Journal of Current Biomedical Reports
Volume 3, Number 3, 2022
jcbior.com
eISSN: 2717-1906

Original research

Differentiation of mesenchymal stem cells isolated from the amniotic membrane and umbilical cord to osteocytes and the expression of RunX2, Osteonectin, ALP genes

Amin Tabasi[1], Nooshin Barikrow[1,*]

[1]*Department of Molecular and Cellular Sciences, Faculty of Advanced Sciences & Technology, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran*

Abstract

Mesenchymal stem cells (MSCs) are multipotent cells and able to differentiate into connective tissues such as bone, fat, cartilage, tendon, and muscle. They show to be very potent tools for tissue engineering and regenerative medicine. Several researches have shown that amniotic membrane mesenchymal stem cells (AM-MSCs) and umbilical cord mesenchymal stem cells (UCB-MSCs) are both multipotent in nature differentiating into several cell types such as adipocytes and osteoblasts. In this study, mesenchymal stem cells were derived from the human amniotic membrane (hAM-dMSCs) and umbilical cord then characterized with their surface antigens using flow cytometry. These cells differentiated to osteocyte and adipocyte in induction medium then the expression of RunX2, Osteonectin, and ALP genes were calculated by Real-Time PCR. We showed that AM-MSCs and UCB-MSCs can discriminate to osteogenic and adipogenic cells in the specific induction medium. The capability of AM-MSCs and UCB-MSCs differentiation to osteogenic cells was confirmed by enhanced expression of RUNX2, ALP and Osteonectin gene and deposition calcium shown by alzerin staning. Given the available evidence, we conclude that AM-MSCs and UCB-MSCs have suitable access, low immunization and lack of medical ethics problems are one of the appropriate sources for differentiation in to osteogenic and adipogenic cells. Also, they can be considered as good choices for treatment of mesenchymal tissue injuries and tissue engineering.

Keywords: Amniotic membrane, Umblical cord, Osteocyst, Osteonectin, RunX2

1. Introduction

Stem cells are predictable the mainly positive agent for the treatment of degenerative and ischemic diseases as of their self-renewal and multiline age differentiation capacity [1]. Mesenchymal stem cells (MSCs) were first discovered in 1968 by Friedenstein as a supporter fibroblast-like population in the bone marrow competent of differentiating into bone [2]. It was then revealed that MSCs can be isolated from different tissues for instance adipose tissue, peripheral blood, placenta, amniotic membrane (AM), and umbilical cord (UC) [3, 4].

The human amniotic membrane (hAM) is the innermost fetal layer considered as an imperative source of MSCs for the treatment of several disorders [5, 6]. Furthermore, hAM is a highly plentiful tissue and willingly available having a single layer of epithelial cells, underlying fibroblasts, and an avascular collagenous stroma [7]. Umbilical cord blood (UCB) is considered one of the youngest

[*]**Corresponding author:**
Nooshin Barikrow, PhD
Department of Molecular and Cellular Sciences,
Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran
Tel/Fax: +98 21 88991117
Email: nooshinbarikrow@gmail.com
http://orcid.org/0000-0001-9367-3885

Received: February, 08, 2021
Accepted: November, 22, 2022

accessible sources of adult stem cells. As well hematopoietic stem cells, UCB has been shown to have endothelial progenitor cells with MSCs [8, 9]. Human umbilical cord derived MSCs isolated from the cord's structures; arteries, vein, cord lining and Wharton's jelly. Various studies have shown that amniotic membrane mesenchymal stem cells (AM-MSCs) and UCB-MSCs are both multipotent in nature differentiating into various cell types such as adipocytes, osteoblasts, hepatocyte, chondrocytes, haemopoetic cells, cardiac and neural cells [10-13]. Umbilical cord stem cells have also been proved promising in probable executive of several diseases and situation [14-16].

Through the past decades, the efficiency of MSCs such as hAM-dMSCs and UCB-MSCs has been widely investigated both in basic and clinical experiments [17, 18]. Due to their anti-inflammatory properties, low immunogenicity and less ethical issues related with their use compared with further sources of stem cells, hAM-dMSCs and UCB-MSCs are alternative resource in the field of tissue engineering [19, 20]. These varieties of MSCs have been studied in clinical trials for currently deadly diseases, for instance bone and cartilage defects, stroke, myocardial infarction, graft-versus-host disease (GvHD) and autoimmune diseases [21]. These cells have a homing aptitude, meaning that they can transfer into injured sites, and they acquire the ability to differentiate into confined components of injured sites and the ability to secrete chemokines, growth factors and cytokines, that facilitate in tissue regeneration [22]. These cells are identified to create trophic factor for tissue repair/regeneration [23].

It has been clarified that many various genes are involved in differentiation of osteoblast cells such as alkaline phosphatase (*ALP*), *Runx2,* and*osteonectin*. ALP is an enzyme which its expression and activity have been evaluated in some mammals like humans to conclude cell fate in developmental studies or even within clinical trials. This enzyme is considered as key markers in the recognizing of embryonic stem cell. Runt-related transcription factor 2 (Runx2) is a member of the Runx family, which is crucial for differentiation of osteoblast and chondrocyte maturation [24]. Expression of RUNX2 in mesenchymal cells has an induction effect on osteoblast differentiation which leads to bone formation [25, 26]. In addition, Osteonectin, also known as secreted protein acidic and rich in cysteine (SPARC) is a calcium-binding matricellular protein considered as an important non-collagenous protein expressed in mineralized and non-mineralized tissue [27, 28].

Thus far, because of MSCs relieve of isolation, culture expansion, multipotential differentiation and immunomodulatory properties, they have the potential for concern in regenerative medicine and have therapeutic purpose [29]. Furthermore as mentioned above, MSCs used in the treatment of bone lesion. Thus, in this study, two available and non-invasive extra-embryonic sources (AM-MSCs and UCB-MSCs) were selected. Also the differentiation potential of these cells to differentiate into osteocytes was compared.In addition, they can be considered as good choices for treatment of mesenchymal tissue injuries and tissue engineering**.** Initially we isolated MSCs from AM and UCB sources. In the next step, differentiation of AM-MSCs and UCB-MSCs into adipogenic and osteogenic cells were carried out in induction mediums. Then, the expression of *ALP, Runx2,* and *osteonectin* genes were evaluated in osteogenic cells differentiated from AM-MSCs and UCB-MSCs.

2. Materials and Methods

2.1 Isolation, MSCs from amniotic membrane

Amniotic membrane samples were collected from mothers who gave informed consent at Milad hospital (Iran). They were then transferred to the laboratory to isolate the MSCs from amniotic membrane. A two-step method was used to isolate MSCs: First the amniotic tissue was treated with trypsin to remove the epithelial cells, which was followed by treatment with Dnase and collagenase IV (Gibco, UK) for 5 minutes at 37˚C. The MSCs extracted from amniotic membrane are adherence cells [30, 31]. AM-MSCs were cultured with DMEM (low glucose) medium and 10% FBS, 1% penicillin streptomycin solution at 37˚C in 5% CO_2.

2.2 Isolation, MSCs from UCB

In this study, MSCs were isolated from UCB after receiving an approval form. Cord blood mononuclear cells (MNCs) were extracted according to the instructions [32]. Briefly, MNCs division was obtained by Ficoll (Sigma-Aldrich, USA) followed by concentration gradient centrifugation and in a 225 cm flask containing 6 ml of DMEM culture medium with

10% FBS (Invitrogen, USA), 1% penicillin and 1% Streptomycin (CinnaGen, Iran) and then cells were cultured at 37 ° C. The, 48 hours after culture, non-adherent cells were removed by altering the culture medium. Replacement of cell culture medium with an interval of 3 days removed non-adherent cells. Finally, mesenchymal stromal cells from the third passage were provided for final experiments.

2.3 Differentiation to osteoblasts and adipocytes

The MSCs were differentiated to osteoblasts and adipocytes. The cells in Passage 5 were cultured in six-well plates. The osteogenic differentiation culture containing DMEM and 10% FBS, ascorbic acid 50 µg/mL, β-glycerolphosphate 5 mM and dexamethasone 10 mM, and the adipogenic differentiation culture containing DMEM and 10% FBS, ascorbic acid 50 µg/mL, Indomethacin 60 mM, and dexamethasone 10 mM were used (all from Sigma-Aldrich, USA). The cells were incubated for 14 days at 37˚ C and 5% CO_2. The differentiation to osteoblasts was observed during staining with Alizarin Red and the differentiation of adipocytes was revealed by staining with Oil Red O (all from Sigma-Aldrich, USA) [33].

For oil-red-O staining, the differentiation medium was first removed and the cells were washed with PBS for stabilization. Cold ethanol 71% was added to the cells. After 1 hour, the ethanol was removed and the cells were washed 2 to 3 times with distilled water. Then 1ml Oil-Red-O 2% solution was added to the cells and incubated for 50 minutes at room temperature. After draining the staining solution, the cells were washed 4 times with distilled water. Finally in order to prevent the cells from drying out, 1 ml of distilled water was added to them and the cells were stained with were evaluated (all from Sigma-Aldrich, USA).

For Alizarin staining, first the differentiation medium was removed and the cells were washed with PBS. Cold ethanol was added to the cells. After 1 hour, remove the ethanol and wash the cells 2 to 3 times with distilled water, then 1 Alizarin Red% 2 solution was added to the cells and for 30- 40 minutes at room temperature Was incubated. After draining the staining solution, the cells were washed 4 times with distilled water. Finally in order to prevent the cells from drying out, 1 ml of distilled water was added to them.

2.4 Flow cytometry

Flow cytometric immune phenotyping was used to determine cell identity and surface markers.

Based on references, after washing with PBS and trypsinizing, we dissolved about 10^5 cells in 1 ml PBS and 100 µl of this suspension was transferred to a tube; 11 µl of monoclonal antibody conjugated to Fluorecein isothiocyanate (FITC) and Phycoerythrin was transferred to the microtube.

After washing with PBS and trypsinized, the cells were stained with monoclonal antibodies against, PerCP-29, FITC-CD44, FITC-CD45 and PE-CD34 for 30 min on ice (BD Bioscience, San Jose, CA, USA), where PerCP-IgG1, PE-IgG1, and FITC-IgG1 were used as controls. Then, they were washed with PBS and analyzed using flow cytometer (FACS Calibur; Becton-Dickinson) for stained cell [34]. Moreover, Flomax and FlowJo (FlowJo, LLC) softwares were applied for analyzing flow cytometrydata.

2.5 RNA isolation and real-time PCR

Total RNA was extracted by YT4500 Total RNA Purification Mini kit (Yekta Tajhiz Azma, Iran) according to manufactured protocol from the samples. Then, cDNA was synthesized using total RNA and cDNA synthesis kit (Stem Cell Technology Co., Iran). To conclude the concentration of DNA, fluorescence color SYBER Green (Sigma-Aldrich, USA) was used. GAPDH was used as the internal control [35]. Finally, real-time PCR (StepOnePlus, Korea) was employed to measure the expression of RUNX2, Osteonestin and ALP genes. Primer sequences are presented in Table 3.

2.6 Statistical analysis

All experiments were performed with at least three repetitions. In this study, standard error is used to describe the data. Using SPSS software, version 23 is used to compare the means of two groups independent of the test and to compare the means of more than two groups of the test. The results are considered statistically significant if P value <0.05. Graphs and tables are drawn using Excel software.

3. Results

3.1 Growth characterization of AM-MSCs and UCB-MSCs

In morphological study by invert microscope it was clarified that MSCs derived from amniotic membrane (Supplementary Figure 1A) and UCB

(Supplementary Figure 1B) were spindle-shaped cells. These kind of cells presented typical properties like having a large rounded nucleus containing nucleolus, and long and short cellular tails. Moreover they were adhesive cells which could maintain a strong mitotic competency to passage 3.

3.2 Surface markers of AM-MSCs and UCB-MSCs

The isolated AM-MSCs and UCB-MSCs were characterized with their surface antigens using flow cytometry. Flow cytometry analysis showed that MSCs derived from amniotic membrane and UCB was positive for mesenchymal associated markers (CD29 and CD44) and negative for the hematopoietic cell antigens (CD34 and CD45). The results displayed that in AM-MSCs CD29, CD44, CD34 and CD45 were expressed 97.4%, 99.6%, 1% and 0.5%, respectively. As well, the expressions of these markers in UCB-MSCs were 95% (CD29), 99% (CD44), 1% (CD34) and 5% (CD45).

3.3 Adipogenic differentiation from AM-MSCs and UCB-MSCs

To evaluate adipogenic differentiation from AM-MSCs and UCB-MSCs, cells of passage 3 were cultured in induction medium and differentiated into adipogenic cells (Supplementary Figure 3). On the other hand, the MSCs cultured in basic culture medium were considered as controls and did not show any differentiation (Supplementary Figure 3A and C). As shown by arrows, red points were lipid vesicles identifying adipogenic differentiation from AM-MSCs (Supplementary Figure 3B) and UCB-MSCs (Supplementary Figure 3D).

3.4 Osteogenic differentiation from AM-MSCs and UCB-MSCs

AM-MSCs and UCB-MSCs of passage 5 were used to culture in osteogenic induction medium, which differentiated into osteogenic cells (Supplementary Figure 4). Nevertheless any differentiation shown in the control MSCs cultured in basic culture medium (Supplementary Figure 4A and C). After 14 days Alizarin Red staining was used to represent the osteogeniccells by inverted microscopy. This kind of staining could illustrate osteogenic differentiated from AM-MSCs (AM-OST) (Supplementary Figure 4B) and UCB-MSCs (UCB-OST) (Supplementary Figure 4D) with calcium deposition.

3.5 Gene expression by RT-PCR analysis in osteogenic differentiated cells

Quality of extracted RNA in osteogenic differentiated cells from AM-MSCs and UCB-MSCs were evaluated by Nano drop. Moreover, agarose gel was run to check the quality of RNA (Supplementary Figure 5).

Expression of *ALP, RUNX2,* and Osteonectin in these osteogenic differentiated cells was analyzed by RT-PCR compared to GAPDH transcript level (as internal control) (Supplementary Figure 6). As presented in Supplementary Figure 6A, expression of *RUNX2* displayed a dramatic enhancement in AM-OST (5-fold) and UCB-OST (6-fold) in comparison to the control. *ALP* gene expression in AM-OST and UCB-OST significantly enhanced 10 and 6.8 times respectively greater than that in MSCs control (Supplementary Figure 6B). In addition, the greatest increase was found in expression level of osteonectinin AM-OST and UCB-OST, which showed 13- and 9-fold increase, respectively, compared to the equivalent control (Supplementary Figure 6C).Since the expression of these genes were equal in AM-MSCs and UCB-MSCs, we considered AM-MSCs as the control.

4. Discussion

MSCs are multipotent cells and capable to discriminate into connective tissues for instance bone, cartilage, tendon, fat and muscle. They show to be very influential role for regenerative medicine and tissue engineering, not just for their multilineage discrimination possible [35]. But frequently for their paracrine activity, exerted during the release of soluble agent [36] for instance anti-inflammatory cytokines, trophic molecules and anti-apoptotic that stimulate tissue renovate and counteract inflammation [37]. These cells are regular and reside in different tissues for self-repair and tissue homeostasis. They can be remote from bone marrow, periosteum, adipose tissue, trabecular bone, synovium, skeletal tissue, blood, spleen, brain, kidney, liver, lung, muscle, bone marrow, thymus, pancreas and deciduous teeth [38]. In this study potential differentiation of AM-MSCs and UCB-MSCs into adipogenic and osteogenic cells were compared. Additionally, the expression of *ALP, Runx2,* and *osteonectin* genes were evaluated in

osteogenic cells differentiated from AM-MSCs and UCB-MSCs.

Before studies showed that AM-MSCs were able to reverse the bone deficiency caused by oxidative stress and inflammation [39]. Furthermore, UCB-MSCs transplants were used in patient with bone marrow failure syndromes, hematologic malignancy, or inherited metabolic disorders, which led to treat them [40].

Several researches have represented that particular surface antigens, such as CD44, CD29, CD90, and CD105, are identified MSCs that originate from UCB, AM, bone marrow and adipose tissue [41, 42]. Consist with Wang et al. and Chamberlain et al., our results showed that MSCs were positive for mesenchymal associated markers CD29, CD51, CD44, CD73, and CD105, showed lacked expression of CD34 and CD45 as hematopoietic markers [7, 32].

These MSCs could differentiate into osteoblasts one of the most important cells correlated to bone repair. Also adipocytes diffrentiaed from MSCs could represent a new therapeutic target for multiple diseases from osteoporosis to leukemia [43]. Former researches have revealed that aging or harmful MSCs have damaged osteogenic differentiation ability and improved adipogenic differentiation capacity, which is a probable cause of osteoporosis in the elderly [44, 45]. The differentiation of MSCs in vitro mostly depends on the culture situation. Growth factors, for example the TGF-β family, result in chondrogenic demarcation [46], whereas MSCs cultured with isobutyl methyl xanthine, dexamethasone, and indomethacin will discriminate down an adipogenic lineage [47]. Osteogenic differentiation of MSCs *in vitro* is induced by the occurrence of dexamethasone, β-glycerol phosphate and ascorbic acid [48]. Alternatively, ascorbic acid 50 μg/mL, Indomethacin 60 mM, and dexamethasone are needed in culture medium for induction of adipogenic demarcation from MSCs [49].

Signaling cascades which expand MSCs into adipogenic and osteogenic lineage differentiation rely on two type transcription factors: PPAR$\gamma$and RUNX2, respectively. Also, PPARγ as the master regulator of adipogenesis has anti-osteoblastogenic effects. These transcription factors are devoted for moderating the effects of diverse cytokines in purpose of adipogenic versus osteogenic MSCs differentiation [50, 51]. On the other hand, in this study we used particular induction medium for adipogenic and osteogenic differentiation from AM-MSCs and UCB-MSCs, which lead to emerge individual adipogenic and osteogenic lineages.

In this study, beside cells differentiation, the expression of some genes, RUNX2, ALP, and Osteonectin, involve in osteogenic differentiation from MSCs were examined. And all genes showed enhanced expression in osteogenic differentiated cells.RUNX2 is a member of the RUNX family, which has the DNA-binding domain runt. This factor is necessary for differentiation of osteoblast and chondrocyte maturation. Expression of RUNX2 in mesenchymal cells has an induction effect on osteoblast differentiation which leads to bone formation. Thus, RUNX2 formed a condensed cell layer of osteoblast progenitors by enhancing their generation and inducing their differentiation into osteoblast lineage cells [52-54]. RUNX2-dependent osteoblast differentiation is associated with bone morphogenetic proteins (BMP) signaling. Actually, BMPs participate a role in induction of numerous target genes expression particularly RUNX2 by activating SMAD proteins and also further signal transduction pathways. In addition, RUNX2 increases the cells responsiveness to BMPs [55, 56]. Through differentiation of osteoblast, RUNX2 shows low expression in mesenchymal cells and the upregulated expression in preosteoblasts. Furthermore, its transcript level attains the highest level in immature osteoblasts, and finally is reduced in mature osteoblasts; which were in agreement with Qin et al [57].

ALP is an enzyme which its expression and activity have been evaluated to conclude cell fate in developmental studies or even within clinical trials. Hydrolysis of phosphate esters is mentioned as a general activity of ALP [58]. This enzyme is considered as key markers in the recognizing of embryonic stem cell. It has been known some isoenzymes of ALP are specific for definite tissue in humans, which are expressed by embryonic cells but also carcinoma cells. Another isoenzyme is ubiquitous, which is not tissue-specific and appears in bone, kidney, and liver [59, 60]. Some of ALPs are responsible in the mineralization of hard tissue by providing free phosphate for the creation of hydroxyapatite crystals and hydrolysing pyrophosphate (an inhibitor of bone matrix formation). Also, they interfere in the metabolism of vitamin B6, and also, γ-aminobutyric acid (GABA, as a

neurotransmitter). These ALP activities are dominant in the developing skeleton; therefore their suppression causes various defects in the mineralization of hard tissue and the expansion of the nervous system [61, 62].

The main modulating pathways regulating osteoblastic differentiation and *ALP* expression are the BMP/RUNX2 (CBAf1, AML3)/Osterix system, and the Wingless type (Wnt) signaling cascade, which correlate to each other [63-65]. Some studies of the BMP/RUNX2 system have indicated that a number of patterning gene products were involved in both inductions of osteogenesis in MSCs and controlling expression of some isoenzymes of ALP via chromosomal remodeling [66]. An additional regulation of ALP expression is applied through the actions of 1, 25-(OH) 2 -vitamin D, and Parathyroid hormone and retinoic acid, which interact with the main modulatory systems [67, 68]. In various studies, osteoblastic differentiation and mineralization were elucidated by determining ALP expression by RT-PCR, and mineralized matrix pattern by Alizarin red S staining which is consist with our study [69]. The results presented here are consistent with previous reports [67, 70].

Osteonectin was a different protein which its gene expression was evaluated in this study. This protein identified as SPARC or basement membrane protein 40 is a 32 kDa calcium-binding matricellular protein is secreted by osteoblasts in bone during bone development [71, 72]. Usually, expression of this protein is related to fibrillar collagens such as collagen type I. It has been proposed that osteonectin binds collagen and hydroxyapatite crystals in the osteoid (pre-mineralized bone matrix), and releases calcium ions rising mineralization of the collagen matrix in bones. Osteonectin can be originate in osseous tissue for instance in active osteoblasts and bone marrow progenitor cells, hypertrophic chondrocytes, odontoblasts (dentin-forming cells), and osteoid, which show high expression of this protein [73].

Various studies on animals have revealed that defection in osteonectin could cause various phenotypic abnormalities and decrease in bone mineral density [74, 75]. These consequences could lead to osteopenia is considered as a forerunner to osteoporosis [76]. Furthermore, these evidences have clarified that osteonectin has an effect on osteoblast and osteoclast activity; and induction of osteoblast differentiation in precursors with osteonectin defects would be impaired [77, 78]. Results showed that osteonectin protein level increased at early stages of differentiation and then declined as cells mature and began to express mature osteoblastic marker. Our results were in agreement with these reports.

In conclusion, our results presented here indicate that MSCs isolated from AM and UCB were constructive for mesenchymal associated markers. This finding supported that MSCs could be obtained from these two various resources, AM and UCB, which are more potent than other sources of MSCs which their numbers decrease by increasing the age. In this study, the capacity of AM-MSCs and UCB-MSCs differentiation to Osteogenic cells was confirmed by enhanced expression of *RUNX2*, *ALP* and *Osteonectin* gene and deposition calcium shown by alzerin staning. Given the presented evidence, we conclude that AM-MSCs and UCB-MSCs which have convenient access, low immunization and lack of medical ethics problems are one of the appropriate sources for differentiation in to Osteogenic and adipogenic cells. Moreover, they can be considered as good choices for treatment of mesenchymal tissue injuries and tissue engineering. It seems that further studies in this field are needed.

Supplementary files

Supplementary file 1.

Authors' contributions

The authors were equally involved in the article. All authors read and approved the final version of manuscript.

Conflict of interests

The authors declare that they have no conflicts of interest.

Ethical declarations

All ethical principles were performed in accordance with ethical standards in the research. Also, the study design was approved by regional ethical committee (IR.IAU.PS.REC.1398.243).

Financial support

Self-funding.

References

1. Hochedlinger K, Jaenisch R. Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. N Engl J Med. 2003; 349(3):275-86.
2. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968; 6(2):230-47.
3. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, et al. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells. 2004; 22(7):1338-45.
4. Hendrijantini N, Hartono P. Phenotype Characteristics and Osteogenic Differentiation Potential of Human Mesenchymal Stem Cells Derived from Amnion Membrane (HAMSCs) and Umbilical Cord (HUC-MSCs). Acta Inform Med. 2019; 27(2):72-7.
5. Li J, Zhou Z, Wen J, Jiang F, Xia Y. Human Amniotic Mesenchymal Stem Cells Promote Endogenous Bone Regeneration. Front Endocrinol (Lausanne). 2020; 11:543623.
6. Huang Z, Nelson ER, Smith RL, Goodman SB. The sequential expression profiles of growth factors from osteoprogenitors [correction of osteroprogenitors] to osteoblasts in vitro. Tissue Eng. 2007; 13(9):2311-20.
7. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. 2004; 22(7):1330-7.
8. Bieback K, Netsch P. Isolation, Culture, and Characterization of Human Umbilical Cord Blood-Derived Mesenchymal Stromal Cells. Methods Mol Biol. 2016; 1416:245-58.
9. Ma J, Wu J, Han L, Jiang X, Yan L, Hao J, et al. Comparative analysis of mesenchymal stem cells derived from amniotic membrane, umbilical cord, and chorionic plate under serum-free condition. Stem Cell Res Ther. 2019; 10(1):19.
10. Mennan C, Wright K, Bhattacharjee A, Balain B, Richardson J, Roberts S. Isolation and characterisation of mesenchymal stem cells from different regions of the human umbilical cord. Biomed Res Int. 2013; 2013:916136.
11. Wang L, Ott L, Seshareddy K, Weiss ML, Detamore MS. Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells. Regen Med. 2011; 6(1):95-109.
12. Kakinuma S, Tanaka Y, Chinzei R, Watanabe M, Shimizu-Saito K, Hara Y, et al. Human umbilical cord blood as a source of transplantable hepatic progenitor cells. Stem Cells. 2003; 21(2):217-27.
13. Chao YH, Wu HP, Chan CK, Tsai C, Peng CT, Wu KH. Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation. J Biomed Biotechnol. 2012; 2012:759503.
14. Lee KO, Gan SU, Calne RY. Stem cell therapy for diabetes. Indian J Endocrinol Metab. 2012; 16(Suppl 2):S227-9.
15. Shrestha C, Zhao L, Chen K, He H, Mo Z. Enhanced healing of diabetic wounds by subcutaneous administration of human umbilical cord derived stem cells and their conditioned media. Int J Endocrinol. 2013; 2013:592454.
16. Chicha L, Smith T, Guzman R. Stem cells for brain repair in neonatal hypoxia-ischemia. Childs Nerv Syst. 2014; 30(1):37-46.
17. Jin YZ, Lee JH. Mesenchymal Stem Cell Therapy for Bone Regeneration. Clin Orthop Surg. 2018; 10(3):271-8.
18. Li M, Gao J, Feng R, Wang Y, Chen X, Sun J, et al. Generation of monoclonal antibody MS17-57 targeting secreted alkaline phosphatase ectopically expressed on the surface of gastrointestinal cancer cells. PLoS One. 2013; 8(10):e77398.
19. Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, et al. Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem Cells. 2008; 26(11):2865-74.
20. Wang Y, Jiang F, Liang Y, Shen M, Chen N. Human Amnion-Derived Mesenchymal Stem Cells Promote Osteogenic Differentiation in Human Bone Marrow Mesenchymal Stem Cells by Influencing the ERK1/2 Signaling Pathway. Stem Cells Int. 2016; 2016:4851081.
21. Sharma RR, Pollock K, Hubel A, McKenna D. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. Transfusion. 2014; 54(5):1418-37.
22. Aubin JE. Regulation of osteoblast formation and function. Rev Endocr Metab Disord. 2001; 2(1):81-94.
23. Hoemann CD, El-Gabalawy H, McKee MD. In vitro osteogenesis assays: influence of the primary cell source on alkaline phosphatase activity and mineralization. Pathol Biol (Paris). 2009; 57(4):318-23.
24. Komori T. Regulation of Proliferation, Differentiation and Functions of Osteoblasts by Runx2. Int J Mol Sci. 2019; 20(7):1694.
25. Huang B, Qian J, Ma J, Huang Z, Shen Y, Chen X, et al. Myocardial transfection of hypoxia-inducible factor-1α and co-transplantation of mesenchymal stem cells enhance cardiac repair in rats with experimental myocardial infarction. Stem Cell Res Ther. 2014; 5(1):22.
26. Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res. 1992; 7(6):683-92.
27. Bradshaw AD. Diverse biological functions of the SPARC family of proteins. Int J Biochem Cell Biol. 2012; 44(3):480-8.
28. Murphy-Ullrich JE, Sage EH. Revisiting the matricellular concept. Matrix Biol. 2014; 37:1-14.
29. Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. J Inflamm (Lond). 2005; 2:8.
30. Mihu CM, Rus Ciucă D, Soritău O, Suşman S, Mihu D. Isolation and characterization of mesenchymal stem cells from the amniotic membrane. Rom J Morphol Embryol. 2009; 50(1):73-7.
31. Ji Y, Zhang P, Xing Y, Jia L, Zhang Y, Jia T, et al. Effect of 1α, 25-dihydroxyvitamin D3 on the osteogenic differentiation of human periodontal ligament stem cells and the underlying regulatory mechanism. Int J Mol Med. 2019; 43(1):167-76.
32. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007; 25(11):2739-49.
33. Zhang L, Su P, Xu C, Chen C, Liang A, Du K, et al. Melatonin inhibits adipogenesis and enhances osteogenesis of human mesenchymal stem cells by suppressing PPARγ expression and enhancing Runx2 expression. J Pineal Res. 2010; 49(4):364-72.
34. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods. 2000; 46(1-2):69-81.

35. von Bahr L, Batsis I, Moll G, Hägg M, Szakos A, Sundberg B, et al. Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. Stem Cells. 2012; 30(7):1575-8.
36. Kalinina N, Kharlampieva D, Loguinova M, Butenko I, Pobeguts O, Efimenko A, et al. Characterization of secretomes provides evidence for adipose-derived mesenchymal stromal cells subtypes. Stem Cell Res Ther. 2015; 6:221.
37. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008; 8(9):726-36.
38. Rey A, Manen D, Rizzoli R, Ferrari SL, Caverzasio J. Evidences for a role of p38 MAP kinase in the stimulation of alkaline phosphatase and matrix mineralization induced by parathyroid hormone in osteoblastic cells. Bone. 2007; 41(1):59-67.
39. Wang Y, Wu H, Shen M, Ding S, Miao J, Chen N. Role of human amnion-derived mesenchymal stem cells in promoting osteogenic differentiation by influencing p38 MAPK signaling in lipopolysaccharide -induced human bone marrow mesenchymal stem cells. Exp Cell Res. 2017; 350(1):41-9.
40. Rafii H, Ruggeri A, Volt F, Brunstein CG, Carreras J, Eapen M, et al. Changing Trends of Unrelated Umbilical Cord Blood Transplantation for Hematologic Diseases in Patients Older than Fifty Years: A Eurocord-Center for International Blood and Marrow Transplant Research Survey. Biol Blood Marrow Transplant. 2016; 22(9):1717-20.
41. Frank O, Heim M, Jakob M, Barbero A, Schäfer D, Bendik I, et al. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem. 2002; 85(4):737-46.
42. Planat-Benard V, Silvestre JS, Cousin B, André M, Nibbelink M, Tamarat R, et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation. 2004; 109(5):656-63.
43. Valenti MT, Garbin U, Pasini A, Zanatta M, Stranieri C, Manfro S, et al. Role of ox-PAPCs in the differentiation of mesenchymal stem cells (MSCs) and Runx2 and PPARγ2 expression in MSCs-like of osteoporotic patients. PLoS One. 2011; 6(6):e20363.
44. Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. J Cell Biochem. 2001; 82(4):583-90.
45. Rodríguez JP, Astudillo P, Ríos S, Pino AM. Involvement of adipogenic potential of human bone marrow mesenchymal stem cells (MSCs) in osteoporosis. Curr Stem Cell Res Ther. 2008; 3(3):208-18.
46. Machado do Reis L, Kessler CB, Adams DJ, Lorenzo J, Jorgetti V, Delany AM. Accentuated osteoclastic response to parathyroid hormone undermines bone mass acquisition in osteonectin-null mice. Bone. 2008; 43(2):264-73.
47. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284(5411):143-7.
48. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem. 1997; 64(2):295-312.
49. Zhou YS, Liu YS, Tan JG. Is 1, 25-dihydroxyvitamin D3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells in vitro? Chin Med J (Engl). 2006; 119(15):1278-86.
50. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. 2000; 113 (Pt 7):1161-6.
51. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002; 418(6893):41-9.
52. Maeno T, Moriishi T, Yoshida CA, Komori H, Kanatani N, Izumi S, et al. Early onset of Runx2 expression caused craniosynostosis, ectopic bone formation, and limb defects. Bone. 2011; 49(4):673-82.
53. Komori T. Roles of Runx2 in Skeletal Development. Adv Exp Med Biol. 2017; 962:83-93.
54. Kawane T, Qin X, Jiang Q, Miyazaki T, Komori H, Yoshida CA, et al. Runx2 is required for the proliferation of osteoblast progenitors and induces proliferation by regulating Fgfr2 and Fgfr3. Sci Rep. 2018; 8(1):13551.
55. Pawłowska E, Wójcik KA, Synowiec E, Szczepańska J, Błasiak J. Expression of RUNX2 and its signaling partners TCF7, FGFR1/2 in cleidocranial dysplasia. Acta Biochim Pol. 2015; 62(1):123-6.
56. Komori T. Molecular Mechanism of Runx2-Dependent Bone Development. Mol Cells. 2020; 43(2):168-75.
57. Qin X, Jiang Q, Miyazaki T, Komori T. Runx2 regulates cranial suture closure by inducing hedgehog, Fgf, Wnt and Pthlh signaling pathway gene expressions in suture mesenchymal cells. Hum Mol Genet. 2019; 28(6):896-911.
58. Štefková K, Procházková J, Pacherník J. Alkaline phosphatase in stem cells. Stem Cells Int. 2015; 2015:628368.
59. Kermer V, Ritter M, Albuquerque B, Leib C, Stanke M, Zimmermann H. Knockdown of tissue nonspecific alkaline phosphatase impairs neural stem cell proliferation and differentiation. Neurosci Lett. 2010; 485(3):208-11.
60. Nguyen L, Malgrange B, Breuskin I, Bettendorff L, Moonen G, Belachew S, et al. Autocrine/paracrine activation of the GABA(A) receptor inhibits the proliferation of neurogenic polysialylated neural cell adhesion molecule-positive (PSA-NCAM+) precursor cells from postnatal striatum. J Neurosci. 2003; 23(8):3278-94.
61. Andäng M, Hjerling-Leffler J, Moliner A, Lundgren TK, Castelo-Branco G, Nanou E, et al. Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation. Nature. 2008; 451(7177):460-4.
62. Yang K, Tang XD, Guo W, Xu XL, Ren TT, Ren CM, et al. BMPR2-pSMAD1/5 signaling pathway regulates RUNX2 expression and impacts the progression of dedifferentiated chondrosarcoma. Am J Cancer Res. 2016; 6(6):1302-16.
63. Franceschi RT, Xiao G. Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. J Cell Biochem. 2003; 88(3):446-54.
64. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, et al. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem. 2005; 280(39):33132-40.
65. Datta P, Ghosh P, Ghosh K, Maity P, Samanta SK, Ghosh SK, et al. In vitro ALP and osteocalcin gene expression analysis and in vivo biocompatibility of N-methylene phosphonic chitosan nanofibers for bone regeneration. J Biomed Nanotechnol. 2013; 9(5):870-9.
66. Hassan MQ, Tare R, Lee SH, Mandeville M, Weiner B, Montecino M, et al. HOXA10 controls osteoblastogenesis by

directly activating bone regulatory and phenotypic genes. Mol Cell Biol. 2007; 27(9):3337-52.
67. Golub EE, Boesze-Battaglia K. The role of alkaline phosphatase in mineralization. Curr Opin Orthop. 2007; 18(5):444-8.
68. Hassan MQ, Tare RS, Lee SH, Mandeville M, Morasso MI, Javed A, et al. BMP2 commitment to the osteogenic lineage involves activation of Runx2 by DLX3 and a homeodomain transcriptional network. J Biol Chem. 2006; 281(52):40515-26.
69. Li K, Han J, Wang Z. Histone modifications centric-regulation in osteogenic differentiation. Cell Death Discov. 2021; 7(1):91.
70. Khorshied MM, Gouda HM, Shaheen IA, Al Bolkeny TN. The osteogenic differentiation potentials of umbilical cord blood hematopoietic stem cells. Comp Clin Pathol. 2012; 21(4):441-7.
71. Krukiewicz K, Putzer D, Stuendl N, Lohberger B, Awaja F. Enhanced Osteogenic Differentiation of Human Primary Mesenchymal Stem and Progenitor Cultures on Graphene Oxide/Poly(methyl methacrylate) Composite Scaffolds. Materials (Basel). 2020; 13(13).
72. Hankenson KD, James IE, Apone S, Stroup GB, Blake SM, Liang X, et al. Increased osteoblastogenesis and decreased bone resorption protect against ovariectomy-induced bone loss in thrombospondin-2-null mice. Matrix Biol. 2005; 24(5):362-70.
73. Delany AM, Hankenson KD. Thrombospondin-2 and SPARC/osteonectin are critical regulators of bone remodeling. J Cell Commun Signal. 2009; 3(3-4):227-38.
74. Mansergh FC, Wells T, Elford C, Evans SL, Perry MJ, Evans MJ, et al. Osteopenia in Sparc (osteonectin)-deficient mice: characterization of phenotypic determinants of femoral strength and changes in gene expression. Physiol Genomics. 2007; 32(1):64-73.
75. Phimphilai M, Zhao Z, Boules H, Roca H, Franceschi RT. BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. J Bone Miner Res. 2006; 21(4):637-46.
76. Delany AM, Amling M, Priemel M, Howe C, Baron R, Canalis E. Osteopenia and decreased bone formation in osteonectin-deficient mice. J Clin Invest. 2000; 105(7):915-23.
77. Delany AM, Kalajzic I, Bradshaw AD, Sage EH, Canalis E. Osteonectin-null mutation compromises osteoblast formation, maturation, and survival. Endocrinology. 2003; 144(6):2588-96.
78. Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. J Cell Biochem. 2011; 112(12):3491-501.