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Comparative study of Mesenchymal Stem Cells derived from Bone Marrow and Wharton's Jelly and the interaction with natural biomaterials

Bachelor thesis

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Abstract

Adult mesenchymal stem cells (MSCs) are non-hematopoietic stromal cells that can be easily isolated from bone marrow's (BM) micro-enviroment and from the connective tissue surrounding umbilical cord vessels, namely Wharton's jelly (WJ). BM-MSCs and WJ-MSCs display typical MSC characteristics but a comparative study is lacking. Because they are culture-dish adherent, they can be expanded in culture while maintaining their trilineage differentiation potential to adipocytes, osteoblasts and chondrocytes.

MSCs were induced to differentiate *in vitro* to adipocytes and osteoblasts using appropriate culture media. MSC differentiation was assessed with cytochemical stains (Oil Red-O for adipocytic, and Alizarin Red/Von Kossa for osteoblastic differentiation). MSCs are identified by the expression of many molecules including CD73, CD90, and CD105 and are negative for the hematopoietic markers CD45, and CD14.

The multipotency of MSCs, the ease of isolation and proliferation *ex vivo* and immunosuppressive properties define them an attractive field of study and potential candidates for regenerative medicine. Their great immunomodulatory potential together with natural biodegradable, biocompatible and non-toxic materials can develop 3D scaffolds *in vitro* for many applications of tissue engineering. This work focuses on the biology of MSCs, including their differentiation and proliferation potential in vitro, gene expression of specific osteocytic and adipocytic factors, and an attempt of combining MSCs with natural materials as scaffolds. Chitosan and gelatin composite scaffolds with regard to stem-cell-based tissue engineering are described.

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1. Introduction

1.1. From embryonic stem cells to adult mesenchymal stem cells

Stem cells are undifferentiated cells with great versatility that give tissue cells and organs of the organization. Under appropriate conditions stem cells can differentiate into specific cell populations, and form various tissues and organs. They have the capacity for self-renewal and dividing for long periods.

There are two categories of stem cells, embryonic and adult stem cells. Embryonic stem cells are pluripotent. These cells are identified in blastocyst and have the potential to differentiate in numerous cellular types. On the other hand, adult stem cells can be found in the adult tissues, mainly in bone marrow hematopoietic niche. Most stem cells are in G0 phase of the cell cycle and only a small number of stem cells are responsible for stem cell maintenance and for producing mature cells at any specific time, for example to repair damaged tissue upon stimulation [Alenzi et al., 2009].

The stem cell niche is a dynamic multi-cellular structural unit consisting of a stem cell and its surrounding cells. It is believed that balances the interactions processes between self-renewal and differentiation of adult stem cells. [Rajasekhar and Vemuri, 2005]

In adult stem cells, hematopoietic stem cells (HSC) are the most designated progenitor cells and are responsible for the initiation and maintenance of hematopoiesis.

Hematopoiesis is the formation of blood cellular components. Hematopoiesis begins in the third week of pregnancy when blood islands are formed on the wall of the yolk sac. At the sixth week of pregnancy the first hematopoietic stem cells appear in the fetal liver and parallel starts the production of enucleated erythrocytes. After the tenth week of pregnancy, HSCs colonize the bone cavities and hematopoiesis is taking place through bone marrow which is the main organ of hematopoiesis after

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the seventh month. So, adult human bone marrow conceives blood cells, monocyte-granulosa cell line and platelets. The cells of hematopoietic system are regenerated from a HSCs population. The HSCs are on the top of the hierarchy of hematopoietic cells and are capable of differentiating into at least eight cell lines. [Alenzi et al, 2009].

Bone marrow's micro-environment possesses an important role in the adjustment of self-renewal and differentiation of HSCs. That micro-environment is synthesized from a heterogeneous cell population which includes fibroblasts, adipocytes, endothelial cells and osteocytes. These cells have a common mesenchymal progenitor, the mesenchymal stem cell (MSC). Besides their role in hematopoiesis, as supportive cells, MSCs have a plethora of properties and a great deal of interest because of their potential use in regenerative medicine and tissue engineering.

Mesenchymal stem cells were first identified in the ground-breaking studies of Friedenstein and Petrakova who isolated bone-forming progenitor cells from rat bone marrow [Friedenstein et al., 1966]. MSCs can be isolated from various sources such as liver, adipose tissue, peripheral blood, fetal blood, cord blood etc., nevertheless the most easily accessible source is the bone marrow. In bone marrow the flexible tissue found in the hollow interior of bones, MSCs represent a very small fraction and the estimated frequency of MSCs in the BM nucleated cell population is 1 in 3.4×10^4 cells [Pittenger et al., 1999], [Wexler SA, et al., 2003], [Hass R et al., 2011].

Another source of MSCs is the umbilical cord that contains two arteries and one vein, which are surrounded by mucoid gelatinous connective tissue, called Wharton's Jelly (WJ). Whartons's jelly is composed of myofibroblast-like stromal cells, collagen fibers and proteoglycans [Mitchel et al., 2003]. In the Wharton's jelly, the most abundant glycosaminoglycan is hyaluronic acid [Sobolewski et al., 1997], which forms a hydrated gel around the fibroblasts and collagen fibrils while simultaneously

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maintain the tissue architecture by protecting it from pressure [Wang HS et al., 2004].

1.2. Immunophenotypical characteristics of MSCs

In contrast to HSCs, cultured MSCs do not harbor a unique identification marker. The International Society for Cellular Therapy (ISCT) has provided the following criteria for defining multipotent mesenchymal stromal cells: plastic-adherent fibroblast-like cells under standard culture conditions, characterized by the expression of a variety of cells surface antigens. It is generally agreed that adult human MSCs do not express the hematopoietic markers (CD45, CD34, CD14, CD11, or HLA-DR) and endothelial markers (CD34, CD31). They also do not express the costimulatory molecules (CD80, CD86, and CD40) but they do express many adhesion molecules (CD18, CD29, CD44, CD56, CD73, CD90, CD105) and monoclonal antibodies SH2, SH3, SH4, STRO-1 [Pittenger et al., 1999] [Conget PA and Minguell JJ. 1999].Phenotypical characteristics of MSCs are summarized in **Table 1** [Dominici et al, 2006].

Markers	Identity
CD45 (-)	pan-leukocyte marker
CD34 (-)	marks primitive hematopoietic progenitors and endothelial cells
CD14, CD11 (-/-)	expressed on monocytes and macrophages
CD31 (-)	platelet/endothelial cell adhesion molecule
CD80, CD86, CD40[1] (-/-/-)	molecules for T cell activation
HLA-DR (-)	a MHC class II cell surface receptor
CD18 (+)	leukocyte function-associated antigen-1
CD29, CD44, CD90 (+/+/+)	express in many cells
CD56 (+)	neuronal cell adhesion molecule-1
CD73 (+)	known as ecto 5' nucleotidase and originally recognized by the MAb SH3 and SH4

[1] Negative markers are stained with red color.

CD105 (+)	known as endoglin and originally recognized by the MAb SH2
Adhesion molecules (+)	Integrins, ICAM-1,VCAM-1
Extracellular matrix (+)	Collagen type I, III, fibronectin, laminin, hyaluronan, proteoglycans

Table 1 : Phenotypic characteristics of Mesenchymal stem cells.

1.3. Differentiation of MSCs

An interest fact is the ability of MSCs to sustain undifferentiated in vitro for a long period of time and give rise to different mesodermal cell lineages, including osteoblasts, chondroblasts, and adipocytes under proper experimental conditions in vitro and/or in vivo [Ye Chen et al., 2008]. This tri-lineage mesenchymal differentiation is the biological property that most uniquely identifies MSCs and can be demonstrated by various staining assays, for example von Kossa and Alizarin Red for osteoblasts and Oil Red O for adipocytes. [Dominici et al, 2006].

Regarding osteblasts, osteoprogenitor cells are derived from mesenchymal stem cells. The key factor that triggers the differentiation is the transcription factor runt-related transcription factor 2 (RUNX-2). This protein stimulates the expression of genes that are characteristic for the phenotype of osteoblasts; RUNX-2 and bone morphogenetic proteins (BMPs) seem to play an important role in osteoblast differentiation. Osteoblasts are the differentiated cells that secrete the extracellular matrix of bone tissue which includes collagen type-I (which is 90% of the bone proteins) and proteins which are the original non-calcified bone. The proteins produced by osteoblasts include calcium binding-proteins such as RUNX-2, osterix, DLX-5, osteopontin, osteocalcin, osteonectin and alkaline phosphatase. The process of calcification starts from osteoblasts via secretion in the extracellular matrix of small vesicles (50-250 nm). These small vesicles secreted only during the period that the cells produce the extracellular matrix components and are rich in alkaline phosphatase. When the osteoblasts are completely surrounded by the matrix then the cells are called osteocytes. The osteocytes are responsible for the maintenance

of the extracellular matrix. One of the main roles of osteocytes is the responses to mechanical stimuli that affect the bone [Ross MH and Pawlina W, 2006].

MSCs can be differentiated in many mesodermal cell types, *in vitro,* using different cultural stimuli. For example differentiation of MSCs to osteoblasts *in vitro* involves incubating a confluent monolayer of MSCs with ascorbic acid, β-glycerophosphate, dexamethasone and fetal bovine serum (FBS).

MSCs can also be differentiated into chondrocytes in serum free medium with the addition of one of the family of growth factors TGF-b. The cells rapidly lose its characteristic morphology of fibroblasts and begin to express a number of cellular constituents of the layer [Li FT et al., 2005].

Adipocytes are specific cell types from undifferentiated mesenchymal stem cells. The transcription factor PPAR-g and C/EBPa binding protein plays a critical role in adipocyte differentiation and initiation of lipid metabolism. C/EBPa and PPAR-g together promote differentiation by activating adipose-specific gene expression and by maintaining each other's expression at high level. This factor induces maturation of early adipoblasts to adipocytes. Early adipoblasts resemble fibroblasts but develop small encapsulated lipids and a thin outer membrane. As adipoblastic differentiation starts, the number of cell vesicles grow while the rough endoplasmic reticulum is reduced, displaying encapsulated lipids and external membrane the presence of which separates the adipocytes from the connective tissue cells. With continued growth, cells acquire oval shape. A characteristic is the extensive concentration of vesicles from the core. Glycogen particles appear at the periphery of droplets of fat and basal membrane vesicles are becoming more apparent. These cells, through differentiation, called adipoblasts. At the end of differentiation adipocytes increase in size and become more spherical. Small fat droplets merge and occupy the central portion of the cytoplasm. These cells are called late adipoblasts. Finally, the lipid mass compresses the core off center forming adipocytes [Ross MH and Pawlina W, 2006], [Rosen ED et al., 2002].

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Adipogenic differentiation is induced through treatment of MSC cultures with dexamethasone, isobutyl methyl xanthine (IBMX) and indomethacin that activates peroxisome proliferation-activated receptor γ (PPAR-γ) and fatty acid synthase [Pittenger et al., 1999]. Successful differentiation occurs when intracellular lipid vesicles are typically observed in large numbers.

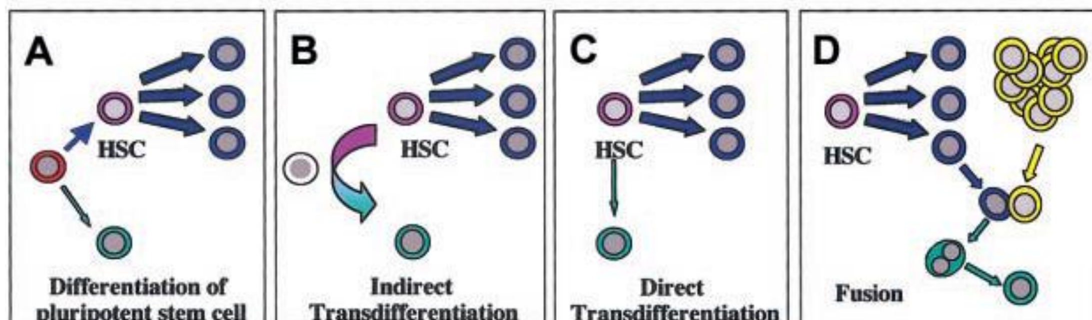

Figure 1: Mechanisms of differentiation.

Adult mesenchymal stem cells have the ability to differentiate into non-related to tissue of origin directions, this property is called plasticity. Four mechanisms have been proposed (**Figure 1**), and proof of one mechanism does not necessarily preclude other possibilities. One mechanism could be that population of cells who may give differentiation into these cell types probably is a pluripotent cell that so far has not be detected (Figure 1A). Alternatively, there have been proposed mechanisms of direct and indirect differentiation (Figure 1B-C). During indirect differentiation one committed cell type has the ability to change its gene expression pattern to that of a completely different cell type. But in direct differentiation, a cell type is differentiated to another without an intermediate state. Finally, the mechanism may be responsible for the range of cell lines that can be derived from a stem cell, is the fusion of progenitor cell with an already differentiated cell (Figure 1D). [Herzog LE, 2003].

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1.4. Immunological properties

One of the most important properties of MSCs is their immunosuppressive properties. As immune-modulators, MSCs may play important roles in many immunological procedures (diseases/disorders); MSCs can exert immunosuppressive effect on T, B and of NK cells and may also negatively affect the function of dendritic cells which are major executors of the adaptive immune response. MSCs express only major histocompatibility complex (MHC) molecules class I, while lack the expression of MHC class II molecules. T cells recognize and bond to antigens that are connected with molecules of MHC which is part of the immune system. MHC class I may activate T cells, but, with the absence of costimulatory molecules (CD80, CD86, CD40) and low expression of MHC class II, a secondary signal would not engage, leaving the T cells anergic and, hence, unable to proliferate. Moreover, studies have shown possible mechanisms providing evidence that MSCs suppress T cells [Caplan AI and Singer NG, 2011], [Di Nicola et al., 2002], [Javason EH, 2004], [Keating A, 2008], [Zhipeng H et al., 2012].

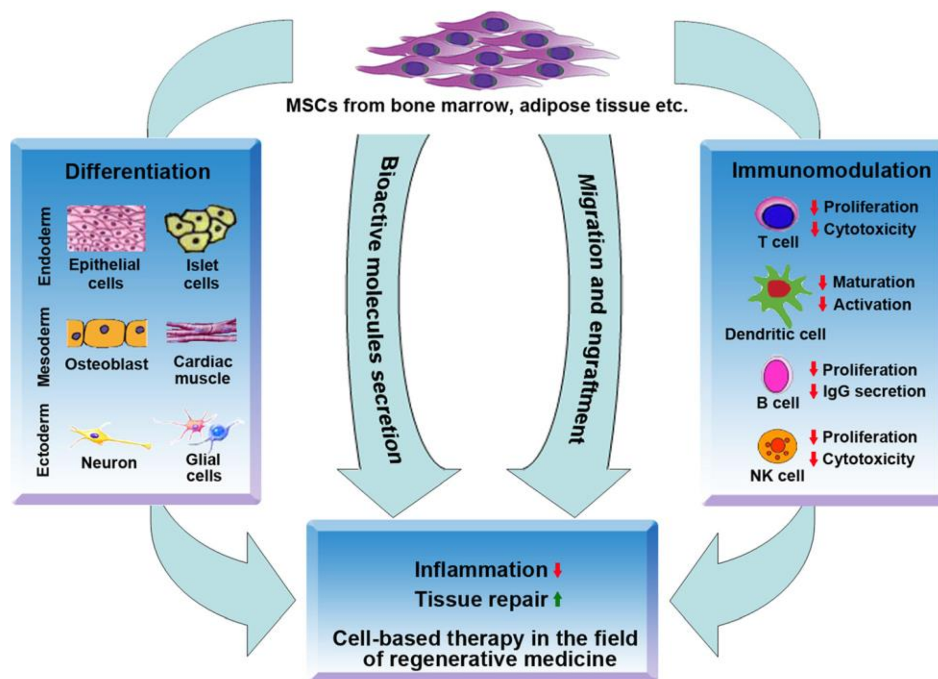

Figure 2: A model for biological properties of MSCs that are associated with their therapeutic effects [Wang S, 2012].

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1.5. Applications of MSCs

Given the above, mesenchymal stem cells can be used for tissue repairing in regenerative medicine and facilitating transplants as their transplantation can minimize the toxicity of the conditions.

An application is the transplantation of MSCs for graft versus host disease (GVHD) treatment where allogeneic MSCs may be used to immunosuppress GVHD and heal damaged intestinal epithelium [Ringden O et al. 2006].

Regarding the treatment of cancer, MSCs can be used as vehicles for cancer gene therapy due to their migratory abilities toward tumors and have been genetically modified to overexpress various anticancer genes, such as IFNs [Studeny M et al. 2002].

The ability of MSCs to differentiate has attracted interest for their use in orthopedics. Studies have shown that can be beneficial in treating bone disorders such as osteogenesis imperfecta (OI) [Horwitz EM et al. 2001], also there have been reports demonstrating the efficacy of MSCs in promoting cartilage repair in which MSCs embedded in collagen gel were transplanted into the knee joints of patients with articular cartilage defects [Wakitani S et al. 2002].

Another potential hypothesis for regeneration and tissue engineering is the combination of MSCs with two and three-dimensional biocompatible scaffolds [Zhao F et al., 2006].

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1.6. Tissue engineering

Tissue engineering is an expanding field where therapies are implemented for the reparation or replacement of damaged tissues or organs. It's a multidisciplinary field that combines applied biology, biomedical engineering and nanotechnology.

Mostly there are three approaches in tissue engineering:

- isolated cells
- acellular biomaterials
- a combination of cells and materials.

In this field, used cells may come from patients themselves (autogenous), from a genetically identical donor (syngenic), from donors (allogenic) and from animal sources (xenogenic). After the collection, proliferation starts and then the right number of cells are seeded on a scaffold and cultured in an incubator. Appropriate bioactive factors can be enhanced in scaffolds before the use of the tissue substitute. When the construct is developed enough, it can be implanted in the area of defect in patient's body (**Figure 3**).

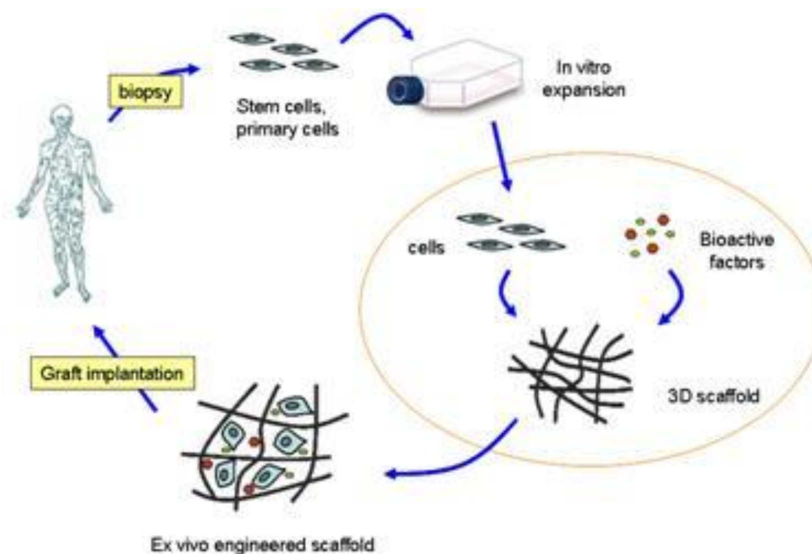

Figure 3: The approach of tissue engineering.

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Applications of tissue engineering include ectodermal (nervous system, cornea and skin epidermis), endodermal (liver, pancreas and tubular), mesodermal which forms mesenchyme (muscle, bone and circulatory systems) derived tissues [Lanza R, 2000].

1.7. Biomaterials

Biomaterials science is the study of biomaterials and their interactions with the biological environment. It is also a multidisciplinary field that combines biology, medicine, engineering and materials science including subjects related to materials, such as mechanical properties or surface modification of implants, as well as biological topics such as immunology, toxicology, and wound healing process.

As biomaterials and the interaction between cell-material are important in tissue engineering, presenting them as scaffolds they should have the following characteristics:

- not cause cytotoxicity
- excellent biocompatibility
- controllable biodegradability
- cytocompatibility
- highly porous microstructure
- mechanical properties should match bone environment properties
- must be capable of promoting cell adhesion
- retaining the metabolic functions of attached cells
- economically affordable.

Biomaterials can be either biological or synthetic. Scaffolds are being composed of natural origin like collagen, hyaluronic acid etc., and also from synthetic polymers such as co-polymer poly lactic acid-co-glycolic acid (PLGA) and polycaprolactone (PCL). PLGA is biocompatible and biodegradable but possesses inflexible mechanical properties. PCL is a bioresorbable and biodegradable ester polymer with potential applications for bone repair because is degraded by hydrolysis of its ester linkages.

Calcium phosphate based scaffolds like hydroxyapatite are used in bone tissue engineering because they are biocompatible, osteoconductive and osteoinductive [Temenoff JS, Mikos AG. 2008], [Wu X et al. 2010], [Zhao F et al. 2006].

Biological biodegradable polymers are a famous category used as scaffolds which include chitosan, a derivative of chitin, and collagen derivatives such as gelatin.

Chitosan is a linear polysaccharide derived from partial deacetylation of chitin, the primary structural polymer in arthropod exoskeletons. In addition, chitosan is a nontoxic, biocompatible, and biodegradable polymer (poly-1,4-D-glucosamine) with structural characteristics similar to glycosaminoglycans (GAGs) and seems to mimic their functional behavior. Gelatin is obtained by a controlled hydrolysis of the fibrous insoluble protein, collagen. Since it has good biocompatibility, high water adsorbing ability and biodegradability *in vivo*, it is often used to produce drug carriers, wound dressing materials and scaffolds for tissue engineering. Its association with chitosan enhances cell adhesion and forms a polyelectrolyte complex (PEC) which can support scaffolds by mimicking the extracellular matrix (ECM) for tissue engineering, [Miranda SCCC et al., 2012], [Thein-Han WW et al., 2009], [Wu X et al., 2010], [Zhao F et al., 2006].

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2. Materials and Methods

2.1. Isolation of mesenchymal stem cells

Umbilical Cord (UC)

Umbilical cord (UC) samples were collected from 4 full-term neonates delivered by normal labour at the University Hospital of Heraklion, after written informed consent of the family. Fifteen-thirty centimeter-long cords were transferred into a sterile jar to the laboratory and kept at room temperature until further process.

Protocol step by step:

1. Cord blood pieces were subsequently cut into smaller 3-5 cm-long pieces and washed vigorously in PBS (Phosphate Buffered Saline, Gibco).
2. Samples were processed within 24 hours after delivery.
3. Each small piece was slit open and cord blood vessels were removed to avoid endothelial contamination.
4. Wharton's jelly (WJ) was manually scrapped off from the inner lining of cord and was subsequently chopped into small pieces and plated in 6-well plates, without any additional enzymatic treatment.
5. Adherent WJ-MSCs outgrew from the tissue within 1-2 weeks.
6. Cells were then expanded in alpha Modification of Eagle's Medium (a-MEM)(Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) 100 IU/ml penicillin-streptomycin and 2 mM glutamine (Gibco).
7. Cultures were maintained at $37^oC/5\% CO_2$ fully humidified atmosphere.
8. On 70-90% confluence, cells were detached using 0.25% trypsin-1mM EDTA (Gibco) subcultured thereafter at a plating density of 1000 cells/cm^2, for 6 passages (P).

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Bone marrow (BM)

Bone marrows were isolated from three healthy individuals undergoing orthopedic surgery for hip replacement. The study was been approved by the Ethics Committee of the University Hospital of Heraklion and informed consent was obtained from all subjects, according to the Helsinki Protocol.

Protocol step by step:

1. Mononuclear cells were isolated from bone marrow Sample dilution in complete culture A-MEM with 10% FBS, 100 IU/ml penicillin-streptomycin and 2 mM glutamine.
2. Sample stacked in Ficoll (Sigma) (ratio 1:1) slowly and angled in order to create two phases.
3. Followed by centrifugation for 30 minutes at 1600 rpm,no brakes, at room temperature.
4. Carefully the mesophase/monolayer of monocytes was isolated and washes in PBS.
5. Centrifugation was followed for 5 minutes at 1500 rpm.
6. Supernatant was removed.
7. Then cell pellet was solubilized in PBS.
8. Cells were transferred to flasks (25 cm^2) with a density of $2x10^5$ cells/cm^2 with A-MEM media and incubated in 37°C/5% CO_2, fully humidified atmosphere.
9. Medium was removed after 24 hours with simultaneous removal of non – detached cells and refresh medium.
10. On 70-90% confluence, cells were detached using 0.25% trypsin-1mM EDTA (Gibco) and were subcultured thereafter at a density of 2000-3000 cells/cm^2, for 6 passages (P).

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Detach of cells from confluent surface with EDTA-trypsin.

Trypsin-EDTA protocol (confluent ~80%)

1. Medium (A-MEM) was removed from flasks.
2. Flask was washed with PBS (two times).
3. PBS was removed.
4. Trypsin-EDTA was added and flasks were incubated (5 min , 37°C).
5. Medium was added to inactivate Trypsin (2:1, A-MEM:Trypsin-EDTA).
6. Centrifugation was followed (5 min, 1500 rpm, 25°C).
7. Cell pellet was solubilized of the in PBS or A-MEM.
8. Cells were counted in Neubauer haemocytometer.
   a) Cells were mixed with Trypan Blue in proportion 1:1 (10 μl Trypan Blue and 10 μl cell solution).
   b) Number of cells in a particular volume can be counted as:

      $$\mathbf{a \times 2 \times 10^4 = N\ cells/ml}$$

      - a = number of cells in Neubauer
      - N = number of cells per ml
9. Cells were reseeded in 75 cm^2 flask A-MEM media and incubated in 37°C/5% CO_2, fully humidified atmosphere.
10. Change of medium every three days until a 80% confluent surface.

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2.2. Differentiations of MSCs

Osteogenic

Mesenchymal stem cells were cultured in 6-well plate dishes until the bottom surface on each well was confluent and then suitable media for differentiation into osteocytes was added. The procedure of osteocytic differentiation kept 14 days.

Osteocytic culture media:

A-MEM media with 10% FBS, 100 IU/ml penicillin-streptomycin and 2mM Glutamine, 0, 1 μM Dexamethasone, 3 mM NaH_2PO_4 (MERCK) and 25mg/Lt ascorbic acid (Sigma).

Histochemical staining

Von Kossa

Protocol:

1. Culture media was removed and samples were washed with PBS twice.
2. Formaldehyde (4%) was added (10').
3. Then formaldehyde was removed and samples were washed with water for injection twice (WFI).
4. Von Kossa (5% w/v Aqueous Silver Nitrate Solution) staining was added for 30 minutes at 25 °C in dark.
5. Von Kossa staining was removed and washed with WFI twice.
6. A 15-30 minute exposure at Ultraviolet light.
7. Observation under microscope.
8. Samples can be retained at 4 °C for 48 h.

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Alizarin Red

Protocol:

1. Culture media was removed and samples were washed with PBS twice.
2. Formaldehyde (4%) was added for 10 minutes.
3. Then formaldehyde was removed and samples were washed with WFI twice.
4. Alizarin Red (2% w/v, pH 4.1-4.3, Sigma) staining was added for 2 – 5 minutes.
5. Alizarin Red staining was removed and samples were washed with WFI twice.
6. Observation under microscope.
7. Samples can be retained at 4 °C for 48 h.

Adipogenic

Mesenchymal stem cells were seeded in 6-well plate dishes until the culture surface be confluent, then suitable media for adipogenic differentiation was added. The procedure of adipocytic differentiation kept 28 days.

Adipogenic culture media:

D-MEM (Dulbecco's Modified Eagle's Medium) low – glucose with 10% FBS, 100 IU/ml peniciline-streptomycin, 60 mM indomethacin (Sigma), 1μM dexamethasone and 0.5 mM IBMX (Before use, incubate at 60 -62°C)

Histochemical staining

Oil Red O

Protocol:

1. Oil Red O (Sigma) stock solution was prepared (0,5% in 99% isopropanol)
2. Culture media was removed and samples were washed with PBS twice.
3. 10 % Formalin (Sigma-Aldrich) was added and samples were incubated for 15 minutes.

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4. Then formalin was removed and samples were washed with PBS twice.
5. At this point working solution of Oil Red O was prepared.
   - Oil Red O working solution:
     i. 6 ml of the stock solution were diluted with 4 ml of distilled water
6. After 5 minutes working solution was filtered through filter paper and right after through a 0.45 mm filter.
7. Filtered working solution was added in samples and following 20-minute incubation.
8. Then samples were washed with WFI.
9. Samples can be retained at 4 °C for 48 h.

Immunophenotype

Immunophenotyping analysis of different cell populations is carried out by flow cytometry. Protocol varies depending on the cell population and the site of the protein we want to detect; either will be intracellular or extracellular. In **table 2** below shows a combination of antibodies which were used for MSCs characterization.

<u>Protocol:</u>

1. Test tubes of cytometry were prepared with appropriate indications.
2. Antibodies were added according to manufacturer's instructions.
3. Cells were washed from excess nutrients, and were added at a concentration about 10^5 cells per tube.
4. Vortex was followed.
5. Samples were incubated at 4 °C for 20 minutes, in a dark place.
6. Samples were washed from the excess amount of unconjugated antibodies, with PBS.

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7. Centrifugation was followed (5 min, 1500 rpm, 25°C).
8. Supernatant was removed.
9. Amounts of 100 - 300 μl PBS were added per tube.
10. Immunophenotype analyzer (Beckman Coulter Cytomics FC 500)

Antibodies	
IgG1 – PE[2] (*Caltac*)	IgG1 – FITC[3]
CD14 – FITC (*Invitrogen*)	CD105 – PE
CD34 – PE	CD29 – FITC
CD45 – FITC	CD73 – PE
CD31 – FITC	CD90 – PE
CD19 – FITC	HLA – DR.PE (*BD Biosciences*)

Table 2: The amount of each antibody is 10 μl, except 7AAD (20 μl)

- All antibodies are from *Beckman Coulter* unless stated.

7-AAD staining

7AAD (7-Amino-Actinomycin-D) staining gives the opportunity to evaluate the phenomenon of apoptosis by determining and separating apoptotic, dead or alive cells in a population. This method is based on the difference in permeability of the membrane of live, apoptotic and dead cells. The permeability of apoptotic cells is greater compared to normal cells, as a result apoptotic cells are appeared firstly as 7-AAD_{dim} (after 7-AAD dye) and then normal cells as negative 7-AAD. Dead cells and those found in advanced apoptosis, lose their membrane integrity and they appear as 7-AAD_{bright}. Its measurement cannot be done after 30 minutes to 1 hour because 7-AAD it's toxic, penetrates cells over time and may result incorrect measurements.

Protocol:

1. Test tubes of cytometry were prepared with appropriate indications.
2. Antibodies were added according to manufacturer's instructions.

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[2] Phycoerythrin (PE)
[3] Fluoroisothyocyanate (FITC)

3. Cells were washed from excess nutrients, and were added at a concentration about 10^5 cells per tube.
4. Vortex was followed.
5. Samples were incubated at 4 °C for 30 minutes, in a dark place.
6. 200 µl PBS were added and immediately proceed to measurement.
7. Immunophenotype analyzer (Beckman Coulter Cytomics FC 500)

MTT assay

MTT assay is about a tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that produces formazan. Dissolving formazan, which is produced through cell metabolism, into isopropanol; the color of the solution in which the cells were incubated varies depending on the amount of formazan which depends on the number of cells that can promote MTT. The tetrazolium salts XTT and WST-1 transform into soluble formazan[4] and used in experiments for measuring cell proliferation, cytotoxicity, enzyme activity, etc.

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Thus, cells proliferation that has been coated into wells can be assessed by photometry in each well. The measurements are performed by the photometer ELISA.

MTT stock solution

- 5 mg/ml MTT (Sigma) were put in sterile PBS.
- Then vortex was followed.
- And was filtered through a 0.2 µm filter and stored at 2-8°C.

[4]Formazan: a group of products resulting from a reducing tetrazolium salt and have bright colors, also insoluble.

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Protocol:

1. Seed 2500-3500 cells / well in appropriate media (48-well plate).

a. Media was removed.

b. Cultures were washed with PBS.

- MTT working solution must be prepared at the same day.

c. Then were added 300 µl working solution (1mg/ml MTT in A-MEM).

d. Sample was incubated at 37 °C for 4h.

e. The converted dye is solubilized in with 300 µl acidic isopropanol. Use 1 N HCl in absolute isopropanol. Pipette up and down several times to make sure the converted dye dissolves completely.

f. Measurement with ELISA reader (EL_x 800 Universal microplate reader, Bio-Teck Instruments), at OD_{630}.

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2.3. Molecular Techniques

Polymer Chain Reaction (PCR)

The polymerase chain reaction is used for the amplification of DNA fragments between two areas with a particular sequence. In reaction needed:

Primers: They have a minimum length of 16 nucleotides, typically 20 to 24 nucleotides are preferred with a concentration of 200ng / l.

Template DNA: use of genomic DNA concentration 1ng / microliter.

DNA polymerase: Usually, Taq polymerase is isolated from the bacterium Thermus aquatiqus. It is thermostable and has a 5 '-> 3' polymerase activity.

Buffer: A solution in which the polymerase acts.

Nucleotides (dNTPs): nucleotides adenine, thymine, cytosine, guanine at a concentration of 10mM.

Water (WFI): fill with water until the desired volume.

As an example in a PCR reaction, the ingredients were placed in the following order:

- 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] 5µL
- 50 mM $MgCl_2$ 1.5 µL,
- 10 mM dNTP Mix 1 µL
- Forward Primer (10 mM) 1 µL
- Reverse Primer (10 mM) 1 µL
- Taq DNA polymerase (5 U/µL) 0.4 µL
- cDNA from first-strand reaction 2 µL
- autoclaved, distilled water to 50 µL

The polymerase chain reaction involves three basic steps:

- **Denaturation** of the parent DNA.

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- Denaturation occurs at 94 °C where two single-stranded molecules created.

- **Annealing** in which the primers recognize their complementary sequences on the template DNA and bind to it. The selected temperature depends on primers and the specific melting temperature (T_m). The melting temperature (T_m, melting temperature) of each primer is calculated from the following equation: $\mathbf{T_m = 2(A+T) + 4(G+C)}$
- **Extension** occurs at 72°C, at this stage the polymerization of DNA fragments is comprised between the primers.

PCR conditions depend on various parameters, like specificity of primers. Generally the principal conditions of a PCR assay are the following:

1. 94 °C 5 min.
2. 94-98 °C for 30 seconds (Melting)
3. 50-65 °C for 1 min (Primer annealing)
4. 72°C for 1 min (Extension)
5. Go to step 2, 30 more times
6. 72 °C for 5 min, the time depends on the length of the track you want to multiply In 1min Taq polymerizes 1000bp (elongation).
7. 4 °C forever

Reverse Transcription – PCR

Reverse Transcription – PCR (RT–PCR) is commonly used in molecular biology to detect RNA expression levels. RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase, cDNA then used as a template for exponential amplification using PCR.

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The process of reverse transcription of mRNA in a cell makes achievable the identification of genes expressed in a cell population. The reaction of reverse transcription begins at 65 °C where undertaken denaturation of double-stranded segments of the chain reaction RNA. Then transported at 4 °C to reduce the mobility of molecules and remain in the denatured state. Followed by a step at 25 °C in which the primers hybridize (random hexamers). At 42 °C operates reverse transcriptase for reverse transcription of the chains and RNAseOUT which is the inhibitor of RNAse. Finally at 70 °C inactivates the enzymes and stops the reaction. Then thermal cycler reduces the temperature to 4 °C where each activity stops, followed with RNAse incubation in order to break the chains of the DNA initially present in the reaction and contributes to the purity of the produced cDNA.

RNA extraction from about 10^6 cells was performed with TRIZON Reagent (Ambion, Life Technologies), according to manufacturer's instructions.

Reverse transcription was operated according to manufacturer's instructions, First – Strand cDNA synthesis SuperScript II RT, Invitrogen (Bio-Rad).

Protocol:

- A 20-μL reaction volume can be used for 1 ng–5 μg of total RNA or 1–500 ng of mRNA.

1. The following components were added to a nuclease-free microcentrifuge tube:
    - Oligo(dT)12-18 (500 μg/mL) or 1 μL
    - 50–250 ng random primers or
    - 1 ng to 5 μg total RNA or x μL
    - 1–500 ng of mRNA
    - 1 μL dNTP Mix (10 mM each) 1 μL
    - Sterile, distilled water to 12 μL
2. Mixture was heated to 65°C for 5 min and quick chill on ice.

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3. Contents were collected and briefly centrifugation of the tube was followed. Then were added:
   - 5X First-Strand Buffer 4 µL
   - DTT 2 µL
   - RNaseOUT™ (40 units/µL) (optional)* 1 µL
   - *RNaseOUT™ (Cat. No. 10777-019) is required if using <50 ng starting RNA.
4. Contents were mixed gently. Then for random primers tubes were incubated at 25°C for 2 min.
5. 1 µL (200 units) of SuperScript™ II RT was added and mixed by pipetting gently up and down.
6. If you are using less than 1 ng of RNA, reduce the amount of SuperScript II RT to 0.25 µL (50 units) and add sterile, distilled water to a 20 µL final volume.
7. For random primers, incubate tube at 25°C for 10 min.
8. Reaction was inactivated by heating at 70°C for 15 min.

- The cDNA can now be used as a template for amplification in PCR. However, amplification of some PCR targets (>1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 µL (2 units) of E. coli RNase H and incubate at 37°C for 20 min.

Real Time – PCR

In Real Time (Corbett) quantitative PCR (qPCR), the amount of PCR product is measured at each cycle. This ability to monitor the reaction during its exponential phase enables users to determine the initial amount of target with great precision.
PCR theoretically amplifies DNA exponentially, doubling the number of molecules present with each amplification cycle. The number of cycles and the amount of PCR end-product can theoretically be used to calculate the initial quantity of genetic material (compared to a known standard), but numerous factors complicate this calculation. In Real-Time PCR, the amount of DNA is measured after each cycle by the use of fluorescent markers that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA (dsDNA)-binding dyes, or dye molecules attached to PCR primers or probes that are incorporated into the product during amplification. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

The advantages of Real-Time PCR include:

- the ability to monitor the progress of the PCR reaction as it occurs in real time
- the ability to precisely measure the amount of amplicon at each cycle
- an increased dynamic range of detection
- the combination of amplification and detection in a single tube, which eliminates post- PCR manipulations.

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Assessment of osteogenesis- and adipogenesis-related gene expression by real-time RTPCR.

P2 MSCs from BM and WJ MSC- cultures were assessed for the expression of genes related to osteogenesis (Alkaline Phosphate, ALP; Osteocalcin, OSC; DLX5; Runt-related transcription factor 2, RUNX2; Osterix, OSX) and adipogenesis (CCAAT/enhancer-binding protein alpha, CEBPA; Peroxisome Proliferator Activated Receptor gamma, PPARG). RNA was isolated and reverse- transcribed as described above, and 20 ng of cDNA were amplified in each PCR reaction. PCR was performed with KAPA SYBR FAST qPCR Kit Master Mix (Kappa Biosystems, Boston, Massachusetts, USA) and 10 μM of each primer. We used a Rotor-Gene 6000 two-step cycling program consisting of 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. A melting curve (62-95°C) was generated at the end of each run to verify specificity of the reactions.

The forward and reverse primer sequences for PCR amplification were:

5'-CCTGCAGCTTCAGAAGCTCAA-3' and 5'ACTGTGGAGACACCCATCCC-3' for ALP,

5'-GAGGGCAGCGAGGTAGTGAAGA-3' and 5'-CGATGTGGTCAGCCAACTCG-3' for OSC,

5'-GCCACCAACCAGCCAGAGAA-3' and 5'-GCGAGGTACTGAGTCTTCTGAAACC-3' for DLX5,

5'-GGCCCACAAATCTCAGATCGTT-3' and 5'-CACTGGCGCTGCAACAAGAC-3' for RUNX2,

5'-AAGAAGTCGGTGGACAAGAACAC-3' and 5'-ACCGCGATGTTGTTGCG-3' for CEBPA,

5'-TCAGGGCTGCCAGTTTCG-3' and 5'-GCTTTTGGCATACTCTGTGATCTC-3' for PPARG.

GAPDH was used as normalization control and the forward and reverse primer sequences were:

5'-CATGTTCCAATATGATTCCACC-3' and 5'-GATGGGATTTCCATTGATGAC-3', respectively

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2.4. Synthesis of biomaterials

Chitosan

Chitosan (Sigma-Aldrich) solution was prepared at 2 % (w/v) by dissolving in 1 % (v/v) acetic acid (Scharlau) in WFI at 37 °C.

Gelatin

Gelatin (Sigma-Aldrich) solution was prepared at 2 % (w/v) in in WFI at 37 °C.

The homogenous mixture (w/w%) with different ratios (1:2, 2:1, 3:1) of chitosan and gelatin were transferred to polystyrene 24-well plates and were refrigerated at 4 °C (24h). Freeze-drying was followed for lyophilization (24h).

Freeze Dryer

In freeze drying there is a phenomenon called sublimation which is the main principle of the procedure. Water passes directly from solid state (ice) to the vapor state overrides the liquid state. Sublimation of water can take place at pressures and temperature below triple point i.e. 4.579 mm of Hg and 0.0099 degree Celsius. Firstly it is frozen and then regulated under high vacuum to heat (by conduction or radiation or both) so that frozen liquid sublimes leaving only solid, dried components of the original liquid.

Prior to cell seeding:

1. The resulting scaffolds were neutralized with 10% NaOH (Sigma) solution to remove acetate.
2. 70% ethanol was added to neutralize the acetic acid.
3. Then scaffolds were washed with WFI.

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4. Samples were immersed into 75% ethanol for 1 h to sterilize them.
5. The ethanol was then removed by soaking for 1 h with three changes of PBS and complete media (A-MEM) with 10% FBS, 100 IU/ml penicillin-streptomycin and 2mM Glutamine was added for incubation.

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2.5. Techniques

Flow cytometry

Flow cytometry measures optical and fluorescence characteristics of single cells. Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis. The resulting information usually is displayed in histogram or two dimensional dot-plot formats.

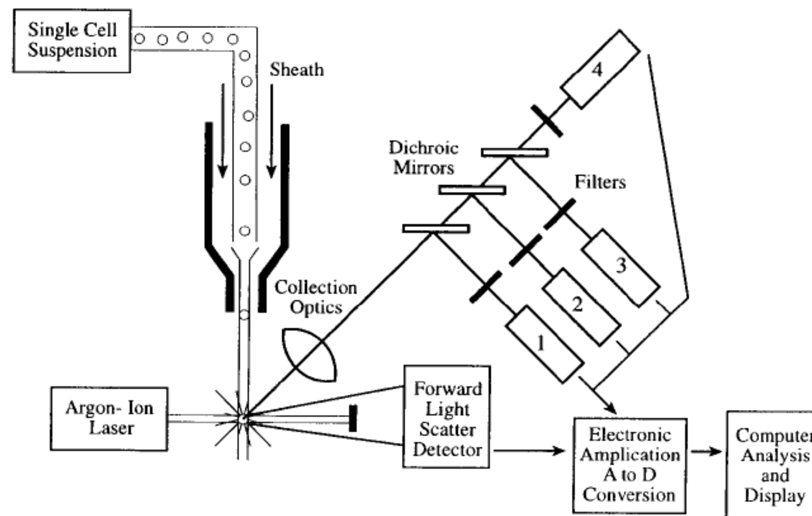

Figure 4: Schematic of flow cytometer [Brown M and Wittwer C. 2000].

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Scanning Electron Microscopy (SEM)

The SEM microscope gives information about the topology of the sample. A beam of electrons is produced at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample. Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen.

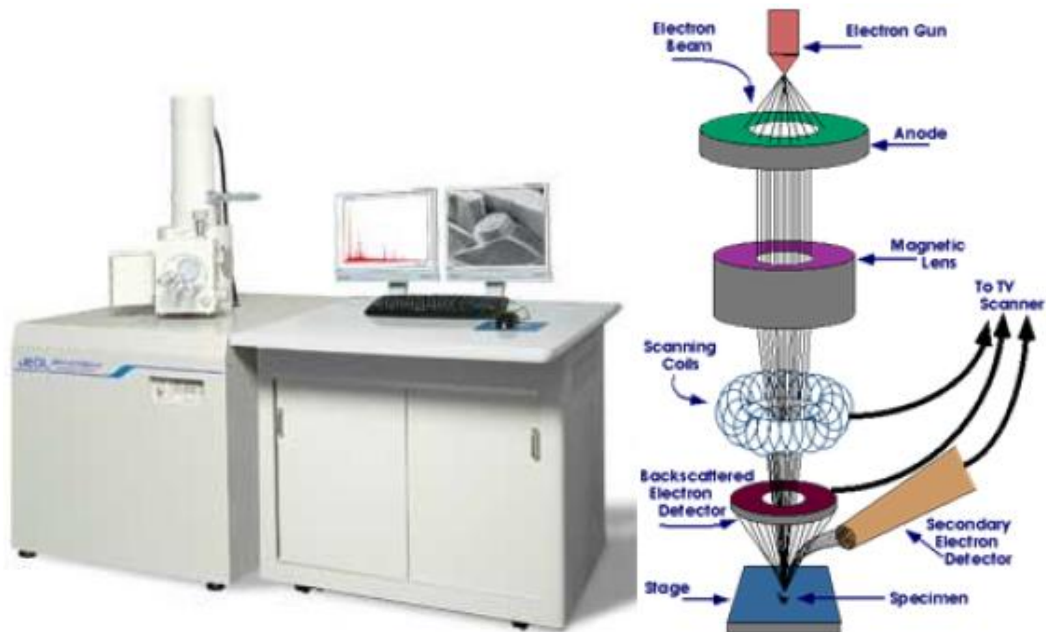

Figure 5 : Scanning Electron Microscopy (http://www.jeol.com, http://purdue.edu)

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3. Results

3.1. Phenotypical characteristics

Morphology of Mesenchymal stem cells

Cultured mesenchymal cells from both tissues, BM and WJ MSCs, showed the characteristic spindle-shape of fibroblasts which retained through the in vitro cultivation, **Figure 7**.

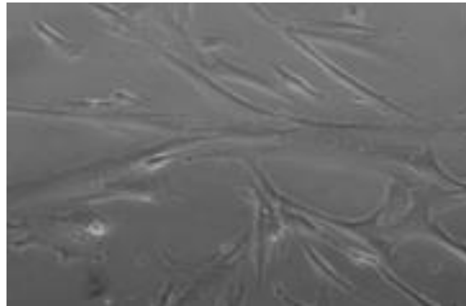

Figure 6: Undifferentiated Mesenchymal Stem Cells

Immunophenotypical characteristics

Immunophenotypic analysis of five samples from each tissue showed that the both MSC-populations do not express hematopoietic markers CD34, CD45, CD14, and for HLA-DR and are positive for CD105, CD90 and CD73 surface antigens at a percentage ≥98%, through the cultivation

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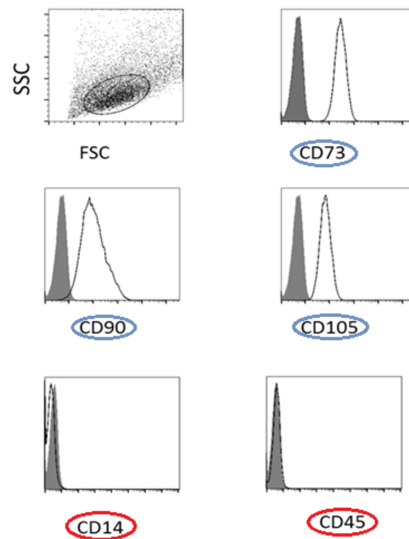

Figure 7: Immunophenotypic analysis

3.2. Proliferation

Doubling Time-DT

Population doubling time (PDT) was calculated from Passages (P) 2 to 6 for MSCs from both BM and WJ. In each case the population double time of WJ-MSCs was significant lower as shown on **Diagram 1**. As a matter of fact, while WJ-MSCs PDT remains almost stable, BM-MSCs have a rising DT through the passages.

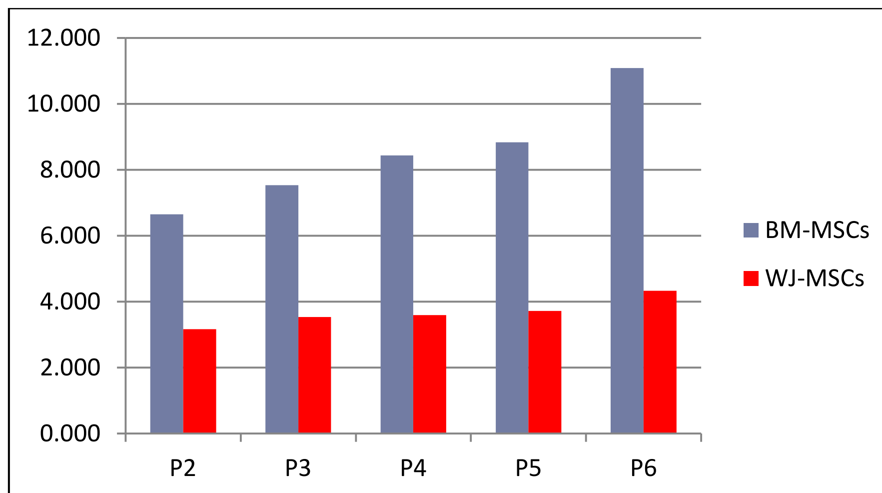

Diagram 1: Doubling Time of MSCs from both derived tissues

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MTT measurement

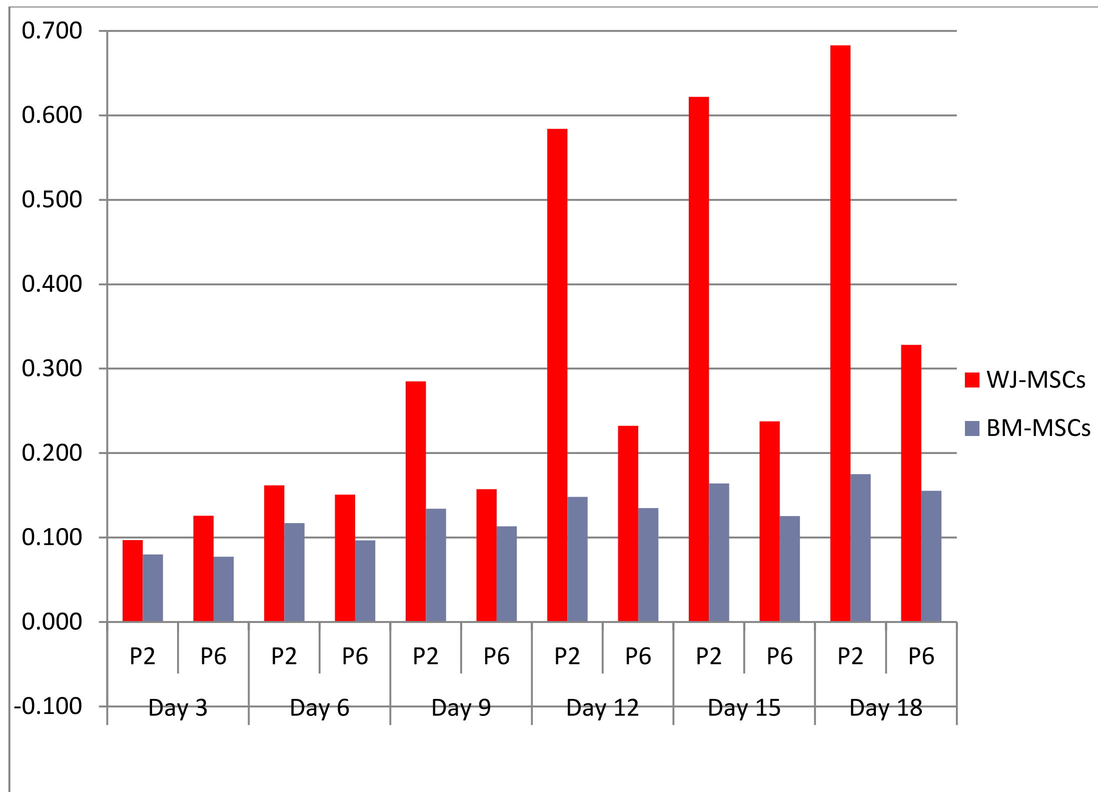

Diagram 2: Proliferative rate of WJ-MSCs and BM-MSCs for P2 and P6

Both WJ-MSCs and BM-MSCs sustain the ability to proliferate. At P2 both types of MSCs proliferate gradually through a cultivation of 18 days. However, at P2 the proliferative capacity of WJ-MSCs is obviously higher than BM-MSCs, at the same days. In a same way at P6 both types of MSCs proliferate gradually through a cultivation of 18 days, but BM-MSCs proliferation capacity increases with a much lower rate than WJ-MSCs, at the same days.

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3.3. Differentiation

MSCs under appropriate culture conditions successfully were differentiated into adipocytes and osteocytes, as shown by histochemical staining.

For adipocytes, successful differentiation occurred and lipid vesicles were observed in large numbers. Apparent accumulation of lipid vesicles of MSCs from bone marrow MSCs and WJ derived tissue appear after treatment with Oil Red O histochemical staining (**Figure 9**).

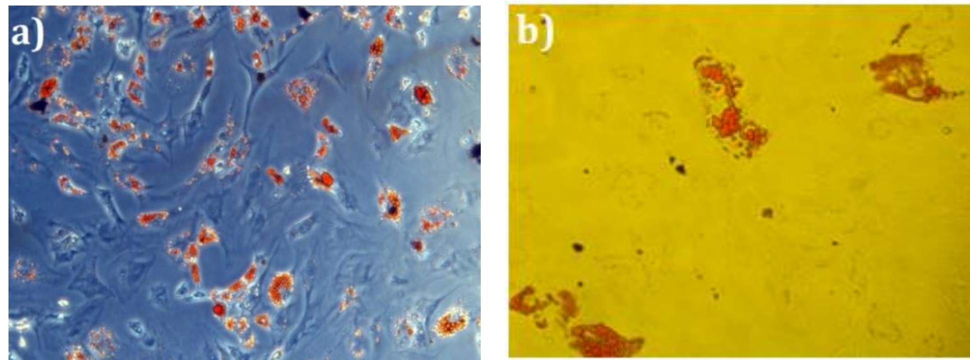

Figure 8: Adipocytes after Oil Red O staining. a) differentiated cells from BM, b) differentiated cells from WJ

Calcium accumulation was observed in differentiation for osteoblasts after treatment with von Kossa histochemical staining. Von Kossa stains the amount of hydroxyapatite that was secreted from osteoblasts during differentiation (**Figure 10**).

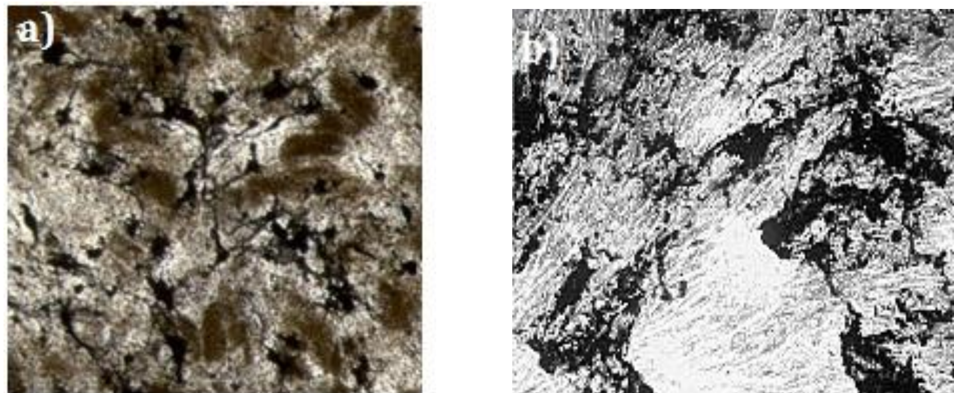

Figure 9: Osteoblasts after von Kossa staining. a) differentiated cells from BM, b) from WJ.

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Osteoblasts stained with Alizarin Red. Alizarin Red stains the calcium accumulation of osteoblasts (**Figure 11**).

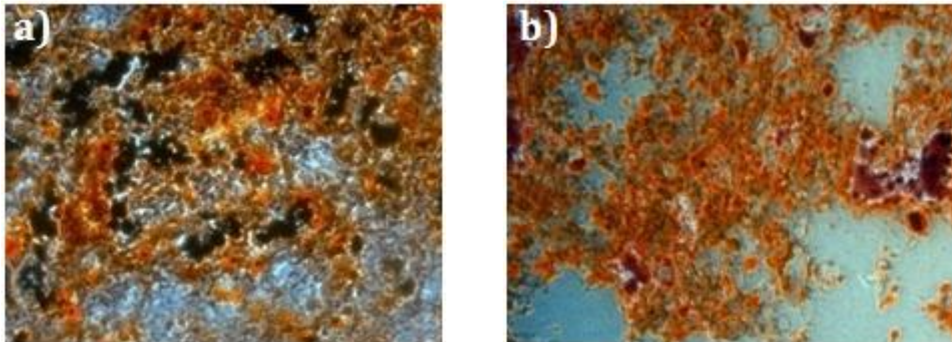

Figure 10: Osteoblasts after Alizarin Red staining. a) differentiated cells from BM, b) from WJ.

3.4. Expression of osteocytic genes

Specific osteocytic genes studied for MSCs derived from both tissues after culture with suitable differentiation media. RUNX2, ALP, DLX5 and OSX genes are shown on the diagrams below.

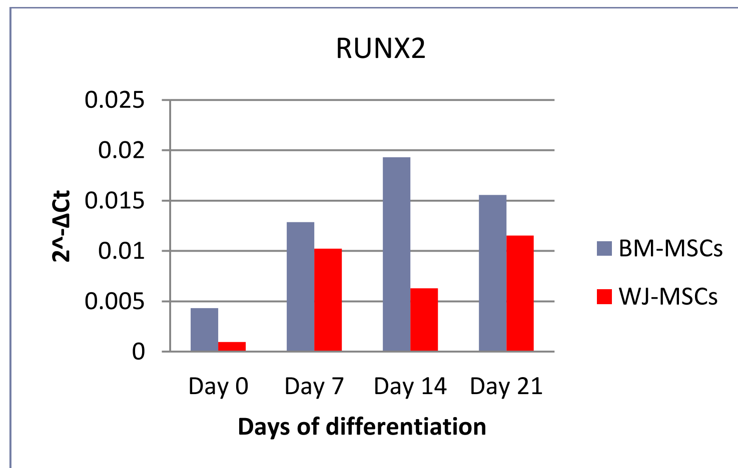

Diagram 3: RUNX2 expression of BM-MSCs and WJ-MSCs

RUNX2 is a key transcription factor associated with osteoblast differentiation and is encoded by the RUNX2 gene.

In 21 days of differentiation MSCs seems to express RUNX2 at some levels. Although MSCs from both sources express the RUNX2 gene, BM-MSCs have greater

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expression. Also, from day 14 to day 21 BM-MSCs expression decreases while WJ-MSCs increases.

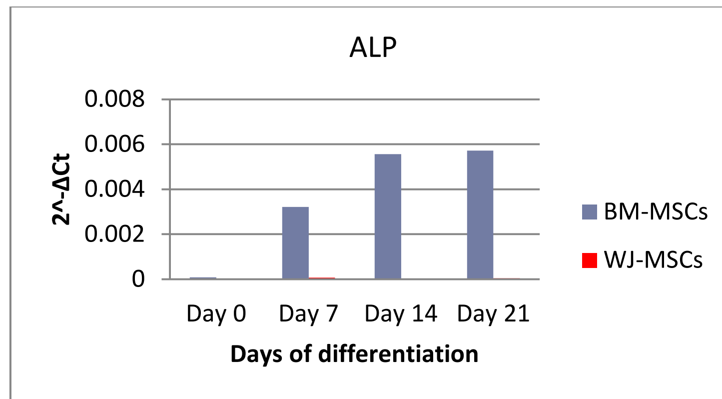

Diagram 4: ALP expression of BM-MSCs and WJ-MSCs

Alkaline phosphatase is a key enzyme that provides high concentrations of phosphate at the site of mineral deposition in active osteoblasts.

Only BM-MSCs express levels of ALP and is taking place after day 7. The absent of WJ-MSCs expression levels is obvious.

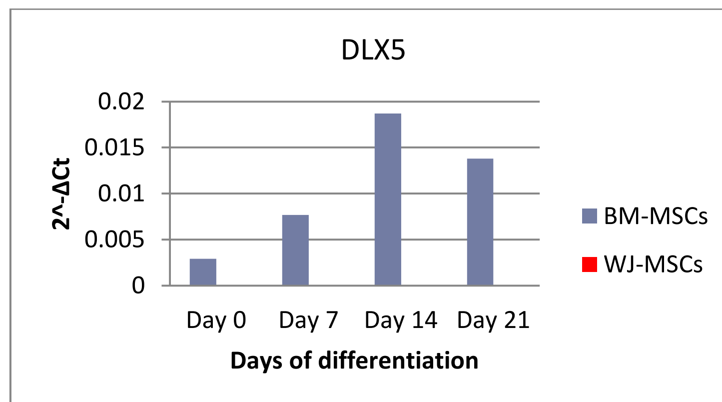

Diagram 5: DLX5 expression of BM-MSCs and WJ-MSCs

Homeobox protein DLX-5 is a protein that in humans is encoded by the distal-less homeobox-5 gene or DLX5 gene, and plays critical role in bone development.

DLX5 is expressible only in MSCs from BM derived tissue. After Day 7 for BM-MSCs expression increased rapidly and then decreased in day 21. In addition, in WJ-MSCs its expression is almost absent.

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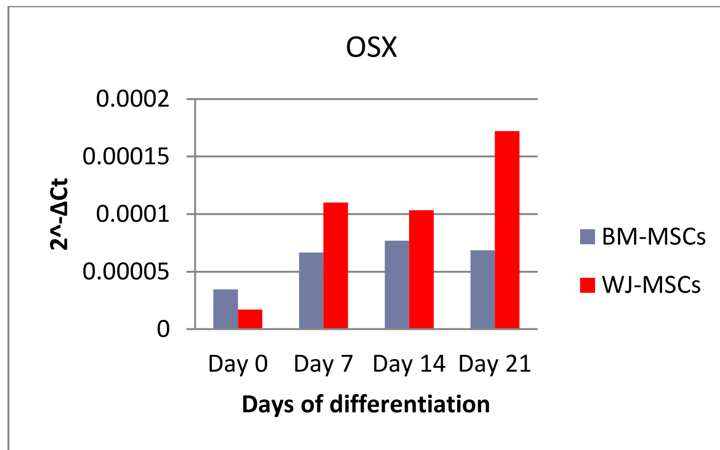

Diagram 6: OSX expression of BM-MSCs and WJ-MSCs

Osterix (OSX) is a novel zinc finger–containing transcription factor that is essential for osteoblast differentiation and bone formation.

OSX gene is expressed from both derived tissues cells. In this case WJ-MSCs have greater expression during the differentiation except day 0. The largest difference is obtained at day 21 where BM-MSCs expression decreases while WJ-MSCs expression almost doubles.

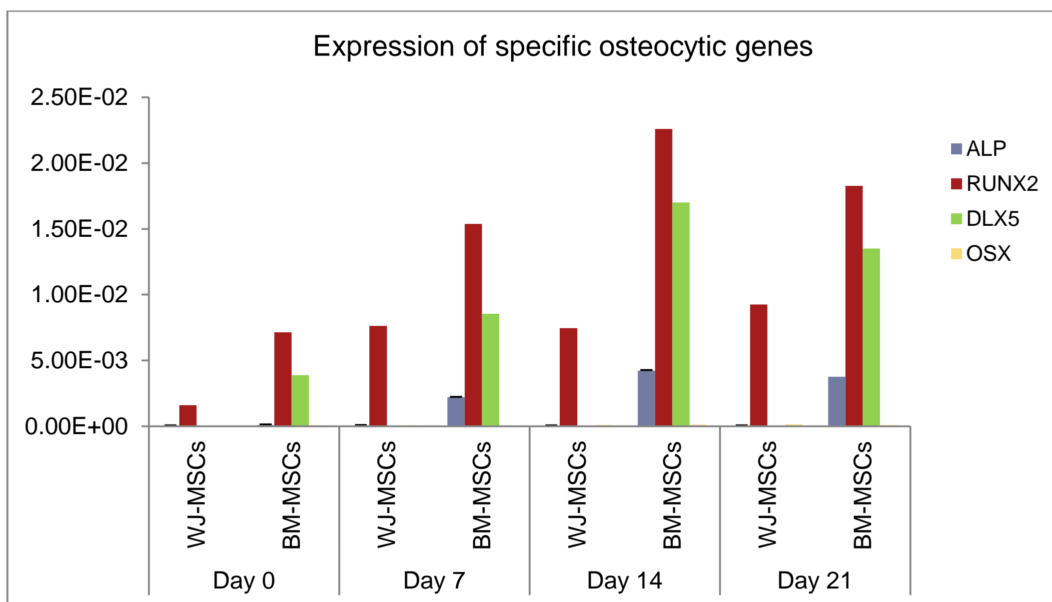

Diagram 7: Expression of ALP, RUNX2, DLX5 and OSX for BM-MSCs and WJ-MSCs

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Summarizing the above, BM-MSCs express all four osteocytic genes at various levels. In contrast, WJ-MSCs are able to express RUNX2 and OSX but the second osteocytic gene expression is very low.

3.5. Expression of adipocytic genes

Comparative study of BM-MSCs and WJ-MSCs also was implemented for adipocytic gene expression after cultured with appropriate media for 28 days. Two genes were studied such as CEBP/a and PPARG.

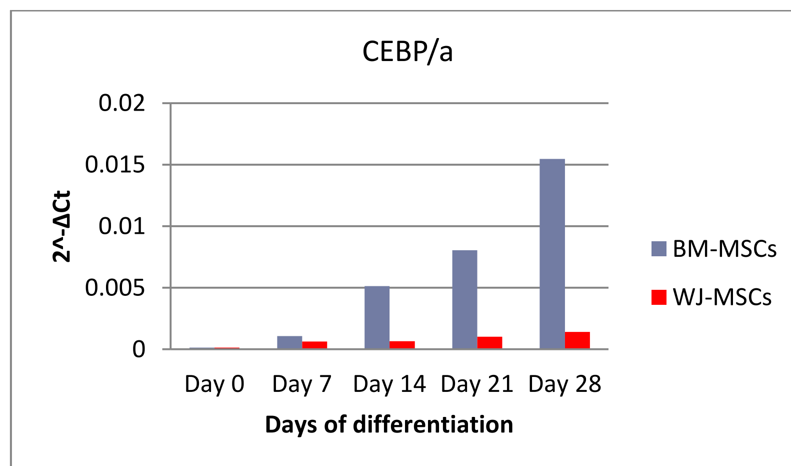

Diagram 8: CEBP/a expression of BM-MSCs and WJ-MSCs

Enhancer-binding protein alpha is a protein that in humans is encoded by the CEBPA gene.

BM-MSCs expression of CEBP/a is apparent, but very low, during the differentiation with a significant increase each 7 days while the expression in WJ-MSCs is minimal and remains so.

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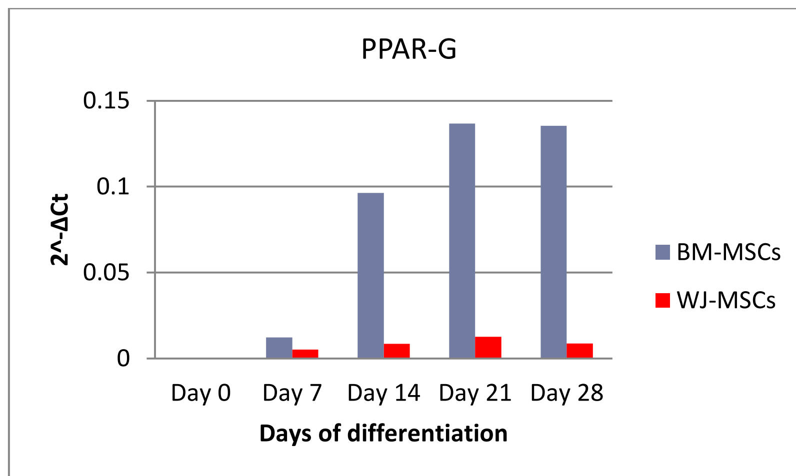

Diagram 9: PPAR-G expression of BM-MSCs and WJ-MSCs

Peroxisome proliferator-activated receptor gamma is a nuclear receptor in humans that encodes gene PPRA-G.

Expression of PPAR-G for both BM-MSCs and WJ-MSCs starts at day 7. In BM-MSCs expression increases significantly and from day 21 to day 28 remains the same whereas for WJ-MSCs the levels of expression are low and continue so.

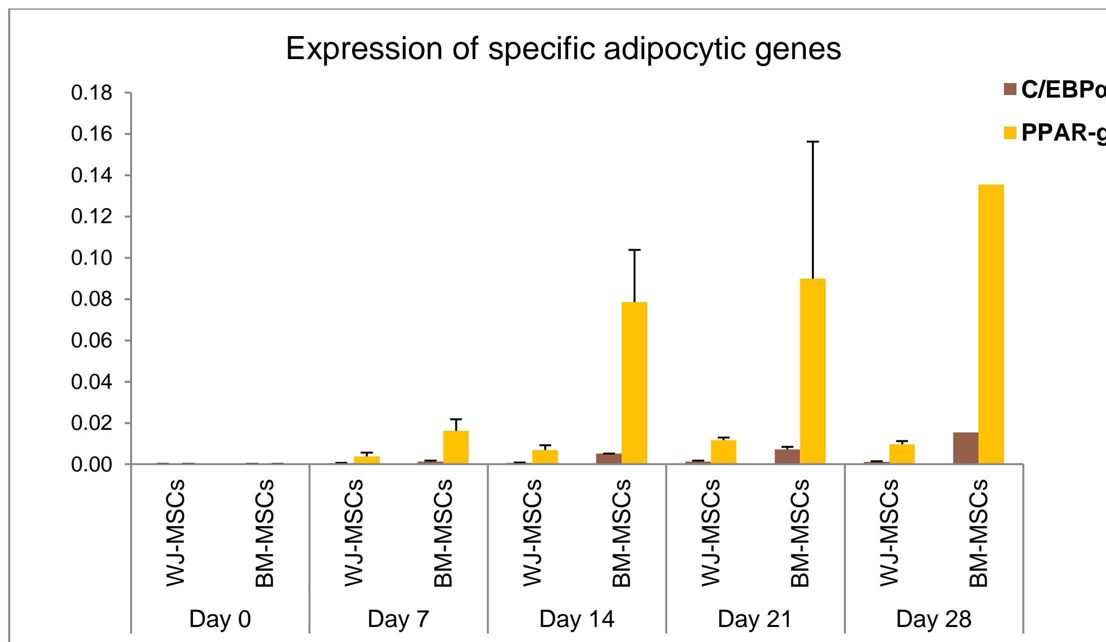

Diagram 10: Expression of CEBP/a and PPAR-g for BM-MSCs and WJ-MSCs

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In conclusion, WJ-MSCs lack of CEBP/a expression and minimal amounts of PPAR-g expression reported. BM-MSCs presenting expression of PPAR-g and low levels of CEBP/a.

3.6. Biomaterials

Three dimensional random structure scaffolds of chitosan-gelatin (CG) were fabricated in different ratios (CG/1:2, CG/2:1, and CG/3:1) for the investigation of porous size and surface morphology after were lyophilized for 24 hours.
CG/1:2, CG/2:1 and CG/3:1 ratio SEM images are illustrated below.

CG/1:2

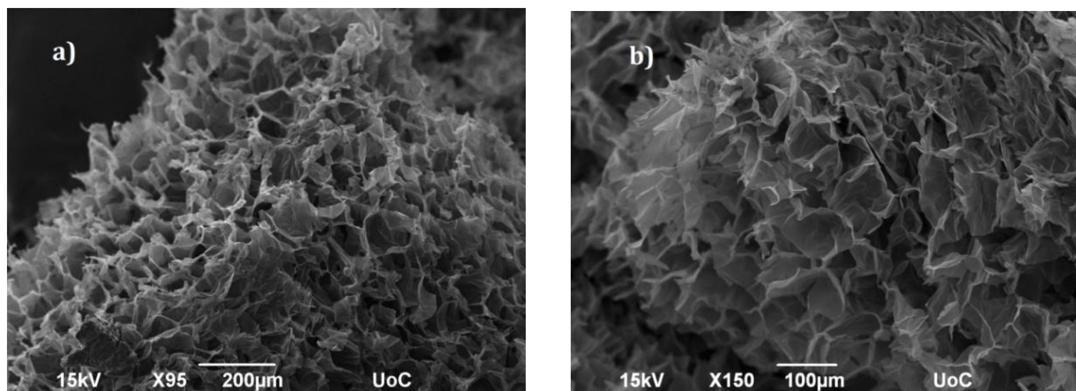

Figure 11: Scanning Electron Microscophy images showing 3D porous chitosan-gelatin scaffolds with 1:2 ratio after lyophilization. a) a 95-fold magnification and b) a 150-fold magnification.

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CG/2:1

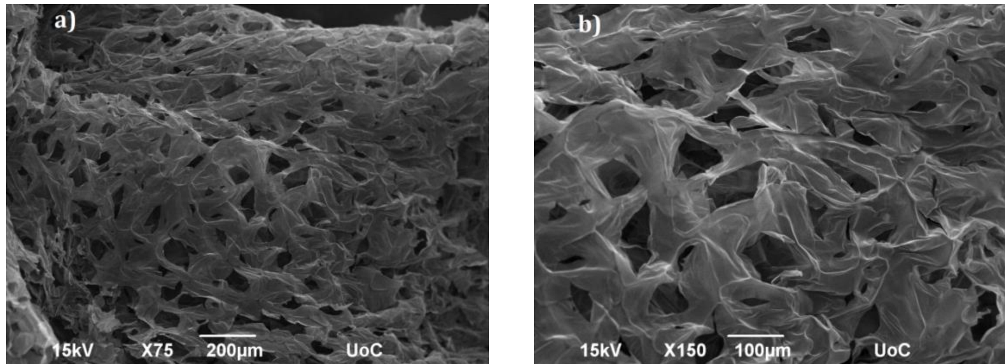

Figure 13: SEM images showing three-dimensional porous chitosan-gelatin scaffolds with 2:1 ratio after lyophilization. a) 75-fold magnification and b) 150-fold magnification.

CG/3:1

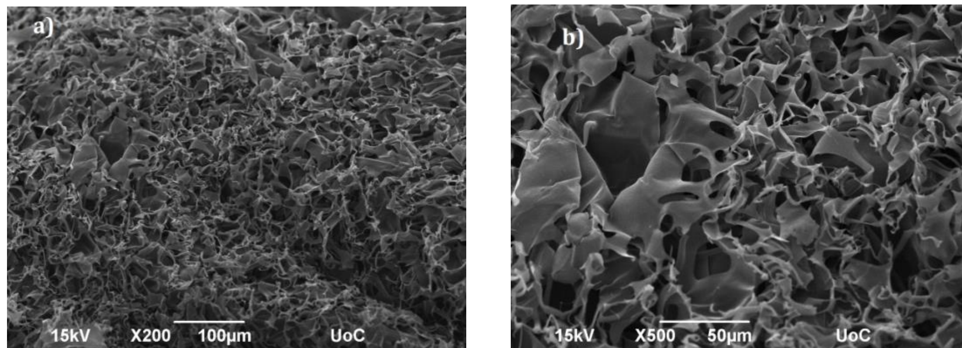

Figure 12: SEM images showing three-dimensional porous chitosan-gelatin scaffolds with 2:1 ratio after lyophilization. a) 200-fold magnification and b) 500-fold magnification.

All three different ratios have shown open pore structure and interconnectivity with big fluctuations in pore diameter, except CG/2:1 ratio which seems to maintain similar pore sizes throughout the surface and better morphological characteristics.

Next, undifferentiated mesenchymal stem cells were seeded in all three different ratio scaffolds to examine cell viability after incubation with A-MEM (10% FBS, 1% P/S, 1% L-Glutamine) in 37 °C/5%CO_2 for 3 weeks and 1 week of air drying. SEM images are illustrated.

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CG/1:2 with MSCs

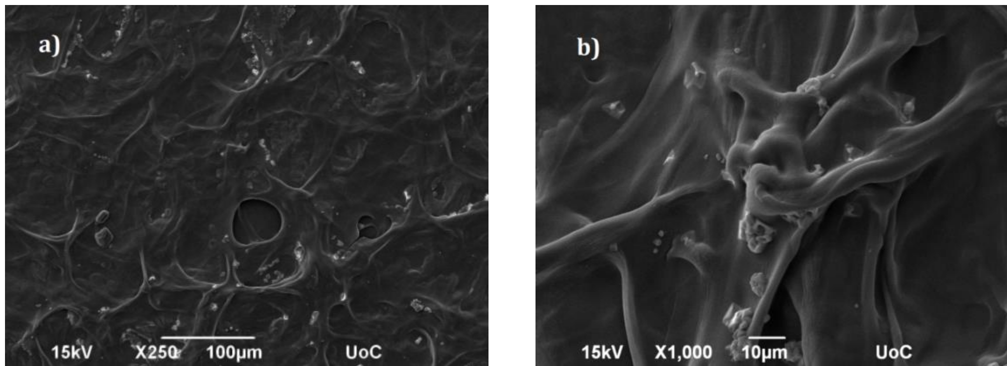

Figure 14: SEM images showing MSCs cultured on chitosan-gelatin scaffolds (1:2 ratio) with A-MEM (10% FBS, 1% P/S, 1% L-glutamine) after air drying. a) 250-fold magnification and b) 1000-fold magnification.

CG/2:1 with MSCs

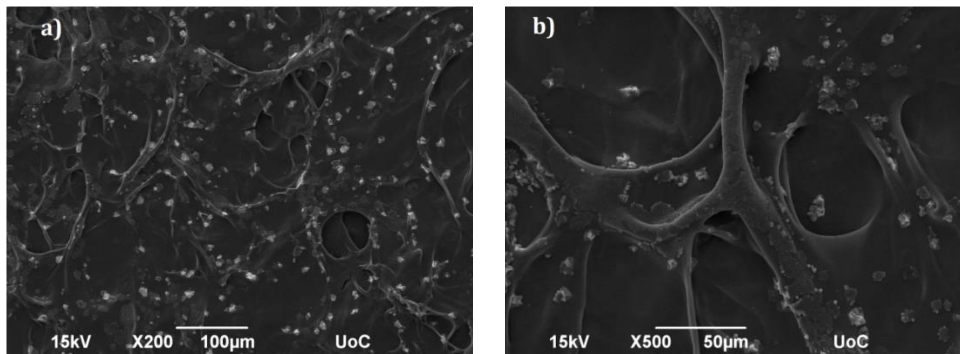

Figure 13 : SEM images showing MSCs cultured on chitosan-gelatin scaffolds(2:1 ratio) with A-MEM (10% FBS, 1% P/S, 1% L-glutamine) after air drying. a) 200-fold magnification and b) 500-fold magnification.

CG/3:1 with MSCs

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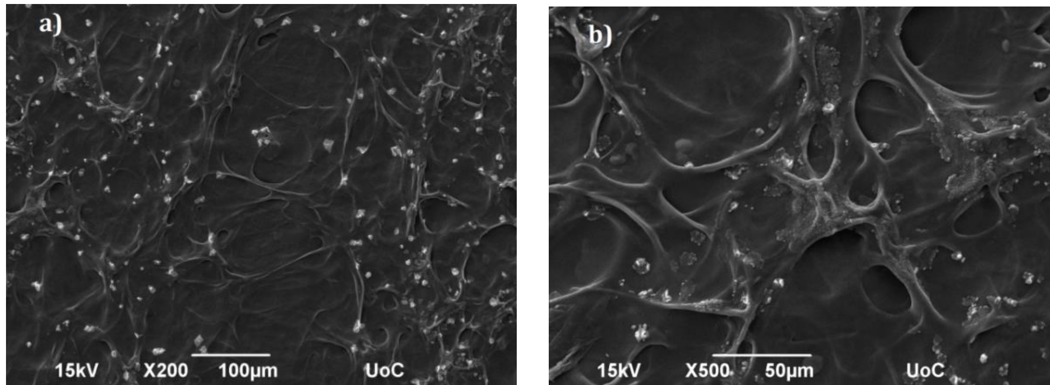

Figure 14: SEM images showing MSCs cultured on chitosan-gelatin scaffolds (3:1 ratio) with A-MEM (10% FBS, 1% P/S, 1% L-glutamine) after air drying. a) 200-fold magnification and b) 500-fold magnification.

After incubation and air drying in every 3D scaffold salt accumulation was observed and a few dead undifferentiated mesenchymal stem cells. From SEM observations best scaffold behavior in ratio was possessed by CG/1:2 where salt accumulation was less.

4. Discussion

This study aimed to the examination and identification of MSCs and specifically MSCs that were isolated from bone marrow's micro-enviroment and Wharton's Jelly, a connective tissue between the two umbilical cord arteries and the umbilical cord vein. Morphological, immunophenotypical characteristics and multipotency were studied in order to determine MSCs from both sources.

According to the results, mesenchymal cells were formed a monolayer surface attached to the plastic and a fusiform shape characteristic of fibroblasts observed.

WJ-MSCs and BM-MSCs displayed identical immunophenotype, as evidenced by the expression of the characteristic positive surface markers (CD90, CD105, CD44, CD29, and CD73), but expression was not observed for hematopoietic surface markers (CD14, CD34, CD45, and CD31) from first recultivation. Also there was no significant difference between WJ-MSCs and BM-MSCs in the proportion of apoptotic cells (7AADbright/dim) at P2 or P6. The MSCs described here have the ability to proliferate extensively, WJ-MSCs displayed superior proliferative potential compared to BM-MSCs throughout passage ($p<0.05$) similar to another study of Li X. et al. Both BM-MSCs and WJ-MSCs maintain the ability to differentiate into osteocytes and adipocytes *in vitro,* establishing their stem cell nature, but WJ-MSCs required longer time to differentiate into adipocytes and osteoblasts as compared to BM-MSCs.

Furthermore, osteocytic and adipocytic gene expression studied for both BM-MSCs and WJ-MSCs in order to examine some specific transcription factors. BM-MSCs seem to express all four factors during the osteocytic differentiation while WJ-MSCs express only RUNX2 and OSX in 21 days of differentiation. Various studies have revealed that RUNX2 may commit cells to osteogenesis though mediating other osteogenic-target genes, such as OSX, Col I, and OCN [Viereck V et al., 2002], [Franceschi RT et al., 2003]. In adipocytic differentiation BM-MSCs express both studied factors CEBP/a and PPAR-g but for WJ-MSCs expression is minimal during the

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28 days of differentiation. Despite our results, studies report that C/EBPa and PPAR-g together promote differentiation by activating adipose-specific gene expression and by maintaining each other's expression at high level [Rosen ED et al., 2002].

Fabrication and selection of random 3D scaffolds was achieved, where natural biodegradable materials chitosan–gelatin combined in different ratios (GC/1:2, GC/2:1, GC/3:1) and open pore structure with interconnectivity was observed for the examination of cell viability and proliferation ability. By SEM images observation preferable surface characteristics seems to maintain CG/2:1 ratio. Similar large pore sizes and open pore structure of the scaffold can provide suitable templates for cell adhesion. Surface structure of scaffold (GC/2:1) and pore size is similar with other reports [Zhao F et al., 2006], [Thein-Han WW et al., 2009].

An attempt of combining undifferentiated MSCs with natural materials presented here. MSCs were seeded in all ratios for cell viability examinations. None of three different ratios managed to support MSCs and, hence, no living cell observed. A possible explanation is that MSCs found an unstable, uncross-linked scaffold for adhesion.

Although this attempt showed no cell viability, studies have reported that once gelatin is chosen as a material to support biocompatible scaffolds, must be cross-linked. The criteria for the selection of the cross-linker during the degradation are firstly low toxicity and secondly high biocompatibility. Natural cross-linkers are preferable due to these criteria and genipin is an excellent natural cross-linker for proteins, collagen, gelatin, and chitosan cross-linking and much less toxic than glutaraldehyde and many other commonly used synthetic cross-linking regents [Choi YS et al., 1999] [Chiono V et al., 2008].

On concusion, mesenchymal stem cells is an under investigation field with great potential in regenerative medicine. Bone marrow is the most common and the most easily accessible source so far, but its acquisition is a painful procedure. On the other hand the acquisition of umbilical cord is a painless procedure. Wharton's Jell is also a

rich source of MSCs and therefore appear to be an inexpensive biological source for the isolation of mesenchymal stem cells thus circumventing the ethical constraint that arises from the use of the embryonic tissue. Further investigation will be needed to elucidate biological mechanisms involved in maintaining active proliferation and maximal cellular plasticity in order to optimize in vitro culturing procedure. Increase the knowledge about the molecular features of WJ-MSCs, may give a great contribution to the field of regenerative medicine. MSCs probably play a role in the rate and extent of the repair and/or regeneration of damaged tissue. Seeding them into 3D scaffolds with appropriate materials for specific tissue regeneration is only the starting point.

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