Global Science Research Journals

ISSN: 2449-1888 Vol. 3 (1), pp. 097-107, June, 2015

http://www.globalscienceresearchjournals.org/

Global Journal of Medicine and Medical Sciences

Full Length Research Paper

Long term culture of CB-MSCs deteriorates their stemness nature through a series of biomolecular changes

[1]Kesmat Maher, [2]Hanan El-Baz, [3]Zeinab Demerdash, [4]Shereen Shawky, *[5]Marwa Hassan, [6]Salwa Mohamed, [7]Faten Mahmoud, [8]Mariam Khalil, [9]Olfat Hammam and [10]Tamer Taha

[1,2,3,5,6,7]Immunology Department, Theodor Bilharz Research Institute.
[4,8]Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University.
[9]Pathology Department, Theodor Bilharz Research Institute.
[10]Reproductive Biology Department, National Research Center, Giza, Egypt.

Acceptance 15 June, 2015

This study was designed to evaluate the effect of long term in vitro culture on morphology, proliferation, telomere length, pluripotency genes expression, and differentiation potentials of cord blood (CB)-derived MSCs. This would allow us to choose the optimum passage number maintaining the MSCs stemness nature and providing the sufficient count intended for their clinical applications. Upon repeated passaging, CB-MSCs telomere length has decreased from 10.0168 kb ± 0.27 to 7.4186 kb ± 0.996 and this decrease was statistically highly significant ($p < 0.01$) which coincided with negative telomerase activity. Pluripotency and proliferation genes expression also decreased at the late passages. The adipogenic and chondrogenic differentiation potentials declined gradually whereas the osteogenic differentiation increased gradually and then dropped at the late passages. Based on the results, *in vitro* expansion attenuated the parameters that were commonly used to define CB-MSCs stemness nature except for the osteogenic differentiation potential which was highest at the intermediate passages. Therefore, we suggest considering CB-MSCs for cell and gene therapy at an early passage, as soon as a clinically sufficient MSCs count is achieved. For orthopaedic applications, the intermediate passages of CB-MSCs will be the best.

Keywords: Mesenchymal stem cells; umbilical cord blood; telomere length; pluripotency genes; osteogenic differentiation; adipogenic differentiation; chondrogenic differentiation.

INTRODUCTION

Mesenchymal stem cells (MSCs) are emerging as a promising therapeutic approach for a wide range of autoimmune disorders and degenerative diseases (Ren *et al.,* 2012). The use of MSCs in clinical applications is mainly attributed to their biological characteristics, the most important of which are, the ability to home to sites of inflammation and tissue injury when injected intravenously, the ability to secrete multiple bioactive molecules capable of stimulating recovery of injured cells and inhibiting inflammation, the ability to differentiate into various cell types, and the lack of immunogenicity (Wang *et al.,* 2012).

As a source of MSCs, bone marrow (BM) may be detrimental due to the highly invasive donation procedure

*Corresponding author. E-**mail**: marwahassan_777@yahoo.com, **Tel**: 01062309003

and the decline in stem cell number and differentiation potential with increasing age. Cord blood (CB) was introduced as an alternative source of less mature stem cells showing a higher proliferation capacity, lower severity and incidence of acute graft versus host disease, lower risk of transmission of infections by latent viruses, absence of risks to donors and faster availability of banked units (Dalous *et al.,* 2012; Rocha and Gluckman, 2007).

The ease of culture expansion of MSCs represents one of their primary advantages and is a necessary component for most clinical strategies using MSCs. However, functional and genetic alterations during culture expansion undoubtedly affect their therapeutic potential. Therefore, the effects of *in vitro* serial propagation on MSCs should be extensively investigated (Zaim *et al.,* 2012).

Telomeres are DNA structures located at the ends of eukaryotic chromosomes. They protect the ends of chromosomes against degradation and fusion, thus maintaining genome stability. The study of telomere biology is important as it is related to the process of aging. In humans, most of somatic tissues lack telomerase, the enzyme responsible for maintaining the telomeric repeats. Consequently, with each cell division telomere shorten due to the incomplete replication of linear DNA strands. In most human somatic cells, the telomere loses~50-150 bp per cell division. When telomeres reach a critical length, cells will stop division known as cellular senescence and may develop abnormal karyotypes (Riethman, 2008).

Oct4, Sox2, and Nanogare master transcriptional regulators which mediate pluripotency in embryonic stem cells (ESCs) through inhibition of tissue-specific genes and promoting self-renewal (Boroujeni *et al.,* 2012). Takahashi and Yamanaka (2006) successfully induced pluripotency in somatic cells and created mouse iPSCs, a process termed cellular reprogramming, using the exogenous expression of four genes, including two pluripotency transcription factors (Oct4 and Sox2) and two proto-oncogenes (c-Myc and Klf4). Also, PDGFRa was found to play an important role in the regulation of proliferation and migration of MSCs (Rodrigues *et al.,* 2010).

Despite growing information concerning MSCs and their use in cell-based strategies, the mechanisms that govern their self-renewal and multilineage differentiation are not well understood. Therefore, research efforts focused on identifying factors that regulate and control MSCs fate decisions are crucial to promote a greater understanding of the molecular and biological characteristics of this stem cell type of high therapeutic potentiality (Baksh *et al.,* 2004).

METHODOLOGY

Isolation and Culture of MSCs

Cord blood samples were collected from full-term babies delivered by cesarean section after informed written consent, as approved by the institutional review board at Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. CB processing was done within 2 hours of collection. Mononuclear cell fraction was isolated by density gradient separation. Cells were initially seeded at a density of 1.5×10^6 cells/cm^2 into cell culture flasks using complete DMEM which consists of low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Visp, Switzerland) supplemented with 30% fetal bovine serum (FBS) (Invitrogen Gibco, Carlsbad, CA, USA), 1% penicillin/streptomycin (Biochrom, Berlin, Germany), and 1% L-glutamine (Lonza) supplemented with dexamethasone10^{-7}M (1µl/1ml media) (Sigma-Aldrich, Steinheim, Germany) followed by incubation in Co_2 incubator at 37°C in an atmosphere of 90% relative humidity and 5% CO_2. The first medium change was done 2 weeks after seeding, and then medium was changed twice weekly.

When cells reached 90-95 % confluency, they were detached with 0.25% trypsin (Euro-lone, Milan, Italy). After initial plating, cells were transferred to the first passage by culturing at density of 5000 cells/cm^2. Medium was changed twice weekly. When cells reached 90-95 % confluency, they were trypsinized and cells were transferred progressively to the next passages.

Immunophenotyping

Cells from the 3rd passage were washed twice in PBS buffer supplemented with 0.5 % FBS. Cells were then suspended at a concentration of 3×10^6 cells in 1 ml PBS. FITC mouse anti-human CD44, CD45, CD105, CD 34, and HLA-DR (BD Biosciences, San Jose, CA, USA), as well as, PE mouse anti-human CD90, and CD73(BD Biosciences, San Jose, CA, USA) were added to flow cytometry tubes (the amount of monoclonal antibodies added was according to manufacturer instructions).

100 µl sample were added to each tube, mixed well, and incubation was done for 20 min at 4°C. Negative control was prepared by addition of 100 µl of sample to an empty flow cytometry tube. Wash was done twice with PBS, and then cells were resuspended in 500 µl PBS. Final analysis was done by the flow cytometer (Beckman Coulter Epics XL-MCL).

Measurement of Absolute Telomere Length

According to O'Callaghan and Fenech (2011),DNA was extracted from MSCs from early and late passages of the same sample, using QIAamp DNA Mini Kit (Qiagen, USA). Standard curves were generated for telomere lengths and the single copy gene (36B4) amplification reactions of synthesized oligomer. PCR reaction was performed with 10 µl 2x QuantiTect SYBR Green PCR Master Mix, 2 µM forward primer, 2 µM reverse primer, 4 µl RNase-free water, 4 µl sample DNA (5 ng/µl). Samples were applied in triplicate. Cycling was initiated with 95°C for 15 min followed by 40 cycles of (95°C denaturation for 15 seconds, 60°C annealing for 1 min), followed by a melt curve. Primers used were provided by Invitrogen (Table 1).

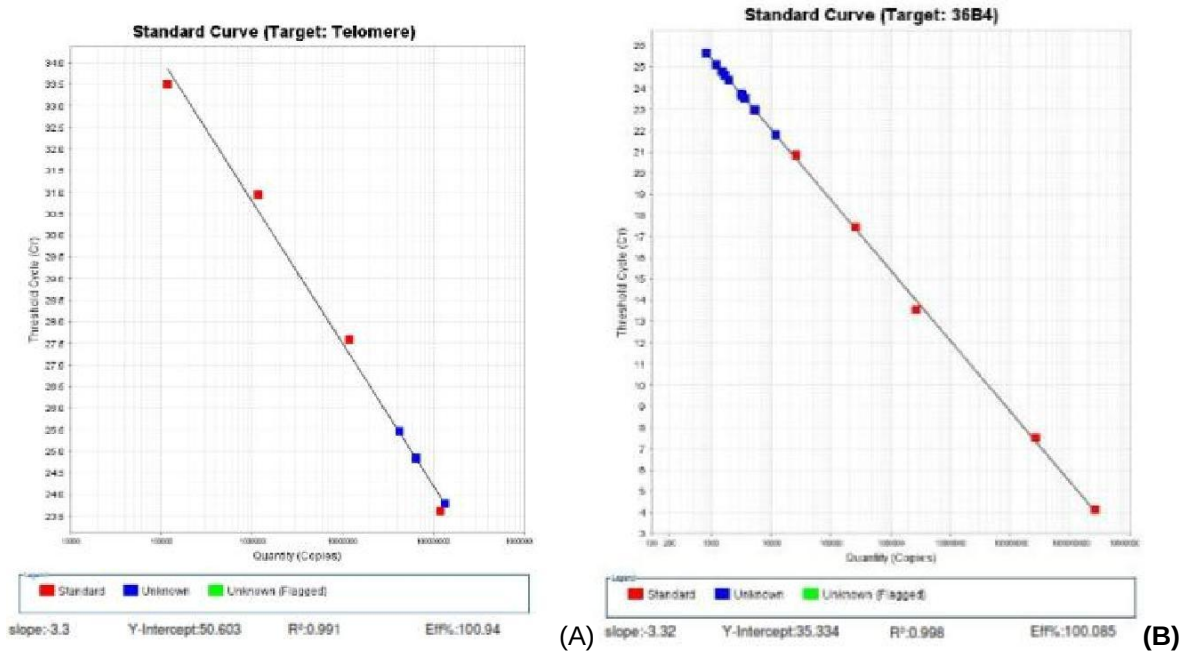

Figure 1: (A) Standard curve of Telomere gene. (B) Standard curve of 36B4 gene.

The slope of the standard curve generated for telomere was -3.3 with a correlation coefficient (R^2) of 0.991 and an efficiency of 100.94% whereas the slope of the standard curve generated for 36B4 was -3.319 with a correlation coefficient (R^2) of 0.998 and an efficiency of 100.085% (Figure. 1).

Gene Expression Analysis of MSCs:

Total RNA was extracted from trypsinized MSCs from early and late passages using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) then reverse transcribed into cDNA using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Then expression of Oct4, Sox2, Nanog, Klf4, c-Myc, and PDGFRa genes was analyzed by real-time PCR (Step One, Applied Biosystems, USA) using QuantiTect SYBR Green PCR kit (Qiagen, USA). The relative quantities of the target genes were calculated against a reference gene, GAPDH. Primers used were provided by Invitrogen (Table 1).

Measurement of Telomerase Activity

Telomerase activity was measured according to telomere repeat amplification protocol (TRAP) by using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche Diagnostics, Germany) according to manufacturer's instructions.

Osteogenic Differentiation

According to Kögler *et al*. (2004), MSCs from different passages were seeded at a density of 6×10^3 cells/cm^{2}into 6-well plates and cultured in complete DMEM. When cells reached approximately 80% confluency, the medium was replaced with osteogenic differentiation medium (Lonza, Visp, Switzerland). DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine was added to negative control wells. The media were replaced every 3-4 days for 14 days. On the 14th day, calcium deposition was assessed by alizarin red staining.

Adipogenic Differentiation

According to Kögler *et al*. (2004), MSCs from different passages were seeded at a density of 5×10^3 cells/cm^2 into 6-well plates and cultured in complete DMEM. When cells reached approximately 80% confluency, the medium was removed carefully. Adipogenic induction medium (Lonza, Visp, Switzerland) was added to the wells for three days followed by adipogenic maintenance medium (Lonza) for three days.Three cycles of induction/maintenance was carried out for optimal adipogenic differentiation. DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine was added to the negative control wells. On the 21st day, adipogenic differentiation was assessed by oil red-O staining.

Chondrogenic Differentiation

According to Kern *et al*. (2006), MSCs from different passages were washed with incomplete chondrogenic medium. The cells were then resuspended in 1 ml incomplete chondrogenic medium (Lonza, Visp, Switzerland) per 7.5×10^5 cells followed by centrifugation.

Table 1: Primers used for gene expression analysis of MSCs by real-time PCR.

Gene	Primer Sequence
Telomere	5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′ 3′-GGCTTGCCTTACCCTTACCC TTACCCTTACCCTTACCCT-5′
36B4	5′-CAGCAAGTGGGAAGGTGTAATCC-5′ 3′-CCCATTCTATCATCAACGGGTACAA-5′
Oct4	5′-TCTCGCCCCCTCCAGGT-3′ 3′-GCCCCACTCCAACCTGG-5′
Sox2	5′-GGCAGCTACAGCATGATGCAGGACC-3′ 3′-CTGGTCATGGAGTTGTACTGCAGG-5′
Nanog	5′-ACTGGCCGAAGAATAGCA-3′ 3′-AAAGCAGCCTCCAAGTCA-5′
Klf4	5′-ACCAGGCACTACCGTAAACACA-3′ 3′-GGTCCGACCTGGAAAATGCT-5′
c-Myc	5′-CAAACCTCCTCACAGCCCACT-3′ 3′-TTCGCCTCTTGACATTCTCCTC-5′
PDGFRa	5′-ACAGTGGAGATTACGAATGTG-3′ 3′-CACATCAGTGGTGATCTCAG-5′

Complete chondrogenic medium was prepared by addition of 1 µl TGF-*β*3 to 2 ml incomplete chondrogenic medium. Then the cells were resuspended in the complete chondrogenic medium at a concentration of 5.0×10^5 cells/ml. Transfer of 0.5 ml (2.5×10^5 cells) of the cell suspension was done into 15 ml polypropylene culture tubes followed by centrifugation. The supernatant wasn't aspirated. The caps of the tubes were loosened and the tubes were incubated at 37°C, in a humidified atmosphere of 5% CO_2. Themedium was completely replaced every 2-3 days. After replacing the medium, the bottom of each tube was flicked to ensure that the pellet is free floating. Chondrogenic pellets were harvested after 14 to 28 days in culture and one pellet was stained with Alcian blue and another pellet of the same was fixed by formalin for histological processing and staining by alcian blue and nuclear fast red stains.

Statistical Analysis

Statistical analysis was performed using the Analysis of Variance (ANOVA) and Duncan's multiple Range Test, to

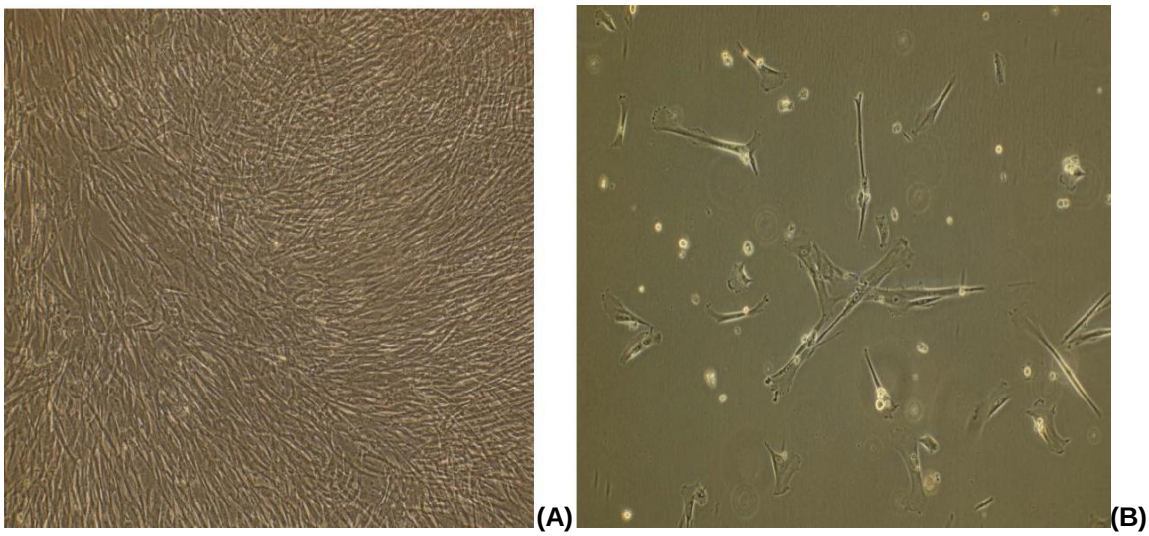

Figure 2: Morphology of CB-MSCs (A) at an early passage MSCs appear as monolayer of spindle shaped fibroblastic - like cells with 90-95% confluency, (B) at late passages MSCs show senescence features (Vacuolation, granulation, and scanty rounded cells).

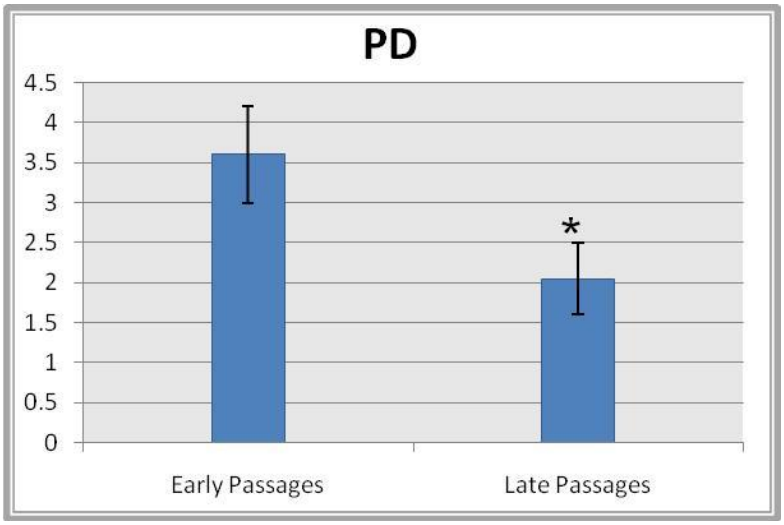

Figure 3: Population doubling of early and late passages of CB-MSCs.
* P < 0.05

Determine differences between groups, means at significance level of 0.05. Standard errors of treatment means were also estimated and regression analyses were also computed.

RESULTS

The minimal requirement for a population of cells to qualify as MSCs, as suggested by the International Society for Cytotherapy, is to meet the three criteria, including: (1) the plastic adherence of the isolated cells in culture, (2) the expression of CD105, CD73, and CD90 in greater than 95% of the culture, and their lack of expression of markers including CD34, CD45, and HLA-DR in greater than 95% of the culture, and (3) the differentiation of the MSCs into bone, fat, and cartilage (Dominici *et al*., 2006).

Isolation and Culture of Cells

MSCs were isolated from 14 out of 50 CB samples and passaged till reaching senescence, when the fibroblastic like MSCs showed vacuolated granular cytoplasm with many cell inclusions, and became rounded detached cells (Figure. 2). Their passaging numbers were variable (7-11). We designated P3 as an early passage, and P7 as the late passage.

To examine long-term growth kinetics of MSCs culture, the population doubling (PD) rate was calculated. It was ranging from 3 to 4.3 at early passages, while it was from 1.6 to 2.5 at late passages (Figure. 3).

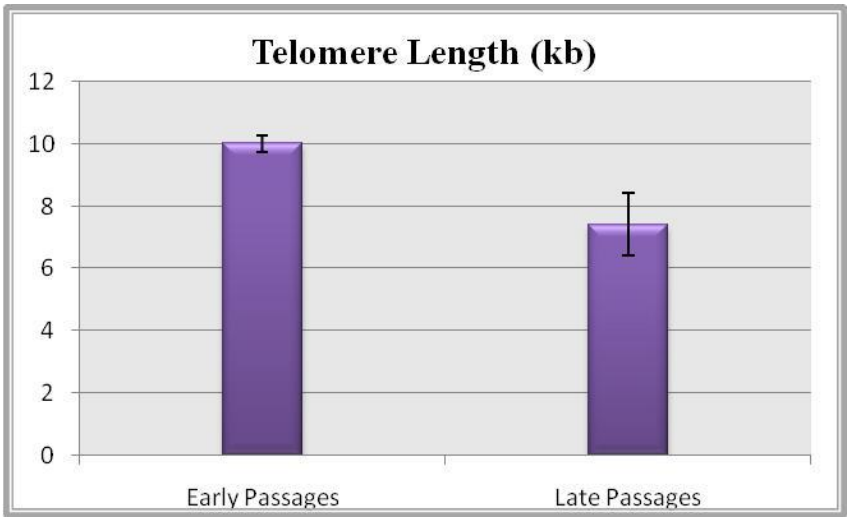

Figure 4: Absolute telomere length of CB-MSCs (mean ± SD). ** P < 0.01

Immunophenotyping

MSCs at P3 showed high expression levels of adhesion marker (CD44), typical mesenchymal markers (CD90 and CD73), endoglin receptor (CD105), and dual expression of CD105/90, whereas they were negative for hematopoietic lineage marker (CD34), leukocyte common antigen (CD45), and human leukocyte antigen class II (HLA-DR) expression.

Measurement of Absolute Telomere Length

The mean ± standard deviation of absolute telomere length of MSCs obtained from early passages was 10.0168 kb ± 0.274749 whereas that of MSCs obtained from late passages was 7.4186 kb ± 0.9958281 and this decrease was statistically highly significant ($p<0.01$) (Figure. 4).

Gene Expression Analysis of MSCs

Self-renewal and multipotency are two defining elements of stem cells. Some types of adult stem cells express Oct4 and Sox2 which are core transcription factors in early embryo development and in pluripotency maintenance of embryonic stem cells (Demerdash *et al*., 2013). CB-MSCs showed expression of transcripts for pluripotency genes (Oct4, Sox2, Nanog, Klf4, c-Myc) and PDGFRa gene which plays an important role in proliferation of MSCs, and the level of expression was decreased in the late passages. The decrease in the level of expression of Oct4 was statistically highly significant ($p < 0.01$), and the decrease in the level of expression of Sox2, Nanog, and Klf4 was statistically significant ($p < 0.05$).

Telomerase Activity

Telomerase enzyme is essential for the maintenance of telomere length. In the current study, telomerase activity was measured in CB-MSCs according to TRAP by using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit. We did not detect telomerase activity at the early passages of culture, nor at later stages during cell expansion.

Osteogenic Differentiation

When CB-MSCs were induced to differentiate into osteogenic lineage, the spindle shape of CB-derived cells became less elongated, and polygonal shaped with the formation of aggregates. The differentiation was confirmed by the formation of mineralized matrix as evidenced by alizarin red staining.

CB-MSCs at early passages showed a low osteogenic differentiation potential, and with repeated passaging, the differentiation potential was increased but at late passages, it became very low (Figure. 5, 6).

Adipogenic Differentiation

Under the influence of adipogenic differentiation conditions, the MSCs became large and rounded with the accumulation of neutral lipid vacuoles indicated by the Oil Red-O stain.

CB-MSCs obtained from early passages showed a high adipogenic differentiation potential but the differentiation potential decreased with repeated passaging (Figure. 7).

Chondrogenic Differentiation

Chondrogenic differentiation of CB-MSCs was assessed by the positive staining of the pellet with alcian blue which

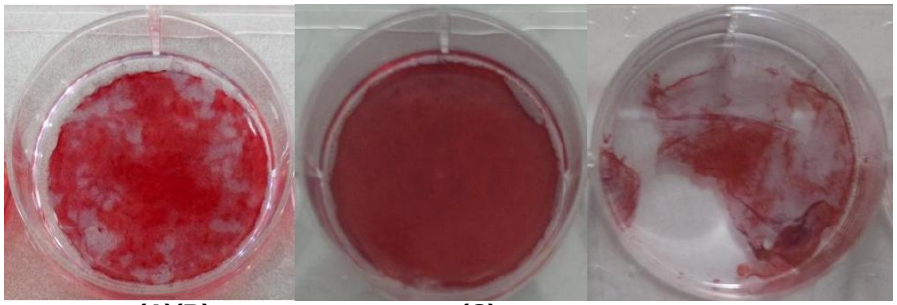

Figure 5: Macroscopic appearance of wells after alizarin red staining of (A) early passage (B) intermediate passage (C) late passage.

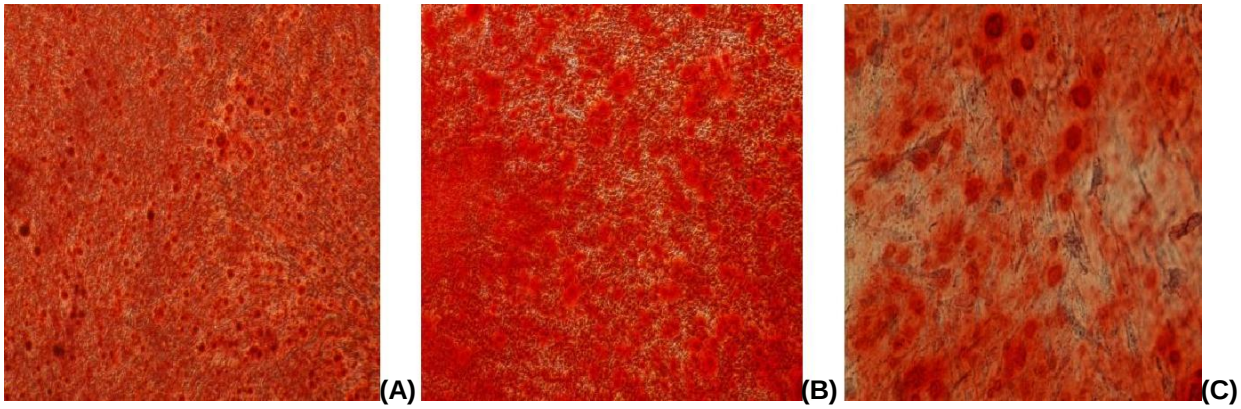

Figure 6: Microscopic appearance of CB-MSCs induced to differentiate into osteogenic lineage after alizarin red staining of (A) early passage (B) intermediate passage (C) late passage.

stains proteoglycans in chondrocytes. Also, the histological evaluation of the pellets showed sheets of mature and immature chondrocytes.

The pellet size was much smaller at late passages with decreased number of mature chondrocytes (Figure. 8, 9).

DISCUSSION

Regenerative medicine is an evolving topic of research involving numerous technological methods to repair damaged tissues. Particularly, MSCs are a promising tool in regenerative medicine because of their ease of accessibility for isolation, extensive capacity for *in vitro* expansion, homing to sites of tissue injury, lack of tumorogenicity and immunogenicity, and ability to perform immunomodulatory as well as anti-inflammatory functions (Taran *et al*., 2014).

Friedenstein *et al*. (1982) first described MSCs in BM as a very rare population (0.01% to 0.001%) and the BM became the most commonly used source of adult MSCs. BM-MSCs have been defined as plastic-adherent, nonhematopoietic, multipotential cells that support hematopoietic stem cells expansion *in vitro*, and studies have shown their ability to differentiate into bone, adipose tissue, muscle, and cartilage. Nevertheless, CB-MSCs are being considered more as an alternative source of MSCs. Their many advantages such as accessibility, extraction procedures that are painless for donors, the lack of ethical controversies, the reduced risk of contamination, the lower incidence of acute graft-versus-host disease, the great expansion capability and the long telomere sequences suggesting a late onset of senescence make them particularly attractive (Dalous *et al*., 2012).

MSCs can be identified by both their capacity to adhere to plastic and their phenotypic characterization through a panel of cell surface molecules, including CD90, CD105, and CD73 while lack expression of CD45, CD34, CD14, or CD11b, CD79 alpha or CD19 and HLA-DR were also considered characteristic of this cell population. However, a unique and specific MSC marker, allowing their exclusive identification, has not yet been found (Dominici *et al*., 2006).

Despite the various sources, concentration of MSCs within tissues is very low, and it is not possible to isolate 50-200 million MSCs (typically used in clinical trials) from a donor for each therapy. Thus, *in vitro* expansion of MSCs has become an inevitable option before putting them to therapeutic use. During this expansion process

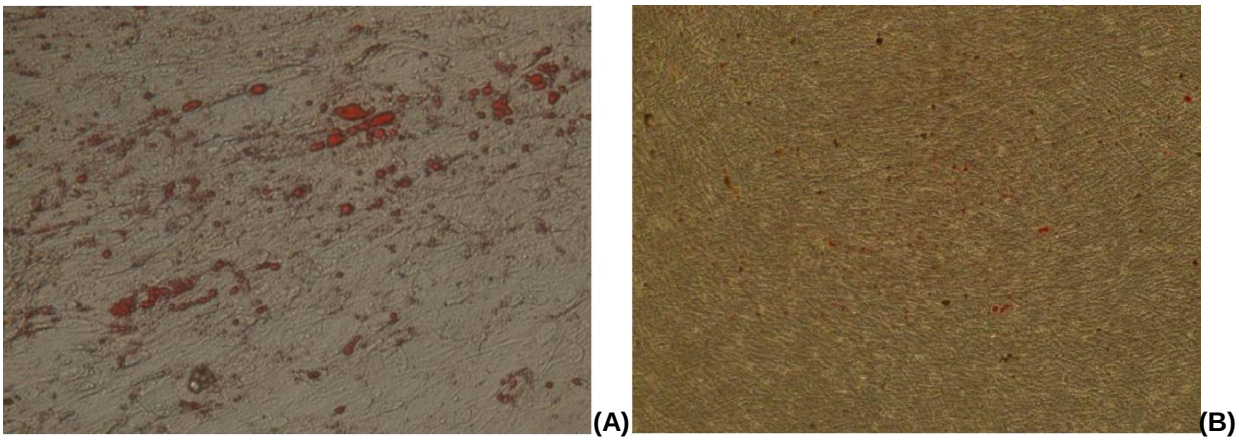

Figure 7: Microscopic appearance of CB-MSCs induced to differentiate into adipogenic lineage after Oil Red-O staining of (A) early passage (B) late passage.

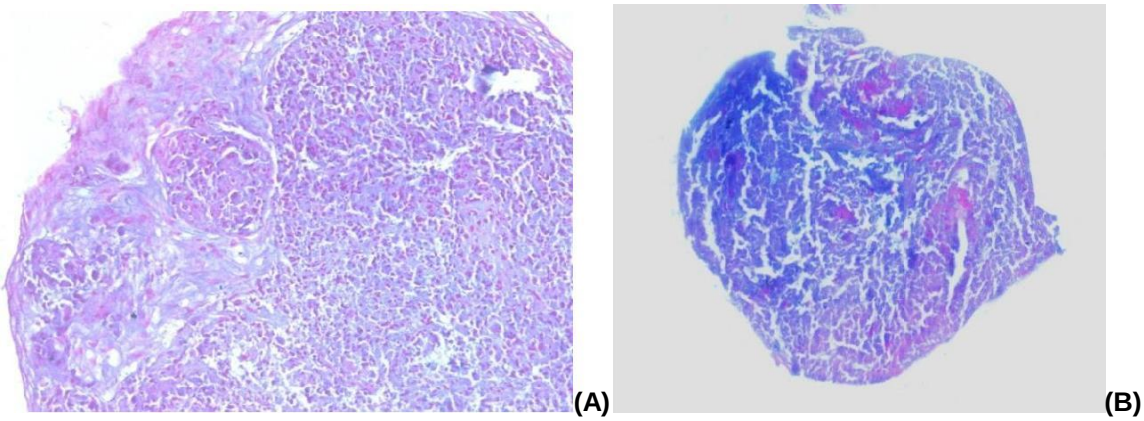

Figure 8: Microscopic appearance of chondrogenic differentiation of CB-MSCs after alcian blue and nuclear fast red staining showing sheets of mature (red color arrow) and immature (black arrow color) chondrocytes (x200) of (A) early passage (B) late passage.

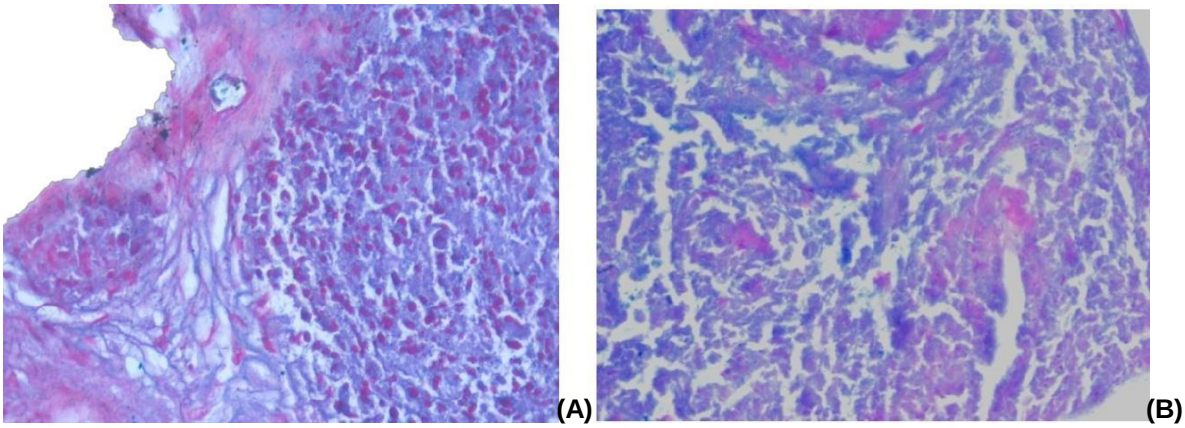

Figure 9: Microscopic appearance of chondrogenic differentiation of CB-MSCs after alcian blue and nuclear fast red staining showing sheets of mature (red color arrow) and immature (black arrow color) chondrocytes(x400) of (A) early passage (B) late passage.

cells enter senescence, which leads to inability to proliferate further. One of the reliable biological markers for cellular senescence and replicative capacity is the telomere length (Haque*et al*., 2013).

Telomeres are specialized structures at the ends of linear eukaryotic chromosomes. The human telomere is a repeat of the nucleotide sequence TTAGGG that occurs about a hundred times. As chromosomes are replicated during cell division, there is incomplete replication of the extreme 3' end of the lagging strand (sometimes referred to as "end replication problem") which results in loss of telomeric material with each cell division. Therefore

without a special mechanism for replicating the ends, chromosomes would be shortened in each cell division. Eventually, telomeres would become extremely short that the cell would stop dividing. The telomerase enzyme solves this problem by adding telomeres to chromosome ends. The telomerase holoenzyme core consists of human telomerase reverse transcriptase that functions as a catalytic subunit, telomerase RNA component that serves as a template, and RNA-binding proteins that are involved in stabilization of the telomerase complex (Sekhri, 2014).

The measurement of telomere length is a genetic tool that is beginning to be employed widely in evolutionary studies as marker of age as telomere length represents a balance between the loss of telomeric repeats, and the addition of telomeric repeats by the RNA-dependent DNA polymerase, or telomerase. Since the telomeric loss is due to an end-replication defect during progressive cell divisions, telomere shortening is regarded as a molecular clock that counts the number of cell divisions and determines the onset of cellular senescence. Thus, the telomere length is an indicator of replicative history and may also determine the replicative potential of cells (Kim *et al*., 2002).

With this in mind, this study was designed to evaluate the effect of long term *in vitro* culture on the morphology, proliferation, telomere length, telomerase activity gene expression and differentiation of MSCs because all advantageous functions may become limited with age.

CB-MSCs isolated in our laboratories have expressed typical cell surface markers at levels in good agreement with results of similar studies (Bernardo *et al*., 2007; Kern *et al.,* 2006; Zuk *et al.,* 2002). They expressed classic MSCs surface proteins (CD44, CD73, CD90, and CD105) and lacked hematopoietic lineage marker (CD34), leukocyte common antigen (CD45), and HLA-DR expression.

These CB-MSCs have been subjected to repeated passaging *in vitro* until reaching senescence where cells became markedly enlarged, varied in size and shape, became granular and vacuolated and lost proliferation. Cells in early passage cultures (P3) had tendency to divide more rapidly than do those in late passage cultures (P7) with a PD rate (3 to 4.3) higher than that of the later passages (1.6 to 2.5). This compares favorably with other studies on MSCs isolated from different sources e.g. bone marrow and hair follicle as Baxter *et al*. (2004) found that MSCs from early passages had a faster expansion rate than those from the late passages, Bonab *et al*. (2006) showed that the average number of PD decreased gradually from 7.7 at the beginning of culture until reaching 1.2 in the last passage, and Bajpai *et al.* (2012) reported that the number of PD declined sharply with repeated passaging from approximately 2.5 to 3.5 divisions reaching less than one doubling.

CB-MSCs from different passages were subjected to measurement of absolute telomere length using the real-time PCR. It was found that the *in vitro* expansion was associated with significant telomere shortening with a mean length of 10.0168 kb at early passages and 7.4186 kb at late passages which was statistically highly significant ($p < 0.01$). This is in agreement with Vidal *et al.* (2012) report which showed that the telomere length has decreased significantly from an average of 10.2 kb in early passage cells to 4.5 kb in senescent cells, whereas previous reports of Terai *et al*. (2005), and Bonab *et al.* (2006) showed a negligible decrease in telomere length from an average of 9.19 kb in the first passage to 8.75 kb in the last passage.

In contrast to embryonic stem cells, which are capable of maintaining telomeres by telomerase, adult stem cells undergo progressive telomere shortening and appear to be mortal, although the activity of telomerase might extend their proliferative capacity (Zimmermann *et al*., 2003). In this study, the activity of the telomerase enzyme in CB-MSCs was examined using the TRAP assay. Similar to previous reports (Bernardo *et al*., 2007; Terai *et al*., 2005; Tichon *et al*., 2009; Zhao *et al*., 2008; Zimmermann *et al*., 2003), it was revealed that these cells are telomerase negative at all passages which explains the progressive telomere shortening during *in vitro* culture.

In the current study, we also investigated changes in molecular profiles of pluripotency, self renewal, and proliferation genes expression associated with young and old cells from the same sample as self-renewal and multipotency are the key hallmarks of stem cells, permitting them to act as the fundamental units maintaining growth, homeostasis and repair of many tissues. We found that Oct4, Sox2, Nanog, klf4, c-Myc and PDGFRa genes expression decreased with repeated passaging of MSCs which confers a decrease in the pluripotency and proliferative capacity as the cells become senescent.

In addition, we investigated the osteogenic, adipogenic, and chondrogenic differentiation capacity of CB-MSCs as MSCs differentiation into other lineages has been used as a marker for the multipotential nature of these cells. In the current study, we induced CB-MSCs from different passages of the same sample to differentiate into osteogenic, adipogenic, and chondrogenic lineages and this differentiation was assessed morphologically and by staining with alizarin red, Oil red O, and alcian blue respectively. We found that the differentiation potential to adipocytes and chondrocytes decreased in the late passages. Our results were in line with similar studies from other groups that demonstrated functional implications on differentiation potential as a result of *in vitro*expansion (Bonab *et al*., 2006; Digirolamo *et al*., 1999; Lee *et al*., 2013; Wagner *et al*., 2008) but the results were contradictory to previous studies (Hua *et al*., 2011; Lee *et al*., 2004; Wagner *et al*., 2008) which showed that MSCs can be extensively clonally expanded *in vitro* while retaining the potential to differentiate, under

in vitro conditions, into multiple lineages of the mesoderm.

An important finding in CB-MSCs differentiation into the osteogenic lineage was the increase in the osteogenic differentiation potential with repeated passaging before this potential became undetectable in the late passages which coincides with Bajpai *et al.* (2012) study which showed that the osteogenesis was maximum at intermediate passages. This observation might have a significant impact on the development of therapeutics aimed at the regeneration of damaged tissues in orthopedic diseases such as osteoporosis and osteoarthritis. Also, Baxter *et al*. (2004) reported that MSCs maintained their osteogenic potential even at late passages in addition to other previous studies (Cheng *et al*., 2011; Wagner *et al*., 2008) which demonstrated that the propensity for osteogenic differentiation increased with repeated passaging. On the other hand, the osteogenic differentiation potential was gradually lost in the late passages (Bonab *et al*., 2006).

CONCLUSION AND RECOMMENDATION:

Taken together, the extensive *in vitro* expansion of CB-MSCs has unfavorable implications on morphology, telomere length, pluripotency genes expression, and finally loss of functions, that is, differentiation potential. This means that*in vitro* expansion attenuates the parameters that are commonly used to define MSCs stemness nature. Considering MSCs of early passages for cell and gene therapy, once reaching their required therapeutic count, is highly recommended. For orthopaedic applications, the intermediate passages of CB-MSCs will be the best.

ACKNOWLEDGEMENT

This work was supported by Science and Technology Development Funds (STDF), Egypt (project 1410), and Theodor Bilharz Research Institute (TBRI) Internal Project K11.

REFERENCES

BajpaiVK, Mistriotis P, Andreadis ST (2012). Clonal multipotency and effect of long-term *in vitro* expansion on differentiation potential of human hair follicle derived mesenchymal stem cells. *Stem Cell Res*. 8(1): 74-84.

Baksh D, Song L, Tuan RS (2004). Adult mesenchymal stem cells: Characterization, differentiation, and application in cell and gene therapy. *J. Cell. Mol. Med.* 8(3): 301-316.

Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuono I (2004). Study of telomere length reveals rapid aging of human marrow stromal cells following *in vitro* expansion. *Stem Cells.* 22 (5): 675-682.

Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F (2007). Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. *Cancer Res*. 67 (19): 9142-9149.

Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B (2006). Aging of mesenchymal stem cell in vitro. *BMC. Cell. Biol*. 7:14.

Boroujeni ME, Gowda J, Johnson J, Rao J, Saremy S *(*2012). The proliferation and differentiation capacity of bone marrow derived-human mesenchymal stem cells in early and late doubling. *Asian J. Biochem.* 7: 27-36.

Cheng H, Qiu L, Ma J, Zhang H, Cheng M, Li W, Zhao X, Liu K (2011). Replicative senescence of human bone marrow and umbilical cord derived mesenchymal stem cells and their differentiation to adipocytes and osteoblasts. *Mol. Biol. Rep*. 38(8): 5161-5168.

Dalous J, Larghero J, Baud O (2012). Transplantation of umbilical cord derived-mesenchymal stem cells as a novel strategy to protect the central nervous system: Technical aspects, preclinical studies, and clinical perspectives. *Pediatr. Res.* 71: 482-490.

Demerdash Z, El Baz H, Mahmoud F, Mohamed S, Maher K, Gaafar T, Shawky S, Hassan M, Abdelhady D, Taha T (2013). Enhancing ex vivo expansion of cord blood-derived unrestricted somatic stem cells for clinical applications. *Cell Prolif*. 46(6): 628-636.

Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ (1999). Propagation and senescence of human marrow stromal cells in culture: A simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol*. 107: 275-281.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 8(4): 315-317.

Friedenstein AJ, Latzinik NW, Grosheva AG, Gorskaya UF (1982). Marrow microenvironment transfer by heterotopic transplantation of freshly isolated and cultured cells in porous sponges. *Exp. Hematol*. 10(2): 217-227.

Haque N, Rahman MT, Abu Kasim NH, Alabsi AM (2013).Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy. *ScientificWorldJournal*. 2013: 632972.

Hua J, Qiu P, Zhu H, Cao H, Wang F, Li W (2011). Multipotent mesenchymal stem cells (MSCs) from human umbilical cord: Potential differentiation of germ cells. *Afr. J. Biochem. Res*. 5(4): 113-123.

Kern S, Eichler H, Stoeve J, Klüter H, Bieback K (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells.* 24(5): 1294-1301.

Kim HR, Kim YJ, Kim HJ, Kim SK, Lee JH (2001). Telomere length changes in colorectal cancers and polyps. *J. Korean Med. Sci*. 17(3): 360-365.

Kögler G, Sensken S, Airey JA, Trapp T, Müschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Müller HW, Zanjani E, Wernet P (2004). A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J. Exp. Med.* 200(2): 123-135.

Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood*. 103(5): 1669-1675.

Lee KS, Cha SH, Kang HW, Song JY, Lee KW, Ko KB, Lee HT (2013). Effects of serial passage on the characteristics and chondrogenic differentiation of canine umbilical cord matrix derived mesenchymal stem cells. *Asian-Australas. J. Anim. Sci*. 26(4): 588-595.

O'Callaghan NJ, Fenech M (2011). A quantitative PCR method for measuring absolute telomere length. *Biol. Proced. Online*. 13: 3.

Ren G, Chen X, Dong F, Li W, Ren X, Zhang Y, Shi Y (2012).Mesenchymal stem cells and translational medicine: Emerging issues. *Stem Cells Transl. Med*. 1(1): 51-58.

Riethman H (2008).Human telomere structure and biology. *Annu. Rev. Genomics Hum. Genet*. 9: 1-19.

Rocha V, Gluckman E (2007). Outcomes of transplantation in children with acute leukaemia. *Lancet.*369(9577): 1906-1908.

Rodrigues M, Griffith LG, Wells A (2010). Growth factor regulation of proliferation and survival of multipotential stromal cells. *Stem Cell Res.Ther*. 1(4): 1-12.

Sekhri K (2014). Telomeres and telomerase: Understanding basic structure and potential new therapeutic strategies targeting it in the treatment of cancer. *J. Postgrad. Med*. 60(3): 303-308.

Takahashi K, Yamanaka S (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 126(4): 663-676.

Taran R, Mamidi MK, Singh G, Dutta S, Parhar IS, John JP, Bhonde R, Pal R, Das AK (2014). *In vitro* and *in vivo* neurogenic potential of mesenchymal stem cells isolated from different sources. *J. Biosci*. 39(1): 157-169.

Terai M, Uyama T, Sugiki T, Li XK, Umezawa A, Kiyono T (2005). Immortalization of human fetal cells: The life span of umbilical cord blood-derived cells can be prolonged without manipulating p16INK4a/RB braking pathway. *Mol. Biol. Cell*. 16(3): 1491-1499.

Tichon A, Gowda BK, Slavin S, Gazit A, Priel E (2009). Telomerase activity and expression in adult human mesenchymal stem cells derived from amyotrophic lateral sclerosis individuals. *Cytotherapy*. 11(7): 837-848.

Vidal MA, Walker NJ, Napoli E, Borjesson DL (2012). Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue. *Stem Cells Dev*. 21(2): 273-283.

Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD (2008).Replicative senescence of mesenchymal stem cells: A continuous and organized process.*PLos One.*3 (5):e2213.

Wang S, Qu X, Zhao RC (2012). Clinical applications of mesenchymal stem cells. *J. Hematol. Oncol.* 5: 19.

Zaim M, Karaman S, Cetin G, Isik S (2012). Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells. *Ann. Hematol.* 91(8): 1175-1186.

Zhao YM, Li JY, Lan JP, Lai XY, Luo Y, Sun J, Yu J, Zhu YY, Zeng FF, Zhou Q, Huang H (2008). Cell cycle dependent telomere regulation by telomerase in human bone marrow mesenchymal stem cells. *Biochem. Biophys. Res. Commun*. 369(4): 1114-1119.

Zimmermann S, Voss M, Kaiser S, Kapp U, Waller CF, Martens UM (2003). Lack of telomerase activity in human mesenchymal stem cells. *Leukemia*. 17(6): 1146-1149.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH *(*2002). Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell.*13(12): 4279-4295.