CytD) was used to temporarily disrupt cytoskeleton in hMSCs, hMSC-chondrocytes (hMSC-Cys) and hMSC-osteoblasts (hMSC-Obs). Fluorescence microscopy revealed a dose-dependent response to CytD.

Kang *et al* (2008) isolated canine AD-MSCs (cAD-MSCs) and induced their development into adipocyte, osteocyte, and neuron-like cells. They then investigated their phenotype and cytokine expression to determine whether they were able to exert an immunomodulatory effect and what the underlying mechanisms of this effect were. cAD-MSCs expressed CD44, CD90, and MHC class I and were also partially positive for the expression of CD34. In addition, they expressed the mRNA of transforming growth factor beta (TGF-β), IL-6, IL-8, CCL2, CCL5, vascular endothelial growth factor, hepatocyte growth factor (HGF), tissue inhibitor metalloproteinase- 1/2, and cyclooxygenase-2 but not that of IL-10. Immonomodulatory factors of MSCs, such as TGF-β, HGF, prostaglandin E2 (PGE2), and indoleamine 2, 3 dioxygenase (IDO), increased significantly in cAD-MSCs that were cocultured with leukocytes. It was suggested that cAD-MSCs have a potential therapeutic use in the treatment of immune-mediated disease.

Neupane *et al* (2008) isolated canine adult MSCs from adipose tissue. Following osteogenic induction the canine adipose-derived mesenchymal stem cells (cAD-MSC) expressed level of markers RUNX2, COL1A, OSTERIX, BSP and OSTEOCALCIN. Chondrogenic transcription factor, SOX9 and markers COL2A, AGGRECAN, COMP, and COL10A were expressed after micromass culture of cAD-MSCs. Adipogenic transcription factors PPARy2 and CEBPa, and adipocyte markers FABP4 and LPL were expressed following adipogenic induction.

Augello *et al* (2010) reviewed the current state of MSC research including the differentiation potency of culture expanded MSCs, expression of chemokines and their receptors in MSCs – both relevant issues for the advocated use of MSCs for tissue repair and their systemic delivery to the affected tissues. It also reviews current knowledge of MSC niches in

their native tissues, addressing the relationship with pericytes. Finally, it provides a scientific basis for the requirement of a thorough characterisation of the endogenous MSC niches within their native tissues *in vivo*. The knowledge of MSC niches will instruct development of innovative therapeutic measures such as producing pharmacological substances that target endogenous MSCs and their niches in order to activate and guide intrinsic repair and to improve disease outcomes.

Manochantr *et al* (2010) studied to isolate, characterize and explore the potential of AM-MSCs in differentiating toward neural lineage in comparison to those of BM-MSCs. The expression profiles of several MSC markers were examined by flow cytometry. The expression of several neural marker genes, including MAP-2, GFAP and β-tubulin III in AM-MSCs was determined by quantitative real time-PCR. The results demonstrated that AM-MSCs could be easily expanded to 18-20 passages while maintaining the undifferentiated state and exhibiting MSC markers (CD73, CD90, and CD105) but do not express the hematopoietic markers (CD34 and CD45).

Martinello *et al* (2010) characterized cAD-MSC and examined the effects of cryopreservation on their stemness features. After the cryopreservation period cells conserved their fibroblast-like morphology, alkaline phosphatase positivity and CD expression. Finally, the cryopreservation protocol did not change the cA-MSC adipogenic, osteogenic and myogenic differentiative potential. Their data demonstrated that stored cA-MSC might represent a promising type of progenitor cell for autologous cellular-based therapies in veterinary medicine.

Zucconi *et al* (2010) collected umbilical cord veins (UCVs) from Golden Retriever newborn dogs and differentiated canne UCV (cUCV) cells into adipocytes, chondrocytes and osteocytes using specific differentiation medium. Expression of typical surface proteins was

examined by immunocytochemistry and flow cytometry. The cUCV cells were positively labeled with CD90 and CD29 adhesion molecules. The expression of FABP4 and LPL was seen in adipose-induced cells, COL2A, SOX9 and aggrecan in chondro-induced cells and osteopontin, COL1A1 and BSP in osteo-induced cells.

Pratheesh *et al* (2011) studied and focused on the advances in the characterization of adult stem cells via selection of unique cell surface markers and regulation of lineage specific differentiation of mesenchymal stem cells.

Boxall and Jones (2012) initially discussed surface and molecular markers that were proposed to serve as the indicators of the MSC potency, in terms of their proliferative potential or the ability to differentiate into desired lineages.

2.6.1 Characterization of MSCs by detection of marker gene expression: Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Stem cells can be characterized by RT-PCR by the expression of specific genes involved in differentiation. Total RNA is extracted from the differentiated stem cells and cDNA is synthesized from total RNA. Polymerase chain reaction (PCR) is performed using specific primers to amplify the target genes. The PCR products were analyzed by agarose gel electrophoresis and visualized under UV light.

Eslaminejad *et al* (2009) isolated and characterized MSCs from goat bone marrow. Fibroblastic cells appeared in goat marrow cell culture were expanded through several subcultures. Passaged-3 cells were then differentiated among the osteogenic, adipogenic and chondrogenic cell lineages to determine their MSC nature. Differentiations were determined by RT-PCR analysis of related gene expression. Fibroblastic cells developed at goat marrow cell

culture are able to differentiate into skeletal cell lineages. They undergo extensive proliferation when being plated at low cell density in 15% FBS concentration.

Karaoz *et al* (2009) studied morphological and immunophenotypic properties of BM-MSCs in detail. Differentiation potential and growth kinetics of adult rat BM-MSCs were also determined. Immunohistochemistry and RT-PCR results indicated that BM-MSCs expressed myogenic (desmin, myogenin, myosin IIa, and a-SMA), neurogenic (c-enolase, MAP2a,b, c-fos, nestin, GFAP and beta III tubulin), and osteogenic(osteonectin, osteocalcin, osteopontin, Runx-2, BMP-2, BMP-4 and type I collagen) markers without stimulation towards differentiation.

Seo *et al* (2009) successfully isolated and characterized MSCs from canine umbilical cord and its fetal blood. cMSCs had stem cell expression patterns with MSCs surface markers by fluorescence activated cell sorter analysis (FACS). After neuronal differentiation study, the cMSC expressed neuronal markers Nestin, GFAP, Tuj-1, microtubule-associated protein 2 and NF160. With osteogenic differentiation, the cMSCs presented osteoblastic differentiation genes by RT-PCR.

2.6.2 Histochemistry

The adult stem cells after 21 days of differentiation are studied by histo-chemistry. Von Kossa/Alizarin Red staining is carried out for osteogenic differentiated which is demonstrated by accumulation of mineralized calcium phosphate (Tharasanit *et al* 2011). Chondrogenic differentiation is confirmed by glycosaminoglycans enriched- matrix stained with Alcian Blue/Toluidine Blue. The presence of adipogenesis is confirmed by Oil Red O staining which detects intracellular neutral triglycerides and lipids (Zucconi *et al* 2010).

Wilschut *et al* (2008) reported the isolation of progenitor cells from pig skeletal muscle tissue fragments. Muscle progenitor cells were stimulated to migrate from protease-digested tissue fragments and cultured in the presence of 5 ng/ml basic fibroblast growth factor. The cells showed adipogenic and osteogenic lineage commitment when exposed to specific differentiation conditions. These observations were determined by Von Kossa and Oil-Red-O staining and confirmed by quantitative RT-PCR analysis. The porcine muscle-derived progenitor cells possess long-term expansion capacity and a multilineage differentiation capacity.

Tharasanit *et al* (2011) isolated, identified and examined the differentiation capability of canine MSCs. Bone marrow aspirates were obtained from 4 dogs. The *in vitro* differentiation of these MSCs into mesodermal lineages (bone, cartilage and adipose tissues) and ectodermal lineage (neuron) was performed using osteogenic, chondrogenic, adipogenic and neurogenic media, respectively. Histological examinations (Von Kossa and alcian blue staining) and mRNA expressions (GLA and COL1A1) were used to examine the bone and cartilage differentiation, while Oil red O staining was used to determine adipogenic differentiation.

2.6.3 Immuno-Histo-chemistry

Immuno-staining is carried out on the differentiated stem cells by antibodies against specific markers involved in differentiation.

Vieira *et al* (2010) studied canine adipose-derived stem cells and found fibroblast like population of cells similar to human adipose-derived stem cells. They carried out immuno-histochemistry and immunoflourescence studies with anti-collagen type II antibody for chondrogenic differentiation and anti-myosin antibody for myosin differentiation. They found that these differentiated cells were positively labeled by these antibodies.

2.7 Clinical applications of Adult Mesenchymal Stem Cells (MSCs)

The specific characteristics of MSCs, including their extensive proliferative potential and the ability to differentiate into various cell types, like bone, fat and cartilage, make them an attractive tool in regenerative medicine. This is especially evident in such fields as cellular biology and gene therapy, resulting in considerable increase in the number of clinical trials based on the use of MSCs.

Apparently, these cells might be simply isolated from various tissues and expanded in culture in large numbers that gives the opportunity to create a tissue-engineered constructs containing these cells and re-introduce them into a patient. MSCs possess the capacity to engraft into various tissues and organs when infused systematically, and this engraftment has been shown to be stable in the long term. Even more, MSCs infused to the peripheral circulation have the ability to migrate to a specific site of injury. This phenomenon has been reported in animal models of bone fracture, cerebral ischemia and myocardial infarction.

MSCs have been also proposed to be an excellent potential tool for gene therapies. They can be subjected to various genetic modifications, such as transduction with viral vectors carrying a therapeutic gene or cDNA for special proteins, serving as molecular transmitters. One of the fields for MSC use in regenerative medicine is the treatment of bone defects. First approach to bone repair relied on biodegradable scaffolds impregnated with recombinant BMPs, and was designed to induce bone formation through the recruitment of local MSCs. With reference to numerous clinical trials using MSCs, a special attention ought to be paid towards Osteogenesis Imperfecta (OI) treatment. Therefore, a treatment strategy for OI is mainly aimed at improving bone strength through ameliorating the structural integrity of collagen. The cell

therapy approach targeted to osteoblast formation from MSCs was first investigated on murine models. Further example of potential clinical MSC usefulness is the possibility to accelerate the reconstitution of hematopoiesis in patients after myeloablative chemotherapy or radiotherapy. In addition, there are also observations indicating the usefulness of MSC transplantation in myocardium regeneration after myocardial infarction.

Nicoll *et al* (1997) summarized recent studies which explore the application of mesenchymal cells as a source of bone and cartilage-forming cells, upon seeding within resorbable polymeric scaffolds in the presence of bioactive growth factors. Such cell polymer-growth factor composites may be fabricated for use as templates for the engineering and repair of bone and cartilage tissues.

Bianco *et al* (2001) discussed bone marrow stromal cells and their potential nature as components of the vascular wall, and the prospects for their use in local and systemic transplantation and gene therapy.

Tuan *et al* (2002) studied the biology of MSCs, including their differentiation potentials *in vitro* and *in vivo*, and the application of MSCs in tissue engineering. MSCs derived from adult tissue present an exciting progenitor cell source for applications of tissue engineering and regenerative medicine.

De kok *et al* (2002) evaluated mesenchymal stem cell (MSC)-based alveolar bone regeneration in a canine alveolar saddle defect model. MSCs were loaded onto hydroxyapatite/tricalcium phosphate (HA/TCP) matrices. Scanning electron microscopic (SEM) evaluation demonstrated greater than 75% MSC coverage of the HA/TCP porous surface prior to placement regardless of MSC donor. Histomorphometrical analysis showed that equivalent

amounts of new bone were formed within the pores of the matrices loaded with autologous MSCs or MSCs from an unrelated donor. Analysis of dyelabelled MSCs in histological sections confirmed that the MSCs persisted in the implants throughout the course of the experiment. They concluded that autologous and allogeneic MSCs have the capacity to regenerate bone within craniofacial defects.

Adel and Mao (2004) outlined several approaches relevant to the isolation and therapeutic use of MSCs, but also presents several examples of phenotypic and functional characterization of isolated MSCs and their progeny.

Zipori Dov (2004) studied that mesenchymal stem cells (MSCs) have a promiscuous gene expression pattern; mesenchymal cells, whether primary or cloned cell lines, express T cell receptor (TCR) β and α genes, along with other components of the TCR complex. These cells may therefore be in a standby state, in which many gene families are expressed at a low level thereby making the cell readily capable of shifting fates.

Shi *et al* (2005) identified and characterized adult human dental pulp (dental pulp stem cells, DPSC), human primary teeth (stem cells from human exfoliated deciduous teeth, SHED), and periodontal ligament (periodontal ligament stem cells, PDLSC) by their capacity to generate clongenic cell clusters in culture. Ex vivo expanded DPSC, SHED, and PDLSC populations expressed a heterogeneous assortment of markers associated with MSC, dentin, bone, smooth muscle, neural tissue, and endothelium. Xenogeneic transplants containing HA/TCP with either DPSC or SHED generated donor-derived dentin-pulp-like tissues with distinct odontoblast layers lining the mineralized dentin-matrix.

Linke *et al* (2005) determined whether the heart in large mammals contains cardiac progenitor cells that regulate organ homeostasis and regenerate dead myocardium after

infarction. They reported that the dog heart possesses a cardiac stem cell pool characterized by undifferentiated cells that are self-renewing, clonogenic, and multipotent. These clonogenic cells and early committed progeny possess a hepatocyte growth factor (HGF)–c- Met and an insulin-like growth factor 1 (IGF-1)-IGF-1 receptor system that can be activated to induce their migration, proliferation, and survival. The results suggested that strategies capable of activating the growth reserve of the myocardium may be important in cardiac repair after ischemic injury.

Hong *et al* (2005) reported that a 14-3-3–binding protein, TAZ (transcriptional coactivator with PDZ-binding motif), coactivates Runx2-dependent gene transcription while repressing PPAR-gamma-dependent gene transcription. By modulating TAZ expression in model cell lines, mouse embryonic fibroblasts, and primary MSCs in culture and in zebrafish *in vivo*, they observed alterations in osteogenic versus adipogenic potential. These results indicated that TAZ functions as a molecular rheostat that modulates MSC differentiation.

Sethe *et al* (2006) examined recent data in support of different theories by means of introducing; a brief overview was given of current MSC definitions and their basic role in tissue regeneration followed by a comparative analysis of gerontological studies involving MSC. Evidence for extrinsic aging and various aging markers relating to morphology, proliferation, signalling, senescence markers, telomeres and telomerase, and other indicators was discussed.

Bobis *et al* (2006) studied and discussed on various aspects such as: sources, surface receptors, basic biology and functions, circulation and niches, growth and expansion, differentiation potential and clinical applications of mesenchymal stem cells (MSCs).

Krampera *et al* (2006) discussed the potential use of MSCs in degenerative or inflammatory diseases involving bone, cartilage, tendon and muscle tissues, on the basis of experimental evidence.

George *et al* (2006) studied the effect of honeycomb collagen scaffolds for the adhesion, differentiation and proliferation of bone marrow-derived mesenchymal stem cells into osteoblasts. The results demonstrated that the honeycomb collagen sponge is an excellent scaffold for the differentiation and proliferation of mesenchymal stem cells into osteoblasts. The data further proved that honeycomb collagen is an effective substrate for tissue engineering applications, and is very useful in the advancing field of stem cell technology and cell-based therapy.

Bosch *et al* (2006) studied 1) to establish porcine MSC (pMSC) cultures; 2) to optimize *in vitro* pMSC culture conditions, 3) to investigate whether pMSCs are amenable to genetic manipulation, and 4) to determine pMSC reprogramming potential using somatic cell nuclear transfer (SCNT). The pMSCs isolated from bone marrowgrew, attached to plastic with a fibroblast-like morphology, and expressed the mesenchymal surface marker THY1 but not the hematopoietic marker ITGAM. pMSCs underwent lipogenic, chondrogenic, and osteogenic differentiation when exposed to specific inducing conditions.

Barrilleaux *et al* (2006) reviewed to examined progress and challenges in ex vivo tissue engineering with adult stem cells. These rare cells are harvested from a variety of tissues, including bone marrow, adipose, skeletal muscle, and placenta, and differentiate into cells of their own lineage and in some cases atypical lineages.

Kolf *et al* (2007) focused on the advances in understanding the cellular and molecular signaling pathways and global transcriptional regulators of adult mesenchymal stem cells have provided new insights into their biology and potential clinical applications, particularly for tissue repair and regeneration, specifically in the context of self-renewal and regulation of lineagespecific differentiation of mesenchymal stem cells.

Chamberlain *et al* (2007) describes what is known about MSCs and their capacity to home to tissues together with the associated molecular mechanisms involving chemokine receptors and adhesion molecules. Harnessing the migratory potential of MSCs by modulating their chemokine-chemokine receptor interactions may be a powerful way to increase their ability to correct inherited disorders of mesenchymal tissues or facilitate tissue repair *in vivo*.

Kucia *et al* (2007) provided evidence that bone marrow (BM) contains a population of stem cells that expresses early developmental markers such as (1) stage-specific embryonic antigen (SSEA) and (2) transcription factors Oct-4 and Nanog. These are the markers characteristic for embryonic stem cells, epiblast stem cells, and primordial germ cells (PGC). The presence of these stem cells in adult BM supports the concept that this organ contains some population of pluripotent stem cells that is deposited in embryogenesis during early gastrulation. They hypothesized that these cells could be direct descendants of the germ lineage that, to pass genes on to the next generations has to create soma and, thus, become a "mother lineage" for all somatic cell lineages present in the adult body.

Phinney and Prockop (2007) evaluated the literature describing the plasticity of MSCs and offer insight into how the molecular and functional heterogeneity of this cell population, which reflects the complexity of marrow stroma as an organ system, may confound interpretation of their transdifferentiation potential. Additionally, they argued that this heterogeneity also provides a basis for the broad therapeutic efficacy of MSCs.

Jackson *et al* (2007) summarized recent studies which explore the application of mesenchymal cells as a source of bone and cartilage-forming cells, upon seeding within resorbable polymeric scaffolds in the presence of bioactive growth factors. Such cell polymer-

growth factor composites may be fabricated for use as templates for the engineering and repair of bone and cartilage tissues.

Laura *et al* (2008) evaluated three bone marrow harvesting sites: the proximal humerus, proximal femur and the wing of the ilium and two isolation and cultivation media: α-MEM and DMEM for mesenchymal cell culture in dogs. The most efficient bone marrow puncture and harvesting sites were the humeral one followed by the femoral one. The α-MEM medium had a higher efficiency rate upon cell plasticity, viability and proliferation capacities.

Arthur *et al* (2008) studied the skeletal regenerative capacity of bone marrow derived MSC alone or in combination with growth factors, biocompatible scaffolds, and following genetic modification.

Eslaminejad *et al* (2008) studied isolation, culture, differentiation and characterization of canine bone marrow derived mesenchymal stem cells. Passaged-3 culture-expanded MSCs of canines bone marrow were suspended in a diluted collagen gel and loaded onto commercially-available HA/TCP ceramics. The cell-loaded scaffolds were then autologously implanted along with the control cell-free scaffolds in masseter muscles of the four mongrel dogs. The implants were appeared to be encapsulated by fibrous tissue within the muscle. No cartilage tissues were observed in implantation site. Histological observation indicated that ectopic bone was formed in both MSCs-loaded scaffolds as well as the control cell-free implants.

García-Castro *et al* (2008) introduced the readers to the biology of MSCs and the mechanisms underlying immune tolerance. They then outlined potential cell replacement strategies and clinical applications based on the MSCs immunological properties. Ongoing clinical trials for graft-versus-host-disease, haematopoietic recovery after co-transplantation of MSCs along with haematopoietic stem cells and tissue repair are discussed. Finally, they

reviewed the emerging area based on the use of MSCs as a target cell subset for either spontaneous or induced neoplastic transformation and, for modelling non-haematological mesenchymal cancers such as sarcomas.

Gundry *et al* (2008) reviewed to provide a context for the use of proteomics in discovering new cell-surface markers for stem cells. Proteomic technologies now offer the possibility to specifically identify and investigate the cell-surface subproteome in a quantitative and discovery-driven manner. Once a cell surface protein marker panel has been identified by MS and the antibodies become available, the panel should permit the identification, tracking, and/or isolation of stem or progenitor cells that may be appropriate for therapeutics.

Docheva *et al* (2008) outlined the MSC surface receptors and focused on receptors that deliver important signals for chondrogenic differentiation of MSCs. Finally, the role of receptors in the progression of cartilage degeneration disorders, such as osteoarthritis (OA), was discussed.

Jang *et al* (2008) evaluated the osteogenic effect of allogenic canine umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) mixed with beta tricalcium phosphate (β-TCP) in orthotopic implantation. Bone formation was observed around β-TCP in longitudinal sections of implant.

Crha *et al* (2009) studied the role of mesenchymal stem cells in bone tissue regeneration and contemporary possibilities of supporting regeneration of damaged bone. They presented possibilities of transplantation of mesenchymal stem cells combined with biomaterials into bone defects, including the results of our own experimental studies dealing with the use of stem cells in the treatment of damaged tissues of the musculoskeletal system in animal models.

Lee and Park (2009) provided basic information about stem cells and explore the role stem cells are playing in the search for effective treatment and consider the medical potential

stem cells may hold in developing a cure for Parkinson's disease. They reviewed recent advances in the therapeutic roles of MSCs in PD and MSA, especially focusing on their neuroprotective properties and use in disease-modifying therapeutic strategies.

Toubai *et al* (2009) reviewed the immunomodulatory function of MSCs in allo-HCT and their potential usefulness in the treatment or prevention of severe acute GVHD.

Astori *et al* (2010) described the regulatory environment surrounding the production of cell based medicinal products and give practical aspects for cell isolation, characterization, production following Good Manufacturing Practice, focusing on the activities associated with the investigational new drug development.

Rastegar *et al* (2010) reviewed basic features of MSC biology including MSC characteristics in culture, homing mechanisms, differentiation capabilities and immune modulation. They highlight some *in vivo* and clinical evidence supporting the therapeutic roles of MSCs and their uses in orthopedic, autoimmune, and ischemic disorders.

Hass *et al* (2011) studied MSC derived from different adult (adipose tissue, peripheral blood, bone marrow) and neonatal tissues (particular parts of the placenta and umbilical cord) and compared them with respect to their cell biological properties, surface marker expression and proliferative capacities. Several MSC functions including *in vitro* and *in vivo* differentiation capacities within a variety of lineages and immune-modulatory properties were also highlighted.

Punwar and Khan (2011) studied the current treatment strategies for articular cartilage, describe use of mesenchymal stem cells for articular cartilage repair along with the results of clinical studies, and describe the future direction that these strategies are likely to take.

Lai *et al* (2011) reviewed the evidences supporting the hypothesis including the recent identification of exosome as a therapeutic agent in MSC secretion. They discussed the potential

and practicality of using this relatively novel entity as a therapeutic modality for the treatment of cardiac disease, particularly acute myocardial infarction.

Jackson *et al* (2011) provided a systematic analysis of recent preclinical and clinical research to evaluate the use of MSCs in wound healing applications. A number of delivery systems have been evaluated and indicate that MSCs could be the basis of a versatile therapy to fulfill the clinical needs for dermal regeneration. The review concludes with a discussion of the translational barriers that are limiting the widespread clinical use of MSCs to enhance wound healing.

Otto *et al* (2011) examined the ability of MSCs to modulate liver, kidney, heart and intestinal repair, and to update their opposing qualities of being less immunogenic and therefore tolerated in a transplant situation, yet being able to contribute to xenograft models of human tumour formation in other contexts. Recent studies showing the clinical safety of MSC in several pathologies are discussed. The possible opposing powers of MSC need careful understanding and control if their clinical potential is to be realised with long-term safety for patients.

Matsumoto *et al* (2011) combined the MSCs into the polymer and fabricated MSC/RADA composites. The composites showed osteogenic differentiation in the culture condition. Supplementation of hydroxyapatite ceramics (HA in the composites (MSC/RADA/HA composites) improved the mechanical property and demonstrated the extracellular bone like matrix formation in the culture condition.

Song *et al* (2011) reviewed the recent progress in controlling MSCs fate and functional activities by small molecules. Small molecules targeting specific signaling pathways have been shown to be key modulators in controlling stem cells' fate and function. Small molecules are also important tools for understanding mechanistic and developmental processes.

Kuroda *et al* (2011) focused on the potential of mesenchymal stem cells, particularly umbilical cord-derived mesenchymal stem cells, to differentiate into functional Schwann cells, and discuss the prospective clinical application of these cells to PNS regeneration.

Juhásová *et al* (2011) studied to characterize the osteogenic differentiation of miniature pig MSCs and markers of this differentiation *in vitro*. Flow-cytometrically characterized MSCs were seeded on cultivation plastic (collagen I and vitronectin coated/uncoated) or plasma clot (PC)/plasmaalginate clot (PAC) scaffolds and differentiated in osteogenic medium. The results indicated similar ability of miniature pig MSCs osteogenic differentiation in 2D and 3D environment, but the expression of osteogenic markers in scaffolds and ECM coated monolayers started earlier than in the monolayers without ECM.

McGinley *et al* (2011) investigated lentiviral vector-mediated genetic modification of rat bone-marrow derived MSCs and examine any functional effect of such genetic modification in an *in vitro* model of ischaemia. The second generation lentiviral vector rHIV-pWPT-EF1-a-GFP-W, was the most efficient and provided the most durable transgene expression of the vectors tested. The data validates the use of lentiviral vectors for efficient *in vitro* gene delivery to MSCs and suggest that lentiviral vector transduction can facilitate sustained therapeutic gene expression, providing an efficient tool for *ex vivo* MSC modification.

Birmingham *et al* (2012) studied to delineate the role of biochemical signalling from osteocytes and osteoblasts, using conditioned media and co-culture experiments, to understand how they direct osteogenic differentiation of MSCs. Osteogenic differentiation of MSCs was quantified by monitoring alkaline phosphatase (ALP) activity, calcium deposition and cell number. Intracellular ALP was found to peak earlier and there was greater calcium deposition

when MSCs were co-cultured with osteocytes rather than osteoblasts, suggesting that osteocytes are more influential than osteoblasts in stimulating osteogenesis in MSCs.

Chauduri and Pramanik (2012) attempted to focus on the crucial beneficial issues of mesenchymal stem cells for the skeletal muscle regeneration and repair. They discussed the overall ideas and future prospects of skeletal muscle regeneration (*in vitro*) using MSCs on 3D scaffold with optimum experimental conditions (use of various media's, growth factors etc.).

CHAPTER III

MATERIALS AND METHODS

3.1 Culture Medium and Supplements

3.1.1 Stock medium

The contents of one Bottle of Dulbecco's Minimum Essential Medium - Low Glucose (DMEM-LG) were dissolved in 700 ml of autoclaved triple distilled water; to this 3.7 g of $NaHCO_3$ was added and then filtered through 0.22µm membrane filter. The filtered stock was incubated at 37ºC for 48 hr to check sterility and then stored at 4ºC till further use.

3.1.2 Growth Medium (GM)

The growth medium (100 ml) for canine mesenchymal stem cell culture was prepared as below:

Stock (DMEM-LG)	70 ml
Fetal Calf Serum	12 ml
Essential Amino Acids	1 ml
Non Essential Amino Acids	1 ml
Vitamins	1.25 ml
Antibiotic (Antimycotic)	200 ul
TDW	upto 100 ml

The pH of GM was adjusted to 7.2 followed by filtration through 0.22µm membrane filter. The growth medium was stored at 4ºC after checking sterility by incubating at 37ºC for 48 hours.

3.1.3 Osteogenic Differentiation Medium (ODM)

The ODM (100 ml) was prepared as mentioned below:

Stock (DMEM-LG)	70 ml
Fetal bovine serum	10 ml
Dexamethasone (100nM)	200 µl
Ascorbic acid (50ng/ml)	5 µl
β- glycerophosphate (10mM)	216 mg
TDW	upto 100 ml

3.1.4 Chondrogenic Differentiation Medium (CDM)

The CDM (100 ml) was prepared as mentioned below:

Stock (DMEM-LG)	70 ml
Fetal Bovine serum	10 ml
TGF-β (10ng/ml)	200 µl
Dexamethasone (100mM)	200 µl
L-Ascorbic acid-2-Phosphate (50ng/ml)	5 µl
TDW	upto 100 ml

3.1.5 Adipogenic Differentiation Medium (ADM)

The ADM (100 ml) was prepared as mentioned below:

Stock (DMEM-LG)	70 ml
Fetal Bovine Serum	10 ml
Sodium pyruvate (10mM)	110 mg
Human recombinant insulin (0.1mg/ml)	100 µl

Dexamethasone (1µM)	2 ml
Indomethacin (0.2mM)	1.098 ml
IBMX (1mM)	2.2 ml
TDW	upto 100 ml

3.1.6 Phosphate buffered saline (PBS)

Sodium Chloride (NaCl)	8.0 g
Potassium Chloride (KCl)	0.2 g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.2 g
Disodium hydrogen orthophosphate (Na_2HPO_4)	1.15 g

Above components were mixed in 800 ml triple distilled water, pH was adjusted to 7.2 with the help of NaOH & HCl, volume was made upto 1000 ml and then sterilized by autoclaving.

3.1.7 Trypsin Phosphate Glucose Versine (TPGV) solution

Trypsin	0.1 g
Versine (EDTA)	0.2 g
Glucose	0.5 g
Phosphate buffered Saline (PBS)	100 ml

The TPGV solution was prepared with the above composition, filter sterilized using 0.22µm membrane filter and stored at 4ºC in 10ml aliquots till further use.

3.2 Collection of bone marrow samples: details of sampling

The bone marrow samples were collected from the fracture cases of dogs brought to the Teaching Vety. Clinical complex, GADVASU, Ludhiana for isolation of cMSC. The dogs were

anaesthetized using i/v aceprom, xylagene and ketamine prior to operation. After 15-20 min, when the dog was sedated and unconscious, the part of the iliac crest (wing of ilium) was shaved with the help of blade. The area was properly cleaned and prepared for the procedure. Once the dog was under complete anaesthesia, sterilized Salah's needle was inserted into the iliac crest inside the bone. Then a 20ml heparinized syringe was attached to the Salah's needle and the bone marrow was aspirated slowly. After collection of the bone marrow sample, it was mixed properly by shaking the syringe, so that it does not form a clot and then immediately transferred to the laboratory for further processing.

3.3 Processing of bone marrow samples for isolation of cMSC

Upon arrival, the bone marrow sample was passed through the 18 gauge & then 20 gauge needle to break the cell clumps. The cell suspension was then carefully layered over four ml of Histopaque- 1077 in a 15ml centrifuge tubes. The tubes were then centrifuged (swing out rotor, REMI) at 1600 rpm (400g) for 35 min. After centrifugation, the mononuclear cell suspension was visible as a buffy coat at the interphase of two solutions which consisted of lymphocytes and stem cells. The buffy coat was carefully removed and put in to a fresh 15ml centrifuge tube having 4-5 ml of washing medium (DMEM-LG (stock) and antibiotic). The tubes were again centrifuged at 1000 rpm for 10 mins to get the cell pellet. Finally, the cell pellet was resuspended in 5 ml of growth medium and cultured in 25cm^2 cell culture flasks with 5% CO_2 in an incubator .

3.3.1 Subculturing of cMSC cells

The processed bone marrow cell suspension cultured in 25 cm^2 cell culture flasks was kept in an incubator at 37^oC, undisturbed for 48-72 hours. Then the flasks were observed under inverted microscope for attachment of MSCs. The GM was replaced at 2-3 days interval with

new GM and these flasks were observed regularly for growth of the cMSCs. Once 70-80% confluent, monolayer of cMSC cells was formed it was subjected to trypsinization for subculture.

Spent GM media was discarded and the cell monolayer was given two washings with PBS (pH 7.2). Then 1 ml of TPGV solution was added to cover the surface of cells and the flask was kept at 37ºC for 30 seconds for cell detachment. GM was added to the flask for neutralizing the effect of trypsin after observing cell detachment from the surface using an inverted microscope. Pipetting was done to detatch cells from the surface of tissue culture flask and to make a uniform suspension. New flasks were seeded with these cells at a split ratio of 1:2. The seeded new flasks were supplemented with 5 ml of GM and incubated at 37ºC in a CO_2 incubator for growth & multiplication of cMSCs.

3.4 Cryopreservation /Freezing of the Cells

The healthy and growing cells in their exponential growth phase were cryopreserved for further future use.

3.4.1 Freezing mixture for cMSC

Growth medium	60%
Fetal calf serum	30%
Dimethyl sulfoxide	10%

The properly mixed freezing mixture was filtered through 0.22µm sterile membrane filter and was stored at 4 º C till use.

3.4.2 Freezing of Cells

The 70-80% confluent, monolayer of cMSC cells was harvested by using TPGV solution as mentioned earlier and then centrifuged at 1200 rpm for 10 mins. The supernatant was

discarded and the cell pellet was resuspended in freezing mixture. This cell suspension was put into the sterile cryovials in one ml aliquots after proper labelling and then the caps were closed tightly. The vials were kept in a gradual cooler (Nalgene), then placed at -80ºC deep freezer for overnight and finally kept in liquid nitrogen container.

3.5 Differentiation of cMSC

cMSC at passage two or three were cultured in 6-well cell culture plates (nunc plates). Once the cMSC reached 70-80% confluence, differentiation medium (osteogenic, adipogenic and chondrogenic differentiation medium) was added to the wells. The medium was changed every 48 hours and the cells were allowed to grow in differentiation medium and differentiation was carried out for 3 weeks. Photographs were taken at various stages of differentiation under Nikon inverted microscope.

3.6 Detection of Cell specific marker expression at mRNA levels by reverse transcriptase polymerase chain reaction (RT-PCR)

3.6.1 Total RNA isolation

Total RNA was extracted from the undifferentiated and differentiated cMSC by Trizol method using the following protocol:

The GM/ differentiation medium was removed from the 6-well cel culture plates.

1. 1 ml of TRI reagent (Ambion) was added to each well of a 6-well culture plate and incubated at room temperature for 5 mins.
2. After vigorous pipetting the suspension was put into 1.5 ml sterile eppendorf tubes.
3. The tubes were centrifuged at 12000 rpm for 10 min at 4^{o}C (optional).

4. The supernatant was transferred to fresh eppendorf tubes and 100µl of chloroform per 1 ml of TRI reagent solution was added to the tubes.
5. The tubes were again centrifuged at 12000 rpm for 10-15mins at 4^{o}C.
6. The aqueous phase was transferred to a fresh eppendorf tube.
7. 500 µl of isopropanol per 1 ml of TRI Reagent solution was added and vortexed for 5-10 sec.
8. The tubes were incubated at room temperature for 5-10 min.
9. The tubes were centrifuged at 12000 rpm for 8 min at room temp. and the supernatant was discarded.
10. 1 ml of 75% ethanol per 1 ml of TRI Reagent Solution was added to the tubes.
11. The tubes were centrifuged at 7500 rpm for 5 min.
12. The ethanol was removed and the RNA pellet was air dried briefly.
13. The RNA was dissolved in 50 µl Nuclease Free Water.
14. The tubes were stored at -80^{o}c.

3.6.2 DNase Treatment of RNA

The RNA was given DNase treatment to remove all the unwanted DNA.

RNA	-	40µl
RNA free DNase I (Invitrogen)	-	1µ (1U/µl)
$MgCl_2$ (50 mM)	-	0.8 µl

1. The RNA reaction mixture was incubated at 37^{o}C for 2 hrs.

2. Then the tube was incubated at 95^{o}C for 10 min to denature the DNAase.

3. Finally, the concentration of RNA was measured using Nanodrop

3.7 First strand cDNA synthesis

Total RNA extracted was converted to cDNA using first srand cDNA synthesis kit (Fermentas,) as follows:

After thawing, all the components of the kit were mixed properly and were kept on ice. In the first step, 10 µl of total RNA extracted from each sample was taken into separate sterilised PCR tubes (200 µl) and 1µl of random hexamer primers were added into each tube, making a total volume of 11ul.

RNA	10 µl
Random hexamer primer	1µl
Total volume	11 µl

This mixture was centrifuge briefly and was incubated at 65°C for 5 min and then snap cooled on ice immediately. After that the following reaction mixture was prepared:

5X Reaction Buffer	4 µl
RiboLock™ RNase Inhibitor (20u/ul)	1 µl
10 mMdNTP Mix	2 µl
M-MuLV Reverse Transcriptase (20u/ul)	2 µl
Total volume	9 µl

These components were mixed properly, briefly centrifuged and 9 µl of above mixture was added in to PCR tubes containing RNA and random hexamer primer making a total volume of 20 µl.

The below mentioned conditions were followed for synthesis of cDNA:

Temperature	Time	No. of Cycles	Remarks
25^{o}C	5 mins	1	Incubation
37 ^{o}C	60 mins	1	Reverse transcription
70 ^{o}C	5 min	1	Stopping the reaction

3.8 Characterization/ Detection of cMSC differentiation by RT- PCR:

Expression of unique specific marker proteins (Osteopontin, SOX9 and LpL) for osteogenic, chondrogenic & adipogenic differentiation confirmation. As differentiated cells express some unique cell specific protein markers.

Therefore, detection of expression of these marker genes by RT-PCR can give indication about the cMSC differentiation.

Primer sequences used for amplification of specific genes are listed as below.

Table 1: Oligonucleotide primers used in this study for amplification of specific genes

Primer	Primer Sequence	Size	Reference
Osteopontin-F	5′-CATATGATGGCCGAGGTGATAG-3′	114 bp	Zucconi *et al* 2010
Osteopontin-R	3′-CAAGTGATGTGAAGTCCTCCTC-5′	114 bp	-do-
Sox9-F	5′-GCTCGCAGTACGACTACACTGAC -3′	101 bp	-do-
Sox9-R	5′-GTTCATGTAGGTGAAGGTGGAG-3′	101 bp	-do-
LPL-F	5′- ACACATTCACAAGAGGGTCAC-3′	132 bp	-do-
LPL-R	5′- CTCTGCAATCACACGGATG-3′	132 bp	-do-

GAPDH-F	5’-CAAGGTCATCCATGACAACTTTG-3’	496 bp	cDNA kit, Fermentas
GAPDH-R	5’-GTCCACCACCCTGTTGCTGTAG-3’	496 bp	-do-

3.8.1 RT-PCR detection of osteogenic differentiation:

In this study, cDNA prepared from the RNA samples extracted from the cells (osteocytes) following osteogenic differentiation was subjected to PCR amplification of osteopontin gene. The following reaction mixture was prepared using osteopontin gene specific primers- osteopontin F & R (Table 1):

Master mixComponent	**Volume/reaction (µl)**
RNase-free water	13.5
10 x buffer (Invitrogen, without MgCl2)	2.5
dNTP Mix (containing 10 mM of each dNTP).	1.0
Osteopontin (forward)	1.0
Osteopontin (reverse)	1.0
Taq polymerase (Invitrogen 5U/µl)	0.2
$MgCl_2$	0.8
Total volume	**20.0**

Above reaction mixture was mixed properly and briefly spun. In PCR tubes, 20 µl of this mixture and 5µl of cDNA of osteocytes was added, spun briefly and then PCR tubes were put in thermocycler with the following cycling conditions:

Temperature	**Time**	**Steps**	**No. of Cycles**
95 ^{o}C	3 min	Initial Denaturation	1
94^{o}C 50^{o}C 72^{o}C	45sec 30 sec 45 sec	Denaturation Annealing Extension	35

72 ^{o}C	10 min	Final Extension	1

PCR products obtained were stored at -20^{o}C till further use.

3.8.2 RT-PCR detection of SOX9 gene expression in the differentiated chondrocytes

cDNA for amplification of SOX9 gene from differentiated chondrocytes was prepared in a similar way as described for Osteopontin gene in section 3.8.1

RT PCR was performed using Fermentas first strand cDNA synthesis kit followed by PCR amplification by Taq polymerase (Invitrogen)**.** Reaction mixture composition for SOX9 gene amplification was prepared as mentioned below:

Component	**Volume/reaction (µl)**
RNase-free water	13.5
10x PCR Buffer	2.5
dNTP mix (10 mM each)	1.0
Primer 1F	1.0
Primer 2R	1.0
Taq Polymerase (5 U/ul)	0.2
$MgCl_2$ (50mM)	0.8
Total volume	**20**

These components were mixed properly, briefly spun and 5µl of cDNA prepared from chondrocytes was added in to it. The tubes were then put in a thermocycler (Eppendorf) with the following cycling conditions.

Cycling Conditions

Temperature	Time	Steps	No. of Cycles
94 ^{o}C	4 min	Initial Denaturation	1
94^{o}C 50^{o}C 72^{o}C	1min 1 min 45sec	Denaturation Annealing Extension	35
72 ^{o}C	10 mins	Final Extension	1

PCR products were stored at -20^{o} C till further use.

3.8.3 RT-PCR detection of LPL (Lipoprotein lipase) gene expression in differentiated adipocytes

The cDNA for amplification of LPL gene from differentiated cMSCs was prepared in a similar way as described for Osteopontin gene in section 3.8.1

RT PCR was performed using Fermentas first strand cDNA synthesis kit followed by PCR amplification by Taq polymerase (invitrogen)**.** Reaction mixture composition for LPL gene amplification was as follows:

Component	Volume/reaction (µl)
RNase-free water	13.5
10x PCR Buffer	2.5
dNTP mix (10 mM each)	1.0
Primer Forward	1.0
Primer Reverse	1.0
Taq Polymerase (5 U/ul)	0.2

$MgCl_2$ (50mM)	0.8
Total volume	**20**

These components were mixed properly, briefly spun, 5µl of cDNA prepared from the RNA of adipocytes was added into the master mix and the tubes were then put into thethermocycler with the following cycling conditions.

Cycling Conditions

Temperature	Time	Steps	No. of Cycles
94 ^oC	4 min	Initial Denaturation	1
94^oC 50^oC 72^oC	1min 1 min 45sec	Denaturation Annealing Extension	35
72 ^oC	10 mins	Final Extension	1

PCR products were stored at -20^o C till further use.

3.8.4 RT-PCR detection of GAPDH as a control from differentiated and undifferentiated cells

cDNA for GAPDH control from differentiated and undifferentiated cMSCs was prepared in a similar way as described for Osteopontin gene in section 3.8.1

RT PCR was performed using Fermentas first strand cDNA synthesis kit followed by PCR amplification by Taq polymerase (Invitrogen)**.** Reaction mixture composition for LPL gene amplification was as follows:

Component	**Volume/reaction** µl
RNase-free water	13.5
10x PCR Buffer	2.5
dNTP mix (10 mM each)	1.0

Primer 1F	1.0
Primer 2R	1.0
Taq Polymerase (2.5U/ul)	0.2
$MgCl_2$(50mM)	0.8
Total volume	**20**

These components were mixed properly, briefly spun and 5µl of cDNA was added in 20µl of master mix then put in a thermocycler with the following cycling conditions.

Cycling Conditions

Temperature	Time	No. of Cycles	Remarks
94 ^{o}C	4 min	1	Initial Denaturation
94^{o}C 58^{o}C 72^{o}C	30sec 30sec 45sec	35	Denaturation Annealing Extension
72 ^{o}C	10 mins	1	Final Extension

PCR products were stored at -20^{o} C till further use.

3.9 Agarose Gel Electrophoresis

3.9.1 Solutions and buffers

Stock solution of Tris Borate EDTA (TBE) buffer (10x).

Tris base	108 g
Boric acid	55 g
EDTA (0.5M)	27.5 g
DW add to	1000 ml

Working solution of TBE buffer (0.5 x)

10X TBE	50 ml
DW add to	1000 ml

Agarose gel

Agarose	0.3 g
0.5x TBE	30 ml
Ethidium bromide (10mg/ml)	3µl

3.9.2 Analysis of PCR products by agarose gel electrophoresis

The PCR amplified products were analysed by agarose gel electrophoresis. One percent agarose was prepared in 0.5 x TBE buffer in conical flask and was heated to dissolve the agarose completely using a microwave. The gel was cooled down to a temperature of around 55-60^{o}C and ethidium bromide solution (stock conc 10mg/ml) was added into the gel to make the final concentration 0.5ug/ml. Gel was poured into a gel casting tray having comb of appropriate number and sizes hung into that and then allowed to solidify at room temperature.

Once solidified, the gel was placed in electrophoresis tank containing 0.5x TBE and then the comb was removed from the gel. PCR products and 6X loading dye (containing 0.03% bromophenol blue, 0.03% Xylene cyanole FF, 0.4% Orange G, 15% Ficoll 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA) were mixed and were loaded in the wells with the help of micropipette. Five µl of DNA ladder (100 base pair plus, Fermentas) was also loaded in one of the wells to access the size of PCR products.

The electrophoresis was carried out for 30-45 mins at 80 volts and the gel was viewed under Geldoc (Bio Rad) system & photograph was taken.

3.10 Cytochemistry

The cMSC following differentiation after 21 days were assessed by histochemistry:

3.10.1 Demonstration of osteogenic differentiation:

This was denoted by accumulation of mineralized calcium phosphate by Von Kossa stain.

Procedure for Von Kossa staining:

1. The differentiated cMSCs in the 6-well plates were fixed using 4% formaldehyde in PBS for 15 min.

2. After fixation, Silver nitrate solution (5%) was added and exposed to direct sunlight, (or ultra-violet lamp, or a 100-watt lamp) light for 30 min.
3. The wells were then rinsed with distilled water.
4. Sodium thiosulphate (5%) solution was added to the wells for 2 mins.
5. The wells were then rinsed with distilled water.
6. Counterstaining was done in nuclear fast red solution for 5 mins.
7. The wells were then rinsed with distilled water.
8. The stained slides were then observed under microscope & photographs were taken.

3.10.2 Demonstration of chondrogenic differentiation.

This was demonstrated by mucopolysaccharide-rich extracellular matrix staining with Alcian Blue.

Procedure for Alcian Blue staining:

1. The differentiated cMSCs in the 6-well plates were fixed using 4% formaldehyde in PBS for 15-20 min.
2. Alcian Blue (0.1%) stain was added to the wells for 30 mins.
3. The wells were then rinsed with distilled water
4. Counterstaining was done with eiosin for 30 secs.
5. The wells were then rinsed with distilled water.
6. The stained slides were then observed under microscope & photographs were taken.

3.10.3 Demonstration of adipogenic differentiation

This was confirmed by intracellular accumulation of lipid-rich vacuoles stained with Oil Red O.

Procedure for Oil Red O staining:

1. The differentiated cMSCs in the 6-well plates were fixed using 4% formaldehyde in PBS for 15-20 min.
2. Absolute propylene glycol was added to the wells for 2 min.
3. Oil red O solution was added to the wells for 1 hr.
4. Differentiation of the wells was done using 85% propylene glycol solution for 5 min.
5. The wells were then rinsed with distilled water, two changes.
6. Counterstaining was done in Mayer's or Harris hematoxylin solution for one minute.
7. The wells were then rinsed with distilled water, two changes.
8. The stained slides were then observed under microscope & photographs were taken.

3.11 Immunocytochemistry

Monoclonal antibody markers CD44 (regeneration technologies), CD105, CD34 & CD45 (Biogenex) specific to cMSCs were used to characterize the cMSC by immunocytochemistry using Alexa Flour 488 anti-mouse IgG as secondary antibody.

Immuno-staining procedure:

1. The cMSCs were allowed to grow upto 70-80% confluence with the addition of fresh GM media in 8-well cell culture chamber slide.
2. The cells were washed thoroughly in PBS for 5X2 min after removing the medium.
3. The cells were then fixed with a solution mixture of 95% ethanol & 5% glacial acetic acid for 3-5 min.
4. The cells were then rinsed in PBS for 5x2 min.

5. Half of the wells were permeabilized with 0.5% Triton X-100 for 5 min.
6. The cells were then rinsed in PBS for 3x5 min.
7. Blocking was done with 1% BSA for 15min.
8. Primary antibody (1:500 dilution) was added and the culture slide was incubated at 37^{o}c for one hour.
9. The cells were then rinsed in PBS for 3x5 min.
10. Secondary antibody Alexafluor–488 anti-mouse IgG (1:1000 dilution) was added into the wells marked and incubated at 37^{o}c for 1 hr.
11. The wells were washed with PBS for three times, PBS was removed from the chamber slides, upper chamber was removed from the slide and allowed to air dry.
12. Immediately, two drops of prolong Gold DAPI mounting solution (Invitrogen) was added over the slide at two different places and one large sized cover slip (24 X 60 mm) was placed over the slide. The cover slip was pressed little bit for uniform distribution of the mounting solution and to remove air bubbles if there was any.
13. The slide was then observed under microscope for fluorescence.

3.12 Immuno-staining for differentiated cMSCs

Antibody markers specific to differentiated (osteocytes, chondrocytes and adipocytes) cMSCs were used to characterize the cMSC for the differentiation property using anti-collagen type 1 antibody, anti-SOX9 antibody and anti-PPAR-gamma antibody, respectively. The secondary antibodies used were Alexa fluor 488 anti-mouse and anti-rabbit IgG.

Immuno-staining procedure:

1. The cMSCs were allowed to differentiate for 21 days by addition of fresh media in 6-well cell culture plates.
2. The cells were washed thoroughly in PBS for 5X2 min after removing the medium.

3. The cells were then fixed with a solution mixture of 95% ethanol & 5% glacial acetic acid for 3-5 min.
4. The cells were then rinsed in PBS for 5x2 min.
5. The wells were permeabilized with 0.5% Triton X-100 for 5 min.
6. The cells were then rinsed in PBS for 3x5 min.
7. Blocking was done with 1% BSA for 15 min.
8. Primary antibody PPAR-gamma (1:200 dilution), anti-collagen type 1 (1:800 dilution) & SOX9 (5µg/ml dilution) was added and the culture plate was incubated at 37^{o}c for one hour.
9. The cells were then rinsed in PBS for 3x5 min.
10. Secondary antibody Alexafluor–488 anti-mouse IgG; Alexafluor anti-rabbit IgG (1:1000 dilution) was added into the corresponding wells and incubated at 37^{o}c for 1 hr.
11. The wells were washed with PBS for three times, PBS was removed from the chamber slides; upper chamber was removed from the slide and allowed to air dry.
12. Immediately, two drops of prolong Gold DAPI mounting solution (Invitrogen) was added over the slide at two different places and one large sized cover slip (24 X 60 mm) was placed over the slide. The cover slip was pressed little bit for uniform distribution of the mounting solution and to remove air bubbles if there was any.
13. The plate was observed under microscope for fluorescence.

CHAPTER IV

RESULTS AND DISCUSSIONS

Stem cells have been extensively studied over the past two decades because these cells have a remarkable potential to develop into various specific cell lineages upon being cultured in appropriate conditions. As they have been considered as a powerful tool for cell or tissue – based engineering in human and veterinary medicine, the present study aimed to isolate, in-vitro differentiate and characterize the canine mesenchymal stem cells (cMSCs).

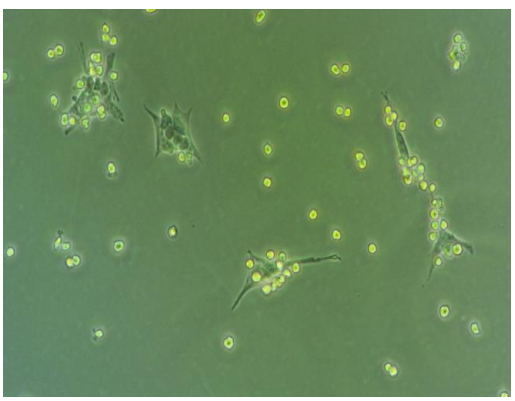
4.1 ISOLATION OF cMSC

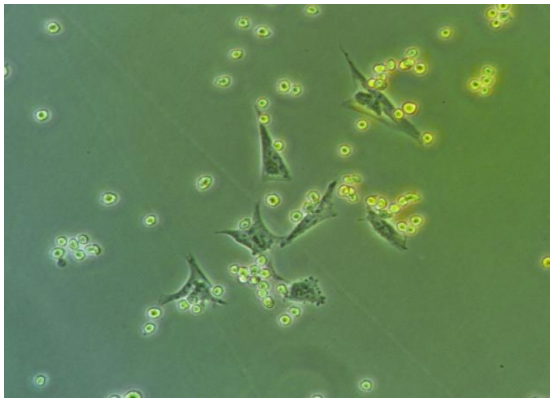
Initially very few cells adhered to the flask surface (fig.1 A & B) but subsequently when the GM medium was changed every 48hr during the culture, the cells started to grow and divide gradually and 60-70% confluent monolayer of the cMSCs was formed following 9-10 days of initial culture (Fig.2). Similar finding have been reported by Tharasanit *et al* (2011) who processed the bone marrow by isolating the cells using Histopaque gradient – 1077.

4.2 Characterization of cMSC

4.2.1 cMSC characterization by immunocytochemistry

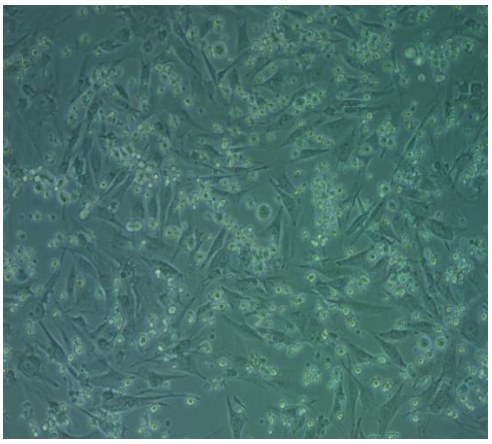
MSCs at the 3^{rd} to 5^{th} passage were observed for their surface marker expression by immunocytochemistry techniques. The cells were fixed using a mixture of 95% ethanol and 5% glacial acetic acid. Immuno staining was carried out using anti-canine CD44 FITC (fourescene Isothiocyanate), CD105, CD34, and CD45 antibodies as primary antibodies and Alexa fluor 488 anti-mouse IgG as secondary antibody and prolong Gold

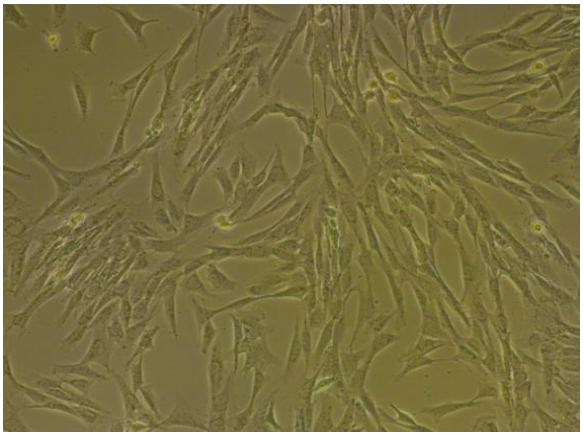




A **B**

Fig: 1 Canine mesenchymal stem cells following 48 hours of culture. (A & B): Initial attachment & growth of few numbers of cMSC.



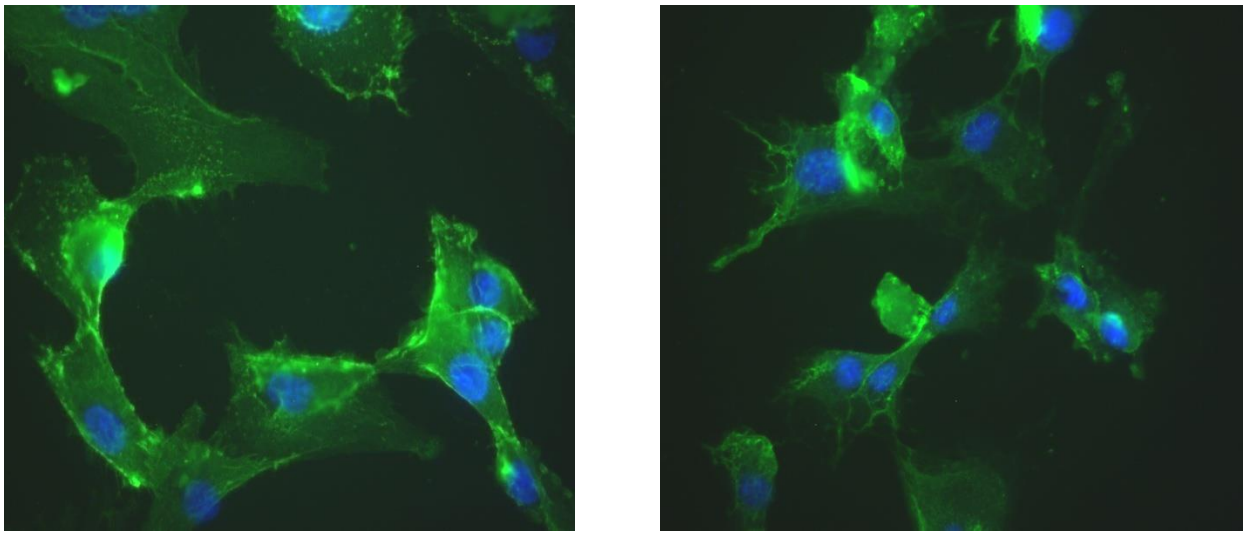


A **B**

Fig. 2 (A) cMSCs in culture after 5-6 days **Fig. 2 (B) cMSCs at 60-70% confluency**

DAPI was used for nuclear staining. MSCs showed positive expression of surface markers CD44 (fig.3 A) and CD105 (fig.3 B) as revealed by green colour flouresence all over the cells with a blue stained nucleus. The hematopoeitic markers CD34 (fig.4 A) and CD45 (fig.4 B) did not show any flourescence indicating absence of these markers in the cMSCs. Csaki *et al* (2007) also found similar immunostaining results against these markers CD105, CD34 and CD45.

cMSCs were evaluated for their differentiation potential by immunocytochemistry. The cells 21 days post differentiation were fixed using a mixture of 95% ethanol and 5% glacial acetic acid. Immuno staining was done using anti- collagen type 1 (for osteocytes), anti - SOX9 (for chondrocytes) and anti – PPAR-gamma (for adipocytes) as primary antibodies and Alexa fluor – 488 anti – mouse and anti – rabbit IgG as secondary antibodies. Prolong Gold antifade reagent DAPI was used to stain the nuclei. Differentiated cMSCs i.e. osteocytes, chondrocytes and adipocytes showed positive labeling for anti – collagen type 1 (fig.5), SOX9 (fig.6) and PPAR- gamma (fig.7) respectively as revealed by green fluorescence over the cells with blue stained nuclei. These results indicated proper osteogenic, chondrogenic & adipogenic differentiation of the cMSC in this *in vitro* study. The undifferentiated cMSCs did not show any fluorescence (fig.5, fig.6 and fig.7) indicating the absence of these markers. Vieira *et al* (2010) did immuno-histochemistry and immunoflourescence with anti-collagen type II antibody for chondrogenic differentiation and anti-myosin antibody for myosin differentiation and found that these differentiated cells were positively labeled by these antibodies.

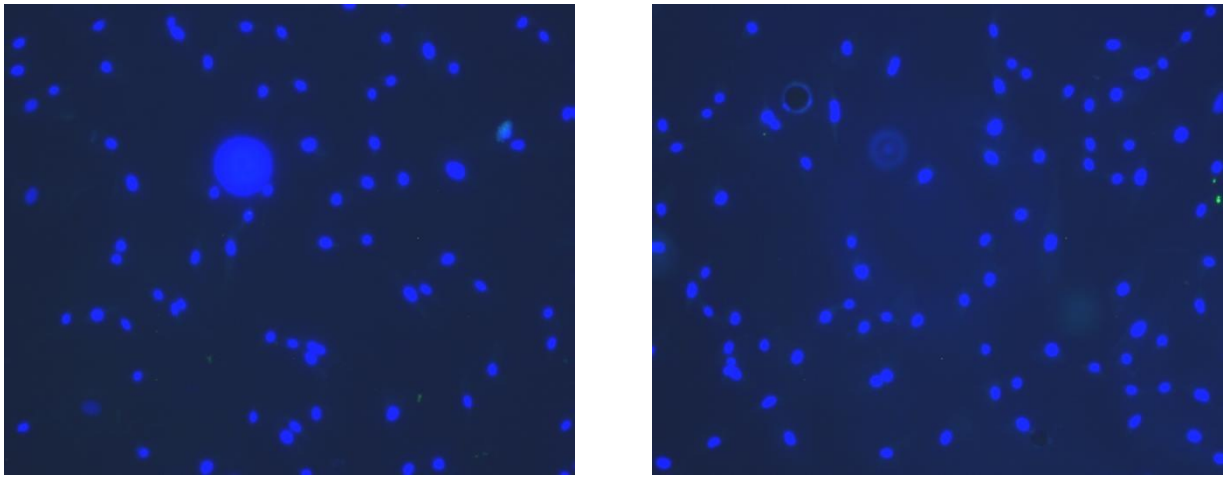


A **B**

Fig. 3. Immunofluorescence labeling of undifferentiated cMSCs with stem cell markers

A. Isolated cMSCs showed a strong positive signal for stem cell specific marker $CD44^+$

B. Isolated cMSCs showed a strong positive signal for stem cell specific marker $CD105^+$

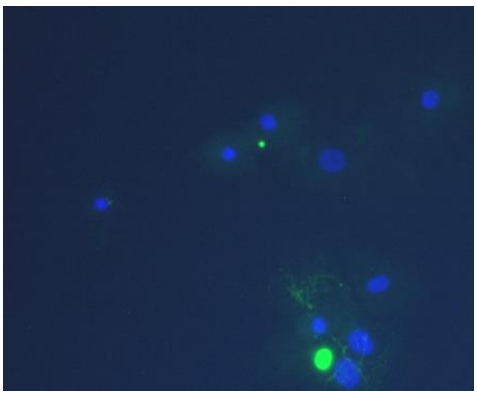


A **B**

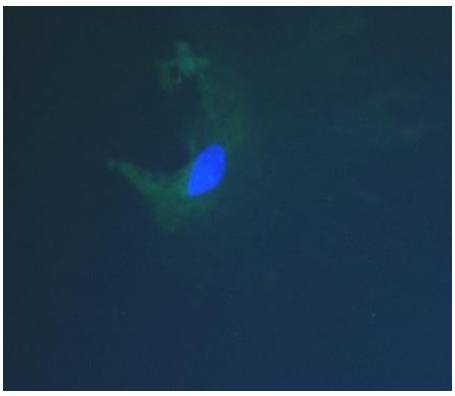
Fig. 4. Immunoflourescence labeling of undifferentiated cMSCs with hematopoietic stem cell markers

A. No fluorescence with hematopoietic stem cell marker CD34

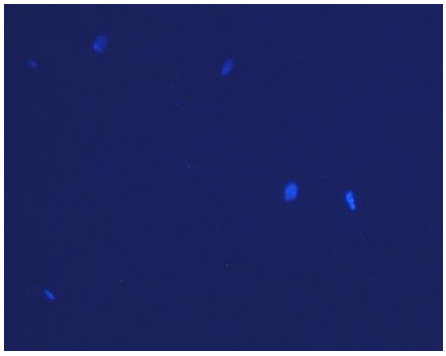
B. No fluorescence with hematopoietic stem cell marker CD45



(A)



(B)

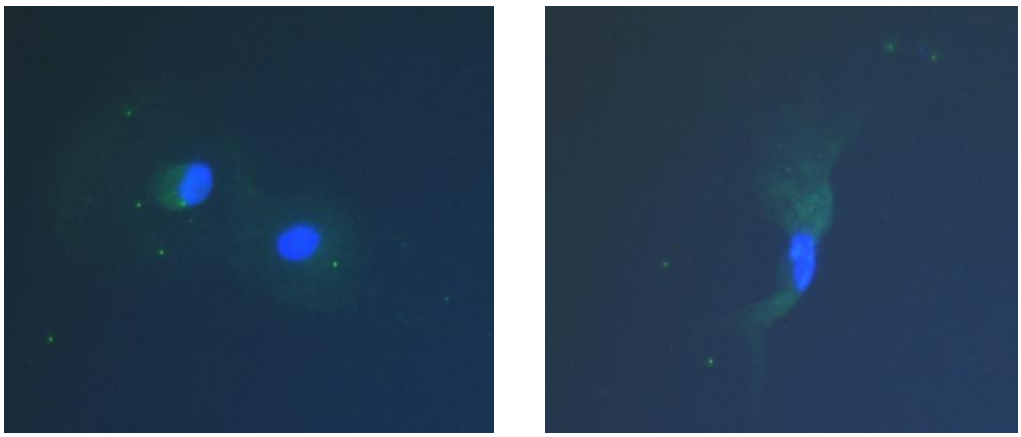


(C)

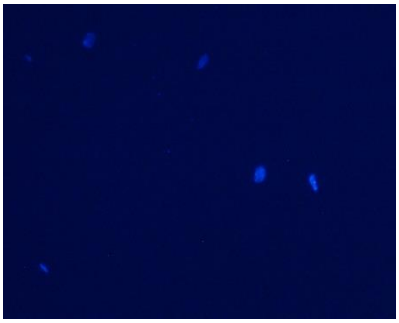
Fig.5 Immunoflourescence detection of osteogenic differentiation by anti-collagen type 1 antibody

A & B. Osteocytes positively labeled showing green fluorescence with nuclei stained blue by anti-collagen type 1

C. No fluorescence in undifferentiated cMSCs with anti-collagen type 1

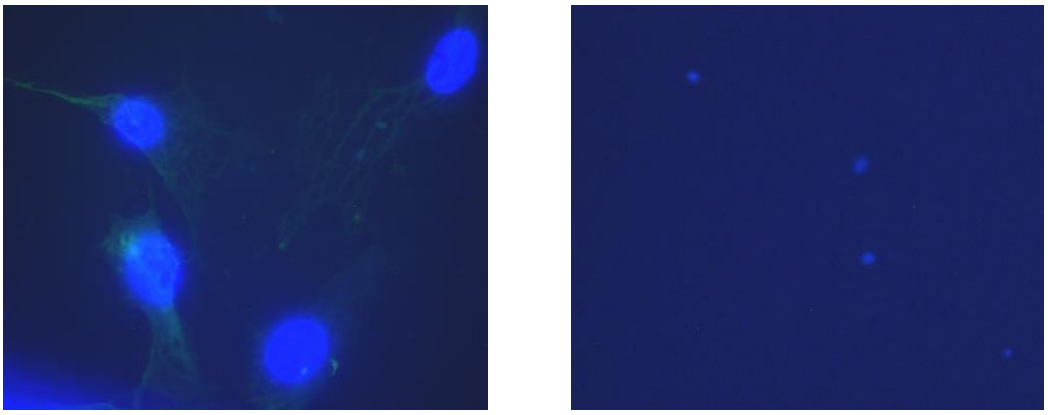


(A) **(B)**



(C)

Fig.6 Immunoflourescence detection of chondrogenic differentiation by anti-SOX9
(A & B) Chondrocytes positively labeled showing green fluorescence with nuclei stained blue by anti-SOX9
(C). No fluorescence in undifferentiated cMSCs with anti-SOX9



A **B**

Fig.7 Immunoflourescence detection of adipogenic differentiation by anti-PPAR-gamma
A. Adipocytes positively labeled showing green fluorescence with nuclei stained blue by anti-PPAR-gamma
B. No fluorescence in undifferentiated cMSCs with anti-PPAR-gamma

4.2.2 RT-PCR – detection of cells specific markers gene expression in the differentiated cMSC.

Total RNA was extracted from the differentiated cells after 21 days of differentiation and also from the non-differentiated cells. Around 200 ng of RNA was used for synthesis of cDNA. The cDNA was used for PCR detection of osteogenic, chondrogenic and adipogenic differentiation by using marker gene osteopontin, SOX9 and Lpl specific primers, respectively. Lpl gene involved in adipogenic differentiation was amplified with an amplicon size of 132 bp in the PCR (fig.8 A). SOX9 gene involved in chondrogenic differentiation was amplified with an amplicon size 101 bp in the PCR (fig.8 B). PCR could amplify Osteopontin gene involved in osteogenic differentiation with an amplicon size of 114 bp (fig.8 C). These marker genes were not detected in the undifferentiated cMSCs. These RT-PCR results further confirm the osteogenic, chondrogenic & adipogenic differentiation of the cMSC in this study. Similar results were also found by Zucconi *et al* (2010); Vieira *et al* (2010) in which these specific marker genes showed similar amplification.

~132 bp

~101 bp

4.3 In-vitro differentiation of cMSC: Characterization of cMSCs differentiation by cytochemistry

4.3.1 Chondrogenic differentiation

Subconfluent cells were cultured in chondrogenic differentiation medium which was replaced every 2-3 days and the cells were fixed on 22nd day with 4% formaldehyde. Chondrogenesis was demonstrated by Alcian blue staining that stained the glycosaminoglycan enriched matrix of the chondrocytes as blue, having a round granular morphology as revealed by the cytochemistry (Fig. 9). Neupane *et al* (2008) also carried

Fig. 8 (A) RT-PCR amplification of LPL gene from the differentiated adipocytes

M – molecular weight marker (100 bp plus)

P – PCR product of Lpl gene with amplicon size of 132 bp

C – control of undifferentiated cMSCs control with no RT-PCR amplification

Fig. 8 (B) RT-PCR amplification of SOX9 gene from the differentiated chondrocytes

M – molecular weight marker (100 bp plus)

P – PCR product of Lpl gene with amplicon size of 101 bp

C – control of undifferentiated cMSCs control with no RT-PCR amplification

Fig. 8 (C) RT-PCR amplification of Osteopontin gene from the differentiated osteocytes

M – molecular weight marker (100 bp plus)

P – PCR product of Lpl gene with amplicon size of 114 bp

C – control of undifferentiated cMSCs control with no am

plification

Fig. 8 (D) GAPDH control for differentiated & undifferentiated cMSC cells

M – molecular weight marker (100 bp plus)

O – osteocyte control for GAPDH with amplification of 496 bp

A – adipocyte control for GAPDH with amplification of 496 bp

C – chondrocyte control for GAPDH with amplification of 496 bp

U – undifferentiated cMSC control for GAPDH with amplification of 496 bp

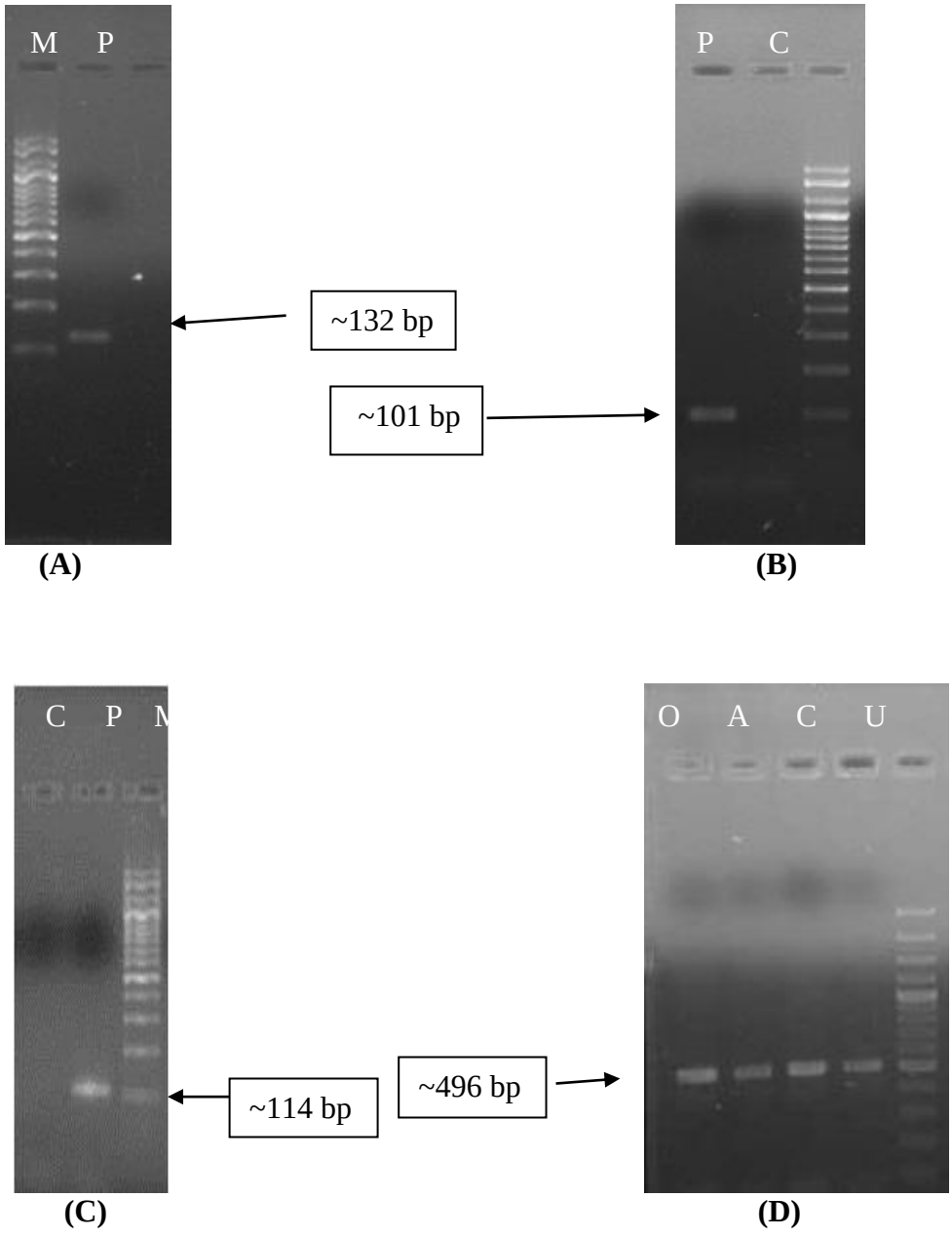
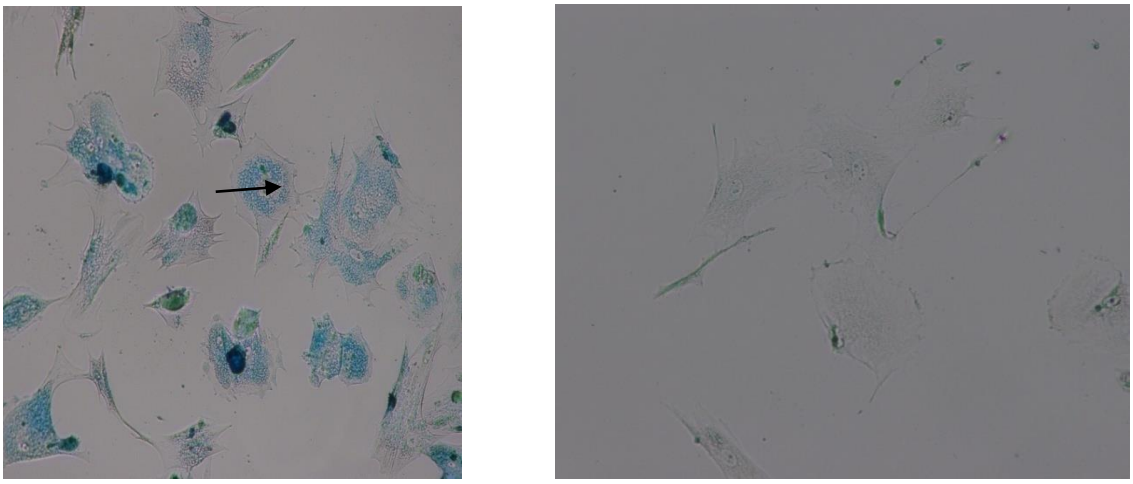


Fig. 8: Detection of cMSC differentiation by RT-PCR

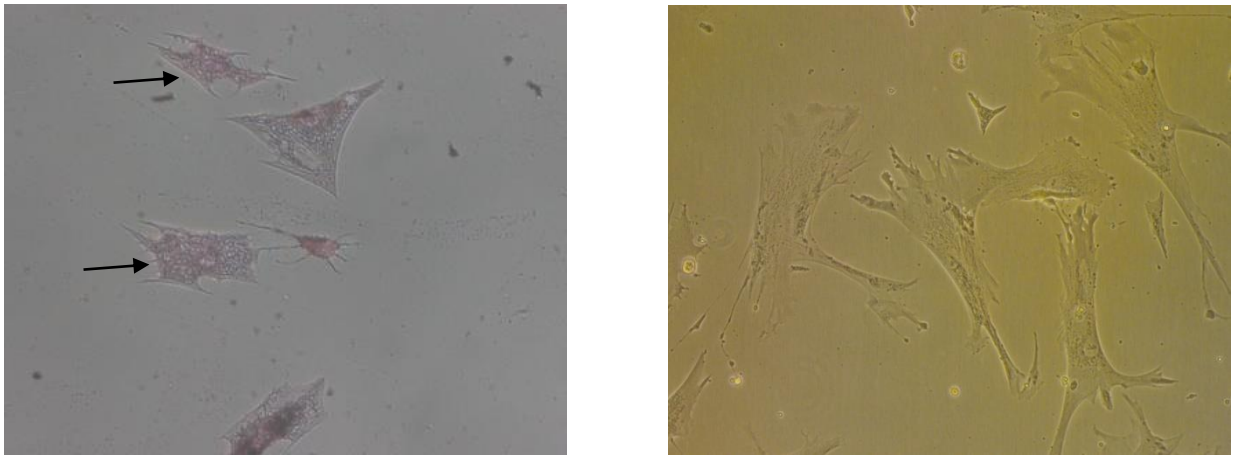


(A) **(B)**

Fig. 9 Chondrogenic differentiation of cMSCs by Alcian Blue staining

A. Glycosaminoglycan enriched matrix of chondrocytes was stained blue.

B. Undifferentiated cMSC did not show any staining reaction with Alcian Blue.



(A) **(B)**

Fig. 10 Adipogenic differentiation of cMSCs by Oil Red O staining

A. Lipid rich vacuoles of adipocytes were stained red.

B. Undifferentiated cMSC did not show any staining reaction with Oil Red O.

out alcian blue staining, showing glycosaminoglycan matrix stained positive for chondrogenic differentiation.

4.3.2 Adipogenic differentiation

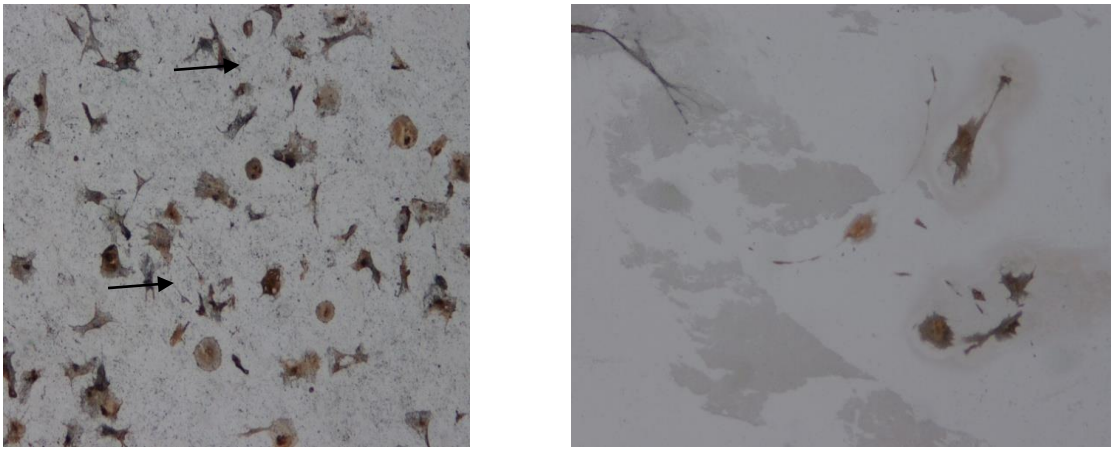
The adipogenic differentiation was performed with 80% confluent cMSCs with adipogenic differentiation medium being replaced every 3 days for 21 days. The adipocytes were fixed using 4% formaldehyde on 22nd day. Adipogenesis was confirmed on day 22 by accumulation of lipid rich vacuoles in the adipocytes showing a triangular morphology as observed under the microscope stained by Oil Red O. (Fig. 10). Viera *et al* (2010) did the Oil Red O staining and found similar results.

4.3.3 Osteogenic differentiation

For osteogenic differentiation, cells were subcultured to reach approximately 80% confluence and the osteogenic induction medium was added to the cells & these cells were kept for 21 days with medium being replaced every 2-3 days. Osteogenesis was confirmed on day 22 by Von Kossa staining that showed accumulation of calcium phosphate stained black in the osteocytes that were observed under microscope having cuboidal morphology. (Fig. 11).

Zucconi *et al* (2010) did Von Kossa staining to confirm calcium mineralization for osteogenic differentiated cells and found similar results.

The cytochemistry study carried out in the differentiated cMSC pretty well detected chondrogenic, adipogenic & osteogenic differentiation in this study.



(A) **(B)**

Fig. 11 Osteogenic differentiation of cMSCs by Von Kossa staining

A. Mineralized calcium phosphate of osteocytes was stained black.

B. Undifferentiated cMSC did not show any staining reaction with Von Kossa.

CHAPTER V

SUMMARY

Mesenchymal stem cells (MSCs), also known as marrow stromal cells or mesenchymal progenitor cells, are recognized by two abilities, first their potential to undergo extensive self – renewal proliferation and second their capacity to differentiate into the mesodermal lineage. These specialized cells are classified as multipotent stem cells because they have the capability of differentiation into the mesodermal lineage. MSCs have been isolated from many tissues of the body such as bone marrow, adipose tissue, umbilical cord and dental pulp. In this study, we successfully isolated and cultured well-defined mesenchymal stem cells derived from canine bone marrow and also differentiated them to osteocytes, chondrocytes and adipocytes.

Bone marrow samples were collected aseptically from the wing of ilium, using the salah's needle from the clinical fracture cases of dogs brought to the Teaching Veterinary Clinical Complex, GADVASU, Ludhiana. The samples were processed first by needle (18 & 20 gaudge) aspiration and then layered over Histopaque-1077 followed by centrifugation to separate the mononuclear (including MSCs) cells from the interphase between the two solutions. The washed cells were then grown in cell culture flask using DMEM-Low Glucose medium (+10% FCS) with 5% CO_2 in an incubator. Initially very few cells were adhered to the flask surface and the cells started to grow and divide gradually. The growth medium was changed every 2-3 days during the culture and the cells reached 60-70% confluence following 9-10 days of initial culture which were then subcultured.

MSCs at the 3rd to 5th passage were observed for their stemness property by using monoclonal antibodies against the specific cell surface markers in immunocytochemistry techniques. Immunostaining was carried out using anti-canine CD44 FITC (fourescene Isothiocyanate), CD105, CD34, and CD45 antibodies as primary antibodies, Alexa fluor 488 anti-mouse IgG as secondary antibody and prolong Gold DAPI for nuclear staining. MSCs showed positive expression of surface markers CD44 and CD105 as revealed by green colour flouresence all over the cells and negative expression of hematopoeitic markers CD34 and CD45, with a blue stained nucleus indicating the intact stemness properties of the cMSCs.

Canine MSCs grown in 6-well cell culture plate at approximately 80% confluent stage were subjected to differentiation studies by growing them in osteogenic, chondrogenic and adipogenic differentioan media for 21 days. The differentiated cMSCs were characterized by RT-PCR detection of the cell specific marker gene expressions, cytochemistry and also by immunocytochemistry using antibodies against these cells specific markers.

Total RNA was extracted from the differentiated cells after 21 days of differentiation and also from the non-differentiated cells and then cDNA was prepared. The cDNA was used for PCR detection of osteogenic, chondrogenic and adipogenic differentiation by using marker gene osteopontin, SOX9 and Lpl specific primers, respectively. PCR could amplify Osteopontin gene involved in osteogenic differentiation with an amplicon size of 114bp, Lpl gene involved in adipogenic differentiation with an amplicon size of 132bp and SOX9 gene involved in chondrogenic differentiation with an amplicon size 101bp indicating proper *in-vitro* differentiation of cMSCs.

The formalin fixed differentiated cMSCs in 6-well plates were subjected to Von Kossa Staining, Alcian Blue staining and Oil Red O staining for determination of osteogenic, chondrogenic and adipogenic differentiation, respectively. Osteogenesis was confirmed by Von Kossa staining that showed accumulation of calcium phosphate stained black in the osteocytes that were observed under microscope having elongated morphology. Chondrogenesis was demonstrated by Alcian blue staining that stained the glycosaminoglycan enriched matrix of the chondrocytes as blue, having a round granular morphology as revealed by the cytochemistry. Adipogenesis was confirmed on day 22 by accumulation of lipid rich vacuoles in the adipocytes showing a triangular morphology as observed under the microscope stained by Oil Red O.

The differentiated cMSC were studied for their differentiation characteristics by immuno- staining techniques using antibodies specific to Collagen type I, SOX9 and PPAR-γ markers for osteoblasts, chondrocytes and adipocytes, respectively. In this study, the Anti-Collagen type I, anti-SOX9 and PPAR-γ antibodies could detect the osteogenic, chondrogenic and adipogenic differentiation of cMSC indicating proper in-vitro differentiation.

CONCLUSION

- Canine mesenchymal stem cells (cMSC) were succesfully isolated and cultured in the laboratory.
- The isolated cMSC showed osteogenic, chondrogenic & adipogenic differentiation potential as revealed by cytochemistry, immunocytochemistry and marker gene expression detected by RT-PCR assays.

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