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Comparison of the osteogenic differentiation potential of mesenchymal cells isolated from human bone marrow, umbilical cord blood and placenta derived stem cells



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ABSTRACT

Bone marrow has been considered for long time as the main source for mesenchymal stem cells. However, bone marrow aspiration is an invasive process that can be associated with morbidity as well as few numbers of obtained cells. Umbilical cord blood and placental tissues are other potential sources for the same type of cells. These sources are abundant, accessible and associated with no harm to the donor. This study aimed at determining the differentiation of the three cell types towards the osteogenic lineage in short term culture and in classical osteogenic conditions. The gene expression profile showed that bone marrow derived cells were the most responsive to the culture conditions while umbilical cord blood derived cells were next, as shown by the expression by the osteogenic key transcription factors 'Runx-2' and osterix. At the meantime, umbilical cord blood and placenta derived cells showed significant enhancement of the gene expression over the study course, which denoted potential response of the cells. Based on these results and the availability of these two sources, umbilical cord blood and placenta should still be considered as potential sources for mesenchymal stem cells in osteogenic research program. However their differentiation potential will need further enhancement.

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1. Introduction

Many patients all over the world are suffering from bone associated problems that cannot be satisfactorily treated by the current management modalities and need physiological replacement, such as non-united fractures, bone cysts and bone defects, which could be congenital or acquired due to blast injuries (Dawson and Oreffo, 2008). The efficient bone regeneration is a hot topic of research based on using different types of stem cells in order to establish the most efficient protocol for induction and consequently could be applied in the future for tissue regeneration programs (Ichinose et al., 2013; Nikukar et al., 2013). Bone marrow derived stem cells (BM-MSCs) are considered as multipotent stem cells that of mesodermal origin (mesenchymal stem cells) that are still considered as the golden standard for this purpose. These cells can preferentially differentiate into mature cells of their lineage, including the osteoblasts (Brooke et al., 2008; Ichinose et al., 2013).

However, the difficulty of obtaining BM-MSCs represents a significant problem. The current approach is to apply a drill in the hip bone under anesthesia; a technique which is invasive, associated with pain and the yield of the cells is variable (Bain, 2001, 2003). Thus looking for other sources of such multipotent cells is an attractive goal of research. These sources include the umbilical cord blood (UCB) and the placental core derived cells. UCB contains a cell population that has the characteristics of mesenchymal stem cells, which have similar properties to BM-MSCs. The differentiation ability of these cells is controversial in different research papers (Jeong et al., 2005; Roobrouck et al., 2008). At the same time, the abundance and accessibility of UCB would make it a promising source of stem cells for research and clinical applications if those cells showed potent differentiation ability (Ali and Bahbahani, 2010).

Both cell types offer a perfectly natural, controversy-free source for acquiring stem cells and can be considered as one of the most abundant sources of non-embryonic stem cells, bearing in mind that the global birth rate is over 200 million per year (McGuckin et al., 2006; McGuckin and Forraz, 2008). In addition, these cells occupy an intermediate stage between the embryonic stem cells and the adult stem cells, which lead to higher proliferating potential and longer telomeres than adult stem cells that are isolated several decades afterwards (Pipes and Ablin, 2006; Slatter and Gennery, 2006). While there are many reports regarding UCB cells, placental stem cells have not received the same attention (Rus et al., 2011).

The differentiation process of the stem cell into the osteogenic lineage had to go across many stages, starting from the commitment of the cells towards the osteogenic lineage and ending by the formation of the osteogenic matrix, in which the cells should be embedded. Such differentiation is associated with activation of a group of genes. Two transcription factors are essential for osteoblast differentiation and skeletal development during the early stages of embryogenesis which are Runt-related transcription factor-2 (Runx-2) and osterix. Runx-2 – also is known as Cbfa-1 – is the key transcription factor during the early stages of embryogenesis (Ducy et al., 1997, 1999), while osterix is a zinc finger-containing transcription factor that is essential for further differentiation and bone formation. In osterix-null mutant mice, neither

endochondral nor intramembranous bone formation occurs, and osteoblast differentiation is arrested (Nakashima et al., 2002). After differentiating to pre-osteoblasts, osterix, and Runx-2 direct the cells to immature osteoblasts, which produce bone matrix proteins, during osteoblast differentiation, Runx-2 up regulates the expression of bone matrix protein genes including type 1 collagen and alkaline phosphatase (Jaiswal et al., 2000; Komori, 2010). Collagen1 is the most abundant protein in animals, makes up about (90–95%) of the organic content of bone; thus collagen is the main constituent of the bone matrix. Mutations in type I collagen gene leads to several forms of bone abnormalities including osteogenesis imperfecta, Ehlers–Danlos syndrome and Marfan syndrome (Kadler, 1995). Alkaline phosphatase (ALP) is an enzyme that is produced in bone during the developmental process. ALP splits pyrophosphate, which is an inhibitor of mineralization, to provide inorganic phosphate that is required as part of the mineralization process. ALP activity typically becomes significantly higher in BM-MSCs cultured in osteogenic conditions compared to cells cultured in basal conditions after 6 days (Mirmalek-Sani et al., 2006).

The aim of the present study is to compare the osteogenic differentiation potential of cells isolated from the UCB and placenta with bone marrow derived stem cells. The verification of such induction was achieved by determining the level of gene expression of the osteogenic master genes Runx-2 and osterix, as well as the osteogenic matrix proteins collagen1 and ALP as the markers of osteogenic induction. The importance of this study was to compare the three sources of stem cells together, and at the meantime to determine which genes should be targeted to investigate early differentiation.

2. Material and methods

2.1. Sample preparation

The following samples were obtained: a) cancellous bone segments removed during hip replacement surgery for bone marrow isolation; b) umbilical cord blood collected after the delivery and clamping of the cord of full-term babies of healthy women from the obstetric emergency room; c) placentas, were immediately collected after delivery. All experiments were conducted with passage zero cells. The study was approved by the research and ethics committees in the Faculty of Medicine, Suez Canal University.

Femoral drill is a routine step performed during hip replacement operation, through which a space is created for inserting the prosthesis, associated with removal of cancellous bony segment. Samples were donated from three patients and the cells were separated from the bony structure by repeated vigorous shaking with media (El-Serafi et al., 2011). The cells were cultured in basal conditions, which are minimum essential medium Eagle's alpha modifications (Sigma–Aldrich Ltd) with 10% fetal calf serum (Invitrogen) and 100 U/ml penicillin and 100 U/ml streptomycin (Sigma–Aldrich Ltd).

Cord blood was diluted with phosphate buffered saline and mononuclear cells were separated using the concentration gradient centrifugation and cultured in basal conditions. All placentas were kept in a mixture of phosphate buffered saline

and antibiotics (penicillin and streptomycin) at 4 °C until processing within 24 h according to the method described by Robin and Dzierzak (2010) and Rus et al. (2011). The placentas were extensively washed to eliminate all blood clumps and the amniotic and deciduas membranes were completely removed. Then each placenta was cut into small pieces and incubated with 1 mg/ml of collagenase IV (SERVA Electrophoresis) at 37 °C for 1–1.5 h with agitation. Cells were separated and cultured in basal conditions.

Stem cells have the property of being adherent to the plastic surface. Non adherent cells were removed after 24 h. When the cell density reached 50% of confluency, the media was changed for the osteogenic induction media. This media consisted of the basal conditions in addition to 100 µM ascorbate-2-phosphate (Sigma–Aldrich Ltd), and 10 mM dexamethasone (Sigma–Aldrich Ltd) (El-Serafi et al., 2011).

3. Molecular biology analysis

RNA was extracted by RNeasy Mini Kit (Qiagen) after the homogenization step using the QIA shredder spin column (Qiagen). RNA was reverse transcribed to cDNA by using QuantiTect Reverse Transcription Kit (Qiagen). Relative expression of genes Runx2, osterix, Alp and Collagen type 1 was determined using the real-time polymerase chain reaction (PCR) technique. The expression level of each gene was determined by a sybergreen based master-mix (Qiagen) and corrected to the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences for different primers are enlisted in Table 1. Data was analyzed using the Applied Biosystem Software. The quantification was performed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The number (n) was 3 per group. Statistical analysis was done using Statistical Package for Social Science SPSS for WINDOWS software (version 14.0; SPSS Inc, Chicago, IL). Statistical significance was determined using one-way ANOVA to compare means between groups, with a p value of less than 0.05 being considered significant.

4. Results

The number of cells isolated from each of the three sources was comparable. After 24 h, the isolated cells were attached to the tissue culture plastic and acquired the classical

Table 2 – Comparison between the numbers of the cells obtained from the three sources and the duration needed till the appearance of spindle-shaped cells.

	No. of isolated cells Mean ± SD in million cells	Appearance of spindle-shaped cells in days
Bone marrow	193 ± 3.37	4.7 ± 1.6*
UCB	142 ± 91.4	6.7 ± 1.6
Placenta	181 ± 9.14	7.1 ± 1.6

morphology. The adherent cells acquired the spindle-shape significantly quicker in BM derived cells, in comparison to the other sources (Table 2).

The expression of Runx-2 was studied in control (before induction) and 24 h after induction. The bone marrow control was set as reference for comparison. Before induction, as shown in Fig. 1, placental derived cells showed the highest expression of Runx-2 in comparison to the other sources. BM-MSCs control expressed significantly higher levels in comparison to UCB cells. Upon induction, the expression of Runx-2 was significantly higher in BM-MSCs in comparison to UCB and placental cells after 24 h. The extension of the culture into 72 h for UCB and placenta derived cells failed to significantly enhance the expression of Runx-2 in comparison to the levels at 24 h.

The expression pattern of the osterix gene was very similar to that of Runx-2. Osterix was significantly enhanced in bone marrow derived cells after 24 h. The expression in BM derived cells was significantly higher than the other cell sources. Extension of the UCB culture for 72 h significantly enhanced the expression to match that of the BM at 24 h. Although osterix expression was enhanced after 24 h of induction of placental cells, the induction was not significantly enhanced with the extension of the culture for 72 h. For both time points, the expression level was significantly lower than bone marrow (Fig. 2).

The ALP gene was induced only in BM-MSCs. The expression of ALP was not detected in UCB or placental derived cells (Fig. 3). Collagen I expression was not detected in all the study groups.

5. Discussion

This study aimed at comparing two possible sources of stem cells as an alternative for bone marrow for osteogenic tissue

Table 1 – Primer sequences for real time PCR.

Primer	Sequence	Reference
GAPDH	Forward 5'CCAGGTGGTCTCCTCTGACTTC 3' Reverse 5'TCATACCAGGAAATGAGCTTGACA 3'	(Hashimoto et al., 2007)
Runx2	Forward: 5' TCTTCACAAATCCTCCGC 3' Reverse: 5'TGGATTAAAAGGACTTGGTG 3'	(Abdallah et al., 2006)
Osterix	Forward: 5'TAGTGGTTTGGGGTTTGTACCGC 3' Reverse: 5'AACCAACACTCTATTCCCTAAGT 3'	(Tsai et al., 2011)
ALP	Forward: 5'AAGGCTTCTTCTGCTGGTG 3' Reverse: 5'GCCTTACCCTCATGATGTCC 3'	(Fujita and Janz, 2007)
Col1	Forward: 5'GCCAAGACGAAGACATCCCA 3' Reverse: 5'CCACACGTCTCGGTATGG 3'	(Yang et al., 2004)

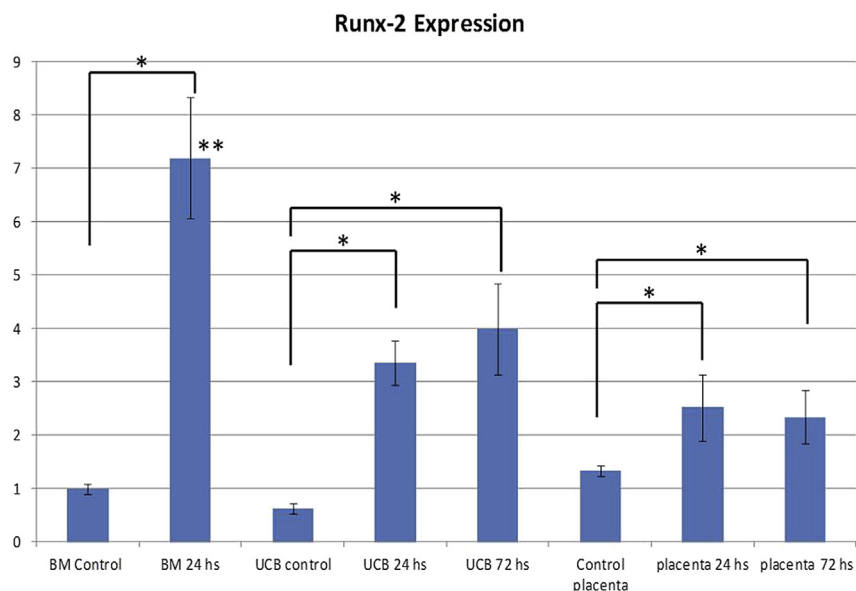


Fig. 1 – Comparison of the expression of Runx-2 between different groups of the study. The BM-MSCs control group was set as reference. Maximum induction was obtained with BM-MSC after 24 h. Extension of the culture to 72 h didn't show comparable results. UCB and placenta showed significant enhancement of Runx-2 expression in comparison to their basal level of expression (n = 3), *p-value is less than 0.05, **p-value is less than 0.05 in comparison to all other groups. ANOVA test is less than 0.05.

engineering. In this study, the isolation efficacy of MSCs from cord blood was comparable to that reported by previous studies (Kern et al., 2006; Kawasaki-Oyama et al., 2008) and the isolation efficacy for placenta was comparable to that reported by Yen et al. (2005); which could indicate relative steadiness of the separation of the cells from the three reported sources. Such reproducibility may encourage other

researchers to use similar protocols for cell separation and consequently expansion of this research field.

Our gene expression profile showed that BM derived cells were superior in the expression of the osteogenic markers in comparison to the other sources. Such finding was in agreement with Shafee et al. (2011), and could be explained by the theory of the stem cell niche. The presence of the BMCs in the

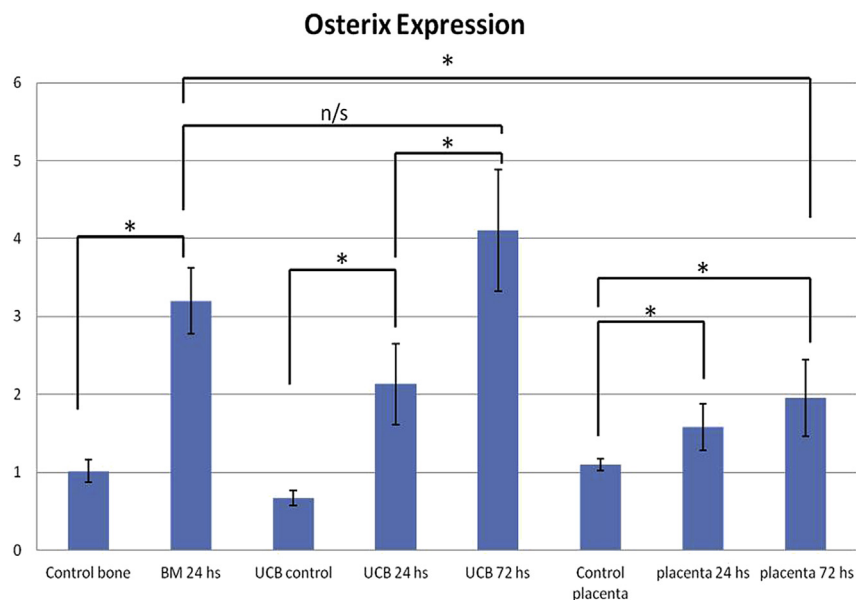


Fig. 2 – Comparison of the expression of osterix between different groups of the study. The BM-MSC control group was set as reference. Maximum induction was obtained with BM-MSC after 24 h as well as UCB-MSC after 72 h. Extension of the culture to 72 h did not show significant enhancement (n = 3), *p-value is less than 0.05, n/s = non-significant, ANOVA test is less than 0.05.

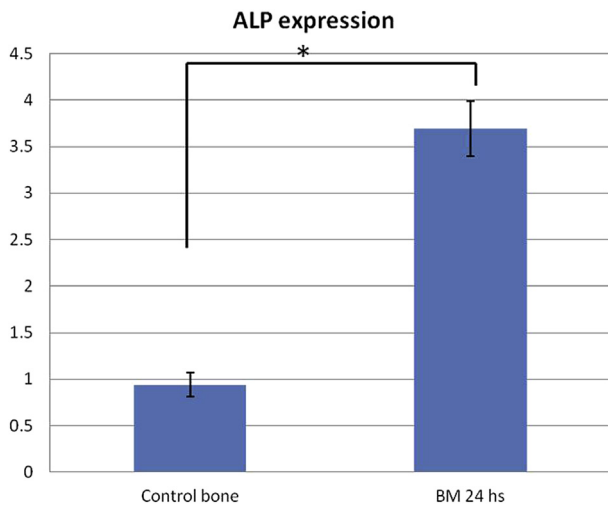


Fig. 3 – The expression of ALP could only be shown with BM-MSCs. There was 3.7 folds increase of the ALP expression after 24 h. Other cell groups failed to show expression of ALP (n = 3), *p-value is less than 0.05.

bony structure of the bone marrow cavity would influence the osteogenic environment around these cells. The physical effects, paracrine secretions, extracellular mediators and the metabolic products play a role in priming the stem cells towards a certain destination, which is the osteogenic lineage in case of BM (Lander et al., 2012). At the meantime, this study reported a trend of gradual increase in Runx-2 and osterix, the transcription factors responsible for the differentiation of stem cells into osteocytes, in the other studied cell types. The expression level of the osteogenic master gene, Runx-2, was crucial for this purpose. The role of Runx-2 has been proved in vitro as well as in some in vivo models (Bhat et al., 2008). Peng et al. (2008) reported that Runx-2 expression reached the peak at 3–7 days in induced BM-MSCs, well ahead of UCB-MSCs. Wang et al. (2010) seeded the UCB-MSCs on polyglycolic acid scaffold and showed gradual increase of Runx-2 over three weeks. The expression level was significantly higher than the controls on every tested time point including after one week, which is a consistent finding with our results.

Osterix is another crucial transcription factor that enhances bone formation during the normal developmental process as well as during stem cell differentiation. Our results showed gradual enhancement of osterix expression in placental and umbilical cord blood derived cells. The gene expression profile in UCB-MSCs encapsulated in different types of carriers showed similar trend over a time-course of seven days (Zhao et al., 2010). The trends for Runx-2 and osterix are very similar, which could denote an early stage of response to induction. In addition, the authors reported the expression of ALP reached the maximum level after seven days.

Although our results showed that UCB had better response to the osteogenic culture conditions than the placenta derived cells, we should emphasize that the time frame for this study was concise, because our aim was to investigate the initial response. The response of the three types of cells on long term induction time-frame will be interesting to illustrate. Thus,

this study highlights the possibility of using UCB and placenta derived cells for osteogenesis protocols and suggested that the response of UCB would be superior to placental derived cells. The addition of other factors that can enhance the process of osteogenesis, such as 5-Aza-dC (El-Serafi et al., 2011) would be another interesting field for studying the response in the different cell sources.

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