Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61532R2
Full Title:	Isolation and Cultivation of Mandibular Bone Marrow Mesenchymal Stem Cells in Rats
Section/Category:	JoVE Medicine
Keywords:	Mandibular bone marrow mesenchymal stem cells (mBMSCs), osteogenesis, chondrogenesis, adipogenesis, cell cultivation, fluorescent cell sorting.
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Shanghai,China

TITLE:

Isolation and Cultivation of Mandibular Bone Marrow Mesenchymal Stem Cells in Rats

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KEYWORDS:

mandibular bone marrow mesenchymal stem cells, mBMSCs, osteogenesis, chondrogenesis, adipogenesis, cell cultivation, fluorescent cell sorting

SUMMARY:

This article presents a method that combines whole bone marrow adherence and flow cytometry sorting for isolating, cultivating, sorting, and identifying bone marrow mesenchymal stem cells from rat mandibles.

ABSTRACT:

Here we present an efficient method for isolating and culturing mandibular bone marrow mesenchymal stem cells (mBMSCs) in vitro to rapidly obtain numerous high-quality cells for experimental requirements. mBMSCs could be widely used in therapeutic applications as tissue

engineering cells in case of craniofacial diseases and cranio-maxillofacial regeneration in the future due to the excellent self-renewal ability and multi-lineage differentiation potential. Therefore, it is important to obtain mBMSCs in large numbers.

In this study, bone marrow was flushed from the mandible and primary mBMSCs were isolated through whole bone marrow adherent cultivation. Furthermore, $CD29^{+}CD90^{+}CD45^{-}$ mBMSCs were purified through fluorescent cell sorting. The second generation of purified mBMSCs were used for further study and displayed potential in differentiating into osteoblasts, adipocytes, and chondrocytes. Utilizing this in vitro model, one can obtain a high number of proliferative mBMSCs, which may facilitate the study of the biological characteristics, the subsequent reaction to the microenvironment, and other applications of mBMSCs.

INTRODUCTION:

Bone marrow mesenchymal stem cells (BMSCs) are non-hematopoietic stem cells derived from bone marrow that manifest strong proliferation capability and multi-lineage differentiation potential[1-4]. Indeed, BMSCs have been considered as an ideal candidate for bone tissue engineering and regeneration ever since they were discovered. For years, the iliac crest or long bones such as the tibia and femur have been the most common source of BMSCs for craniofacial regeneration. However, orofacial BMSCs, such as mandibular BMSCs (mBMSCs), display some differences from long bone BMSCs, such as different embryonic origin and development pattern. Mandibles arise from neural crest cells of the neuroectoderm germ layer and undergo intramembranous ossification, while axial and appendicular skeletons are from the mesoderm and undergo endochondral ossification. Furthermore, clinical observations and experimental animal studies have consistently indicated that there are functional differences between orofacial and iliac crest BMSCs[5-8]. Reports have shown that BMSCs derived from craniofacial bone such as mandible, maxillary bone, and alveolar bone exhibited superior proliferation, life span, and differentiation capability than those from axial and appendicular bones[9]. mBMSCs, therefore, are considered to be the preferred resources for future therapeutic applications of craniofacial diseases such as cherubism, jaw tumor, osteoporosis of jaw bone, and periodontal tissue defect[10-12]. To understand the treatment potential in preclinical experiments, it is essential to establish a method for rapidly isolating and culturing mBMSCs in vitro.

In this study, the aim was to obtain purified mBMSCs by whole bone marrow adherence and flow cytometry sorting. The anatomical morphology of rat mandible, clearly observed through micro computed tomography (Micro-CT) and histological sections, showed that the trabecular bone of the mandible was between the incisor medullary space and the alveolar bone. The bone marrow from trabecular bone was flushed to obtain mandibular marrow cells, but the cells cultured in this way were not pure mBMSCs and were likely to consist of multiple types of cells with uncertain potencies and diverse lineages such as cells from bone, fat and endothelial cells[13,14]. The next step of cell purification was particularly important. Flow cytometry filters cells by recognizing a combination of cell-surface proteins and has been widely adopted in the enrichment of mesenchymal stem cells. Cell homogeneity is the main advantage of flow cytometry, but the process does not determine cell viability and can result in a limited cell yield. In this study, the P0 mBMSCs obtained from whole bone marrow adherence were sorted by flow cytometry to obtain mBMSCs with high purity and strong proliferation capacity.

This study introduces a reproducible and reliable protocol for isolation, culture, and differentiation of rat mandibular BMSCs using a combination of whole bone marrow adherence and flow cytometry sorting. It is a reliable and convenient method for researchers in related fields to use.

PROTOCOL:

All animal experimental procedures in this paper were approved by the Animal Care Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

1. Preparation

1.1. Use two 5-week-old male Sprague Dawley rats for the experiment.

1.2. Sterilize all the instruments, including needle holders, tweezers and scissors at high temperature or immersed in 75% ethanol for 10 min.

NOTE: Ethanol immersion should not be too long to avoid cell damage.

1.3. Prepare culture media beforehand, the composition of which is provided in **Table 1**. Supplement each medium as described below.

1.3.1. Preparation of α-MEM culture medium (with 10% FBS): Supplement minimum essential medium alpha (α-MEM) with 10% fetal bovine serum and 1% penicillin and streptomycin.

1.3.2. Preparation of osteogenic differentiation medium: Supplement osteogenic differentiation basal medium with 10% fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin, 0.20% ascorbic acid, 1% β-glycerophosphate and 0.01% dexamethasone.

1.3.3. Preparation of osteogenic induction medium: Mix 70% α-MEM culture medium (with 10% FBS) and 30% osteogenic differentiation medium.

1.3.4. Preparation of adipogenic differentiation medium A: Supplement adipogenic differentiation basal medium with 10% fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin, 0.20% insulin, 0.10% IBMX, 0.10% Rosiglitazone and 0.01% Dexamethasone.

1.3.5. Preparation of adipogenic differentiation medium B: supplement adipogenic differentiation basal medium with 10% fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin and 0.20% insulin.

1.3.6. Preparation of chondrogenic differentiation medium: Supplement chondrogenic differentiation basal medium with 0.01% Dexamethasone, 0.30% Ascorbic acid, 1% ITS, 0.10% Sodium pyruvate, 0.10% Proline and 1% TGF-β3.

NOTE: The osteogenic differentiation medium must be used up within a month after configuration. The effective period of the configured chondrogenic differentiation medium is 12 h.

2. Isolation and cultivation of rat mBMSCs

NOTE: All experimental operations should be performed on ice as much as possible to maintain cell viability.

2.1. Harvesting rat mandibles

2.1.1. Euthanize two 5-week-old male Sprague Dawley rats by CO_2 asphyxiation. Ensure that the animal's breathing is stopped before proceeding with the experiment.

2.1.2. Rinse these rats in a beaker containing 75% ethanol for 3 min.

2.1.3. Place the rat inside a clean fume hood to harvest mandibles.

NOTE: Sterilize the fume hood by ultraviolet light radiation for 30 min before use.

2.1.4. Place the rat in a supine position. Incise the skins and buccinator muscles from bilateral angulus oris to the posterior region of the mandible, which is the only movable bone with lower incisor.

NOTE: It is recommended to make a 2 cm incision on each side of the angulus oris to get a clear operation field.

2.1.5. Open the mouth of the rat by pushing the maxillary and mandibular incisors towards the opposite direction with both thumbs to expose the mandibular teeth, which are attached to the mandible.

2.1.6. Completely disconnect the buccal muscles and the tendons attached to the coracoids and the inferior border of mandible.

NOTE: An increased mobility of the mandible is observed during this step because of the lack of muscle tension.

2.1.7. Press the mandibular posterior teeth and rotate back and downwards until the condyles on both sides are clearly exposed.

NOTE: This step is performed to artificially open the mouth of the rat to the maximum extent possible to dislocate the condyle. The mandible is connected to skull through bilateral condyles, so the exposure of the condyles leads to the disconnection between the skull and mandible.

2.1.8. Separate the mandible body from the skull.

2.1.9. Clean the adherent soft tissue on the bone surface using a wet gauze.

2.1.10. Place the bone in a 10 cm sterile glass dish filled with precooled minimum essential medium-α (α-MEM) or phosphate buffer saline (PBS) on ice to preserve the viability.

2.2. Isolation and cultivation of the mBMSCs

2.2.1. Cut off the anterior bone along the mesial edge of the first molar from the mandibular body. Remove the mandibular ramus including coracoid and condyle along the distal edge of the third molar to expose the marrow cavity.

2.2.2. Fill a 10 cm sterile glass dish with 10 mL of α-MEM (with 10% FBS).

2.2.3. Use 1 mL syringe to aspirate α-MEM culture medium. With the syringe needle inserted into the bone marrow cavity, repeatedly flush the bone marrow into the dish. Flush the bone cavity at least 3 times from both mesial and distal sides of the bone, respectively, until the bone turned white.

NOTE: This is the most critical step, as the bone marrow cavity of a rat mandible is not as obvious as in long bone and the proper point to insert the needle needs to be determined empirically. All experimental samples above should be stored on ice to maintain cell viability and so the operation time must be no longer than 2 h.

2.2.4. Transfer the media containing the flushed cells into a 15 mL centrifuge tube and centrifuge at 800 *x g* in 4 °C for 5 min. Discard the supernatant.

2.2.5. Resuspend the cells with 3 mL of α-MEM (with 10% FBS). Plate the cells in a new 10 cm culture dish and incubate at 37 °C in a 5% CO_2 incubator.

2.2.6. Check the morphological changes and growth of these cells on the 3rd day of culture. Remove the culture medium with nonadherent cells and tissue fragments. Gently add 10 mL of fresh α-MEM (with 10% FBS).

2.2.7. After 7 days of culture, P0 mBMSCs reached 70% to 80% confluence.

2.3. Flow cell sorting

NOTE: P0 mBMSCs were phenotypically identified and primarily purified through flow cell sorting.

2.3.1. Aspirate and discard the culture medium and then wash the dish with PBS. Discard the PBS.

2.3.2. Add 1 mL of 0.25% trypsin with 0.02% EDTA in the dish. Digest the cells at 37 °C for 5 min, then add 2 mL of α-MEM (with 10% FBS) to stop the reaction.

2.3.3. Transfer the cells suspension into a 15 mL centrifuge tube and centrifuge at 800 *x g* for 5 min.

2.3.4. Resuspend the cells in 120 µL of PBS (with 10% FBS) after centrifugation.

2.3.5. Block these cell suspensions with 1 µL of antibody against CD16/CD32 at 4 °C for 15 min.

2.3.6. Transfer 100 µL of the cell suspension into a new microcentrifuge tube, stain the cells with Phycoerythrin (PE)-conjugated antibody against CD45, fluorescein-isothiocyanate (FITC)-conjugated antibody against CD90 and Allophycocyanin (APC)-antibody against CD29 at 4 °C for 1 h in the dark[13,15]. The concentration of antibodies used in this experiment is shown in **Table 2**. Use the other 20 µL of cell suspension as unstained negative control.

2.3.7. Then centrifuge the tubes at 800 *x g* for 5 min, discard the suspension, and resuspend the cells in 0.5 mL of PBS (with 10% FBS).

2.3.8. Add 10 µL of 0.01 mg/mL DAPI for 10 min before analysis.

2.3.9. Use 40 nm filters placed on centrifuge tubes to filter the cells.

2.3.10. Analyze the cells on Fluorescence-activated cell Sorter. Set the panels as follows. Firstly, remove dead cells from total cell count by gating DAPI$^-$ cells, then gate CD29$^+$CD90$^+$CD45$^-$ in the selected cells as targeted mBMSCs.

2.3.11. Collect the sorted CD29$^+$CD90$^+$CD45$^-$ cells into a 15 mL centrifuge tube with 3 mL of α-MEM (with 10% FBS) pre-prepared.

2.3.12. Centrifuge the tubes at 800 *x g* for 5 min. Remove the collection buffer and add 1 mL of fresh α-MEM (with 10% FBS) to resuspend the cells. Then plate them in a 6 cm culture dish.

3. Colony formation capability

NOTE: This step was performed to check for the division ability of mBMSCs.[15]

3.1. Select second passage mBMSCs (P2) for this experiment. When the cell confluence reached 80% to 90%, reseed the cells in a serial gradient in a 6 well plate to evaluate their clonal proliferation ability.

NOTE: It is recommended to set the gradient from 1 x 10^2 to 1 x 10^3 cells per well, but the final dilutions of different cell lines of human or animal origin were empirically determined.

3.2. Culture the cells in α-MEM (with 10% FBS) for approximately one week until a good number of the colony forming units could be seen under light microscope. Refresh the culture medium every 3 days.

3.3. Aspirate and discard the culture medium, wash the wells with PBS twice for 5 min at a time. Next add 4% paraformaldehyde solution to each well and fix for at least 10 min.

3.4. Remove the paraformaldehyde and rinse the wells twice with PBS.

3.5. Stain the cells with crystal violet staining solution and incubate them in 37 °C for about 10 min.

NOTE: The staining time adjusted appropriately until the desired shade is achieved.

3.6. Remove the staining solution and wash the samples with distilled water to stop the reaction.

3.7. Image under a stereomicroscope and count scattered cell colonies which consisted of at least 50 cells.

4. Multilineage differentiation of mBMSCs

NOTE: The P2 mBMSCs were used for subsequent experiments unless otherwise described.

4.1. Osteogenic induction of mBMSCs

4.1.1. Digest the mBMSCs as described above. Seed cells at a density of 2.5 x $10^5/cm^2$ in a 12 well plate supplemented with 1 mL α-MEM (with 10% FBS).

4.1.2. When the cell confluence reached 60-70%, change the medium into 30% osteogenic induction media. Culture the cells with α-MEM (with 10% FBS) as a negative control and change the medium every 2 days.

NOTE: After a great good number of calcium nodules appear during osteogenesis, it is recommended to change the medium exchange pattern to a half volume medium exchange every 2 days, to prevent osteoblasts from floating.

4.1.3. After culturing for seven days, assess the calcification of these cells by alkaline phosphatase staining[16,17].

4.1.4. Remove the culture medium and fix the cells with 4% paraformaldehyde for 10 min. Rinse the cells using 1 mL of PBS per well for 3 min twice.

4.1.5. Then stain the cells with alkaline phosphatase staining solution and incubate at 37 °C for 10-30 min.

NOTE: Incubation can be performed overnight at room temperature to obtain the required color.

4.1.6. Wash the wells with distilled water to stop the reaction. Take photographs under light microscope. Red pigmented granules represent alkaline phosphatase (ALP) activity.

4.1.7. After culturing the remaining cells for another 7 days, perform alizarin red staining to evaluate the mineralization capacity of the cells.

4.1.8. Stain the cells with 0.5% alizarin red staining solution after cell fixation for 10 min[16,18]. Stop the chromogenic reaction with distilled water after 3-5 min.

NOTE: The reaction time can be extended depending on the color developed.

4.1.8. Finally, place the culture plate under the light microscope to observe the effect of osteogenic staining; red nodules indicate calcium deposits of these cells.

4.2. Adipogenic induction of mBMSCs

4.2.1. Seed P2 mBMSCs in a 12 well plate as described above.

4.2.2. After the well become 90% confluent, culture the cells with adipogenic differentiation medium A. Use cells cultured in α-MEM culture medium (with 10% FBS) as negative control.

4.2.3. After 2 days of induction, aspirate the medium A from the well and add 1 mL of adipogenic differentiation medium B in each well.

4.2.4. After one day, remove medium B and start the cycle again with medium A added back into the well. Culture the cells alternately using medium A and B.

4.2.5. Apply the differentiation medium A and B alternately for three cycles and detect the adipogenesis of these cells by oil red O staining[16,18].

NOTE: Lots of round and big lipid droplets can be observed by culturing these cells with differentiation medium B for an additional 2 to 3 days.

4.2.6. Remove the medium and fix the cells with 4% paraformaldehyde for 10 min. Wash out the cells with PBS.

4.2.7. Stain the cells with oil red O staining solution and incubate at 37 °C for approximately 30 min.

4.2.8. Remove the staining solution and wash the wells with distilled water.

4.2.9. Observe adipogenesis under light microscope.

4.3. Chondrogenesis induction of mBMSCs

4.3.1. Seed P2 mBMSCs in 15 mL centrifuge tube at a density of 5 x 10^5/cm^2.

4.3.2. Centrifuge the tubes at 300 *x g* for 5 min. Discard the supernatant.

4.3.3. Resuspend the cells with 0.5 mL of chondrogenic differentiation medium. Centrifuge the cells at 300 *x g* for 5 min.

4.3.4. Loosen the cap of the centrifuge tube and incubate it at 37 °C in a 5% CO_2 incubator. Renew the medium every 2 days.

NOTE: Cells start pelleting after 24 h. It is recommended not to move the centrifuge tube for 48 h. Be careful not to aspirate the cell pellet when changing the medium.

4.3.5. After 21 days of induction, fix the cell pellets with 4% paraformaldehyde, followed by dehydration, paraffin embedding and sectioning.

4.3.6. After deparaffinization and hydration, stain the slides in Alcian blue solution for at least 15 min[18-21], then wash with distilled water to stop the reaction. Capture the photographs under light microscope.

4.3.7. For immunofluorescent staining of collagen type II, perform the steps below.

4.3.7.1. Incubate the slides with 5 µg/mL proteinase K for 15 min at 37 °C after the deparaffinization and hydration step.

4.3.7.2. Incubate the slides in blocking buffer for 1 h at 37 °C.

4.3.7.3. Apply 1 µL of collagen type II antibody in 200 µL of blocking buffer to the slides and incubate overnight at 4 °C.

4.3.7.4. The next day, wash the slides with PBS twice and incubate with secondary antibodies at 37 °C for 1 h.

4.3.7.5. Incubate the slices with 40,6-diamidino-2-phenylindole (DAPI) for 10 min.

4.3.8. Take photographs of each slides under fluorescence microscopy.

5. Real-time PCR

5.1. Extract total RNA from mBMSCs using a guanidium isothiocyanate based commercially available reagent.

5.2. Perform reverse transcription of RNA into complementary DNA using a commercially available kit. The reaction conditions of reverse transcription were as follows: 65 °C for 5 min, 37 °C for 15 min, 85 °C for 15 s.

5.3. Perform real-time PCR to detect osteogenesis and adipogenesis specific genes using primers listed in **Table 3.** The PCR amplification procedure was as follows: 95 °C for 5 min. 95 °C for 5 s, 60 °C for 30 s for 40 cycles.

REPRESENTATIVE RESULTS:

Using this protocol, a large proportion of cells adhered to the plate on the third day after the initial culture. Typically, after an additional 3-4 days of culture, the cell confluence reached to 70 to 80% **(Figure 1B)**. With fluorescent cell sorting, $DAPI^{-}CD29^{+}CD90^{+}CD45^{-}$ mBMSCs were purified[18,22], which accounted for about 81.1% in the P0 cells **(Figure 1C)**.

After seeding P2 mBMSCs at 100 cells in each well of 6 well plate for a week, a significant amount of colony forming units were observed, which suggested the significant colony forming capability of mBMSCs **(Figure 1D)**.

To assess the multi-lineage differentiation ability, the mBMSCs were induced into osteo-, chondro- and adipo-lineages, respectively, in 12 well plates. The mBMSCs displayed strong osteogenic differentiation capability. Increased activity of ALP, red calcific nodules distributed sporadically under alizarin red staining, and increased expression of osteogenic specific genes *Runx2*, *Alp, Bsp* and *Ocn* **(Figure 2)** indicated oestogenic induction. For adipogenesis, identified by Oil-red-O staining, numerous lipid-rich vacuoles were evident after 9 days of induction. Likewise, the expression of adipogenic specific genes *Pparγ1* and *Cebpa* showed a significant increase **(Figure 3)**. For microscopic observation of chondrogenic differentiation slides, the samples showed positive staining for Alcian blue. In addition, immunostaining with anti-type II collagen antibody showed enhanced accumulation of cartilage matrix **(Figure 4)**.

FIGURE AND TABLE LEGENDS:

Table 1: Components of culture medium and differentiation medium.

Table 2: Antibody concentration used in this study.

Table 3: Primers used in Real-time PCR.

Figure 1: Isolation and culture of mBMSCs. (**A**) Schematic diagram of the protocol. mBMSCs were isolated and plated on day 0 and incubated with α-MEM culture medium. On day 7, the P0 mBMSCs were purified through flow cytometry sorting and the sorted cells were plated on a new culture dish. On day 14, P1 mBMSCs were collected and plating on 12-well plate. On day 15, P2 mBMSCs were induced into osteoblasts, adipogenic cells and chondroblasts under corresponding induction medium. (**B**) Schematic model of rat mandibular bone marrow and microscopic

observation of P0 mBMSCs. (**C**) Flow cytometry sorting of rat mBMSCs. The flow cytometry analysis shows these cells were positive for CD29 and CD90, but negative for CD45, which is congruent with BMSC characteristics. Of these, 1.4×10^6 cells were sorted, which accounted for appropriately 80% of the total cells. (**D**) Representative image of crystal violet stained P2 mBMSC clones.

Figure 2: Osteogenic differentiation potential of mBMSCs. (**A**) After 7 days of osteogenic induction, the change of ALP activity was visualized. Large numbers of mineralized nodules were stained under alizarin red staining at 14 days after induction of osteogenic differentiation. (**B,C**) The positive area of ALP and alizarin red staining were evaluated using Image J software. (**C-G**) The mRNA expression of osteoblast-specific markers *Runx2, Alp, Bsp* and *Ocn* increased significantly after 7 days of osteogenesis.

Figure 3: Adipogenic differentiation potential of mBMSCs after nine days of induction. (**A**) A large quantity of lipid droplets form and adipocytes were stained by oil-red-O. (**B,C**) The mRNA expression of adipogenic markers *Cebpa* and *Pparγ1* increased remarkably after 9 days of adipogenesis.

Figure 4: Stereoscope view of chondrogenic differentiation effect. (**A**) mBMSCs after 21 days chondrogenic induction showed positive for alcian blue staining. (**B**) Immunofluorescence image of chondrogenic aggregate stained with anti-type II collagen.

DISCUSSION:

This protocol describes a method to isolate BMSCs from rat mandibles in vitro by combining whole bone marrow adherence and fluorescent cell sorting, which is a simple and reliable way to obtain proliferative mBMSCs with strong differentiation ability. This method could preliminarily purify mBMSCs by flow cell sorting, but if there are higher requirements for cell homogeneity, more precise purification methods may be required.

Currently, there are four main techniques used for isolating mBMSCs, including whole bone marrow adherence, density gradient centrifugation, fluorescent cell sorting and magnetic activated cell sorting[22]. Whole bone marrow adherence and density gradient centrifugation are the most common and easy methods used to obtain mBMSCs in a short time, however, the low purity of harvested mBMSCs is their main disadvantage. The last two methods can isolate highly purified mBMSCs through immunological techniques, but have the shortcomings of being expensive, taking long time and impaired cell viability. In this study the advantages of whole bone marrow adherence and the fluorescent cell sorting method were combined to obtain enough numbers of proliferative mBMSCs in a short time.

Doubtlessly, one of the most critical steps in this protocol is dissection of rat mandible, which is quite distinct from those of axial and appendicular bones. It is essential to understand the anatomy of rat mandible to obtain an intact sample. Similar to human, rat mandible sits beneath the maxilla, holds the lower teeth in place and is connected to the skull by bilateral condyles. Since the mandible is the only bone that can move in the skull, there are many muscles attached to mandible, which control its movement. Only by removing these soft tissues completely and turning the mouth open maximally can the condyles connecting with the skull be exposed. It is also worth mentioning that the condylar neck is a physical weakness in mandible and is easy to fracture. If excessive resistance is found when rotating the mandible downwards, it means that the masticatory muscles may not been completely removed. When this is observed, do not rotate it constrainedly, otherwise it is easy to break the condylar neck thereby leading to cell contamination. Other difficulties in separation and culture of mBMSCs include low content in bone marrow, delicate cell activity, low purity, low cell frequency and contamination of hematopoietic cells[18,23]. To obtain mBMSCs with good growth and relatively high differentiation potential, ensuring the activity of mBMSCs is of crucial importance. There are several key steps, including the use of four-week old rats for this and subsequent experiments, as young rats are preferred to maintain good viability. Many studies have confirmed that the activity of mBMSCs is related to the age of experimental animals. Those mBMSCs from older donors may result in lower proliferation activity, differentiation potential and life span[2]. All the operations during cell harvest need to be completed on ice and the operation time should be as short as possible, preferably within 2 hours. In addition, keep the trypsin digestion time no longer than 3 minutes. Finally, still another challenge in this protocol is the process of harvesting mBMSCs. It may be troublesome to flush mBMSCs from the bone cavity because the cavity of rat mandible is very small, so it is very important to be familiar with the anatomical structure. Micro-CT images can be of great help in this regard. Besides, it is worth noting that as juvenile bones are slender and brittle, breakage can result in contamination.

Referring to immunophenotypic characterization, BMSCs express several phenotypes, but none of which is specific to them[24]. It is generally accepted that BMSCs do not express CD11b, CD14, CD34, or CD45, but they have a high expression of Sca-1, CD29, CD90, and CD105. This study chose the widely accepted markers of CD29, CD90, and CD45 for fluorescent cell sorting[13,14,25]. It found that $CD29^{+}CD90^{+}CD45^{-}$ cell accounted for appropriately 80% of total cells, which was enough for subsequent cell culture and research.

For decades, stem cell therapy is widely used in the treatment of various diseases, such as immune system diseases, hematological systemic diseases, cancers, or trauma. Undoubtedly, mBMSCs, as a substitute for BMSCs, can be used as a safer and more powerful tool in stem cell therapy due to their superior characteristics. Cell culture and expansion of mBMSCs, therefore, become particularly important to obtain sufficient number of cells for treatment.

In summary, this study demonstrated a promising and reliable protocol to harvest abundant mBMSCs with high homogeneity and multi-differentiation capability in a short period of time.

ACKNOWLEDGMENTS:

We thank for the assistance of Laboratory for Digitized Stomatology and Research Center for Craniofacial Anomalies of Shanghai Ninth People's Hospital. The work of this manuscript is supported by grants from the National Natural Science Foundation of China (NSFC) [81570950,81870740,81800949], Shanghai Summit & Plateau Disciplines, the SHIPM-mu fund from Shanghai Institute of Precision Medicine, Shanghai Ninth People's Hospital, Shanghai Jiao

Tong University School of Medicine [JC201809], the Incentive Project of High-level Innovation Team for Shanghai Jiao Tong University School of Medicine. And L.J. is a scholar of the Outstanding Youth Medical Talents, Shanghai "Rising Stars of Medical Talent" Youth Development Program and the “Chen Xing” project from Shanghai Jiaotong University.

DISCLOSURES:

All authors state that they have no conflicts of interest.

REFERENCES:

1 Jin, C. et al. Stem cell education for medical students at Tongji University: Primary cell culture and directional differentiation of rat bone marrow mesenchymal stem cells. *Biochemistry and Molecular Biology Education.* **46** (2), 151-154 (2018).

2 Chu, D. T. et al. An Update on the Progress of Isolation, Culture, Storage, and Clinical Application of Human Bone Marrow Mesenchymal Stem/Stromal Cells. *International Journal of Molecular Sciences.* **21** (3), 708 (2020).

3 Jin, Z., Chen, J., Shu, B., Xiao, Y., Tang, D. Bone mesenchymal stem cell therapy for ovariectomized osteoporotic rats: a systematic review and meta-analysis. *BMC Musculoskeleton Disorder.* **20** (1), 556 (2019).

4 Jiang, Y. et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* **418** (6893), 41-49 (2002).

5 Musina, R. A., Bekchanova, E. S., Belyavskii, A. V., Sukhikh, G. T. Differentiation potential of mesenchymal stem cells of different origin. *Bulletin of Experimental Biology and Medicine.* **141** (1), 147-151 (2006).

6 Lloyd, B. et al. Similarities and differences between porcine mandibular and limb bone marrow mesenchymal stem cells. *Archives in Oral Biology.* **77**, 1-11 (2017).

7 Zhang, W. et al. Comparison of the use of adipose tissue-derived and bone marrow-derived stem cells for rapid bone regeneration. *Journal of Dental Research.* **92** (12), 1136-1141 (2013).

8 Stefanik, D. et al. Disparate osteogenic response of mandible and iliac crest bone marrow stromal cells to pamidronate. *Oral Disorders.* **14** (5), 465-471 (2008).

9 Aghaloo, T. L. et al. Osteogenic potential of mandibular vs. long-bone marrow stromal cells. *Journal of Dental Research.* **89** (11), 1293-1298 (2010).

10 Kaigler, D. et al. Stem cell therapy for craniofacial bone regeneration: a randomized, controlled feasibility trial. *Cell Transplantation.* **22** (5), 767-777 (2013).

11 Li, C., Wang, F., Zhang, R., Qiao, P., Liu, H. Comparison of proliferation and osteogenic differentiation potential of rat mandibular and femoral bone marrow mesenchymal stem cells in vitro. *Stem Cells Development.* **2020**, 1941629 (2020).

12 Matsubara, T. et al. Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells. *Journal of Bone and Mineral Research.* **20** (3), 399-409 (2005).

13 Chan, C. K. F. et al. Identification of the Human Skeletal Stem Cell. *Cell.* **175** (1), 43-56, (2018).

14 Bianco, P., Riminucci, M., Gronthos, S., Robey, P. G. Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells.* **19** (3), 180-192 (2001).

15 Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., van Bree, C. Clonogenic assay of cells in vitro. *Nature Protocol.* **1** (5), 2315-2319 (2006).

16 Xu, H. et al. Icariin prevents oestrogen deficiency-induced alveolar bone loss through promoting osteogenesis via STAT3. *Cell Proliferation.* **53** (2), e12743 (2020).

17 Zhang, P., Wu, Y., Jiang, Z., Jiang, L., Fang, B. Osteogenic response of mesenchymal stem cells to continuous mechanical strain is dependent on ERK1/2-Runx2 signaling. *Internation Journal of Molecular Medicine.* **29** (6), 1083-1089 (2012).

18 Zhu, H. et al. A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. *Nature Protocol.* **5** (3), 550-560 (2010).

19 Lach, M. S. et al. Chondrogenic Differentiation of Pluripotent Stem Cells under Controllable Serum-Free Conditions. *International Journal of Molecular Sciences.* **20** (11), 2711 (2019).

20 Huang, X., Zhong, L., Hendriks, J., Post, J. N., Karperien, M. The Effects of the WNT-Signaling Modulators BIO and PKF118-310 on the Chondrogenic Differentiation of Human Mesenchymal Stem Cells. *International Journal of Molecular Sciences.* **19** (2), 561 (2018).

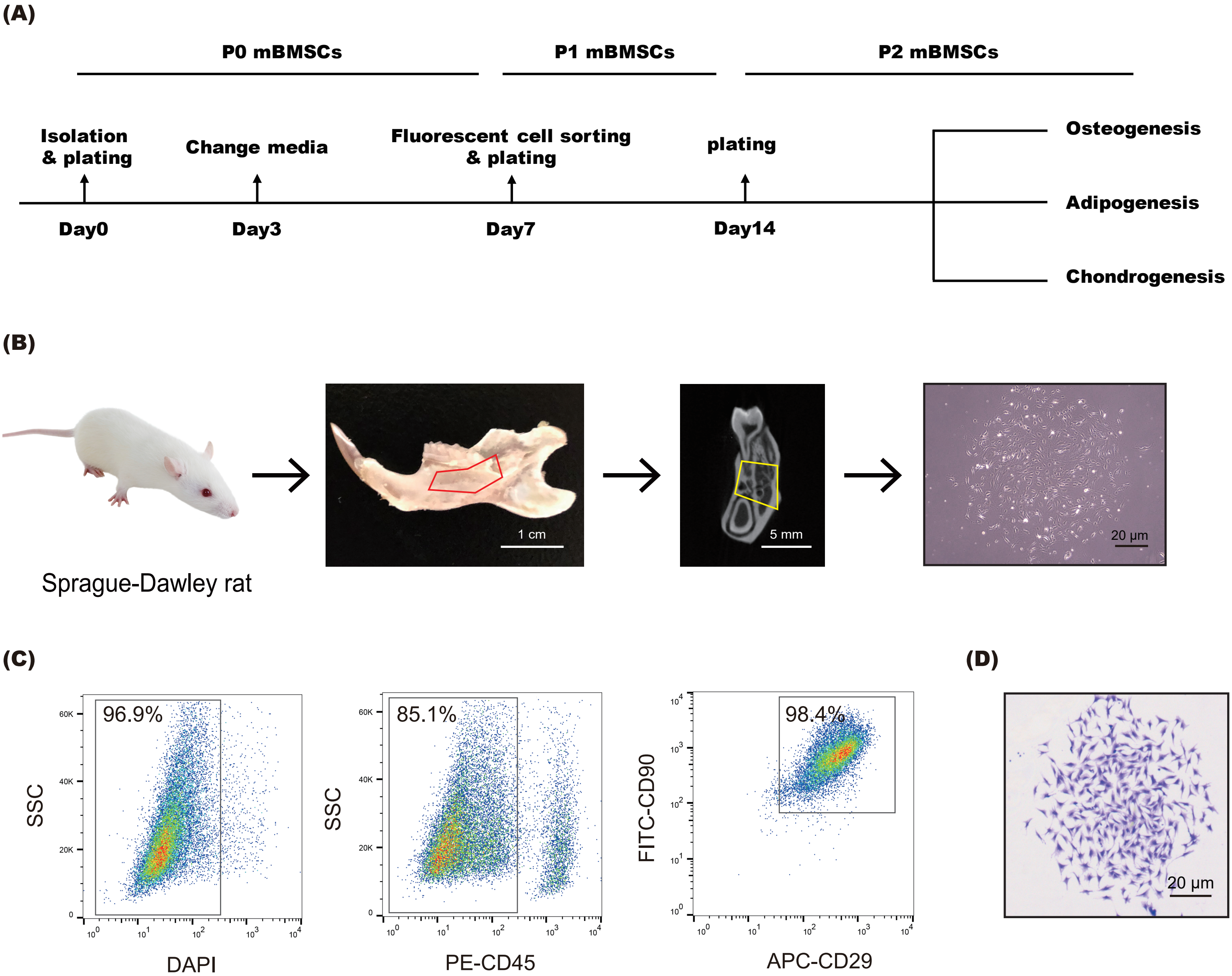
21 Gale, A. L., Linardi, R. L., McClung, G., Mammone, R. M., Ortved, K. F. Comparison of the Chondrogenic Differentiation Potential of Equine Synovial Membrane-Derived and Bone Marrow-Derived Mesenchymal Stem Cells. *Frontiers in Veterinary Sciences.* **6**, 178 (2019).

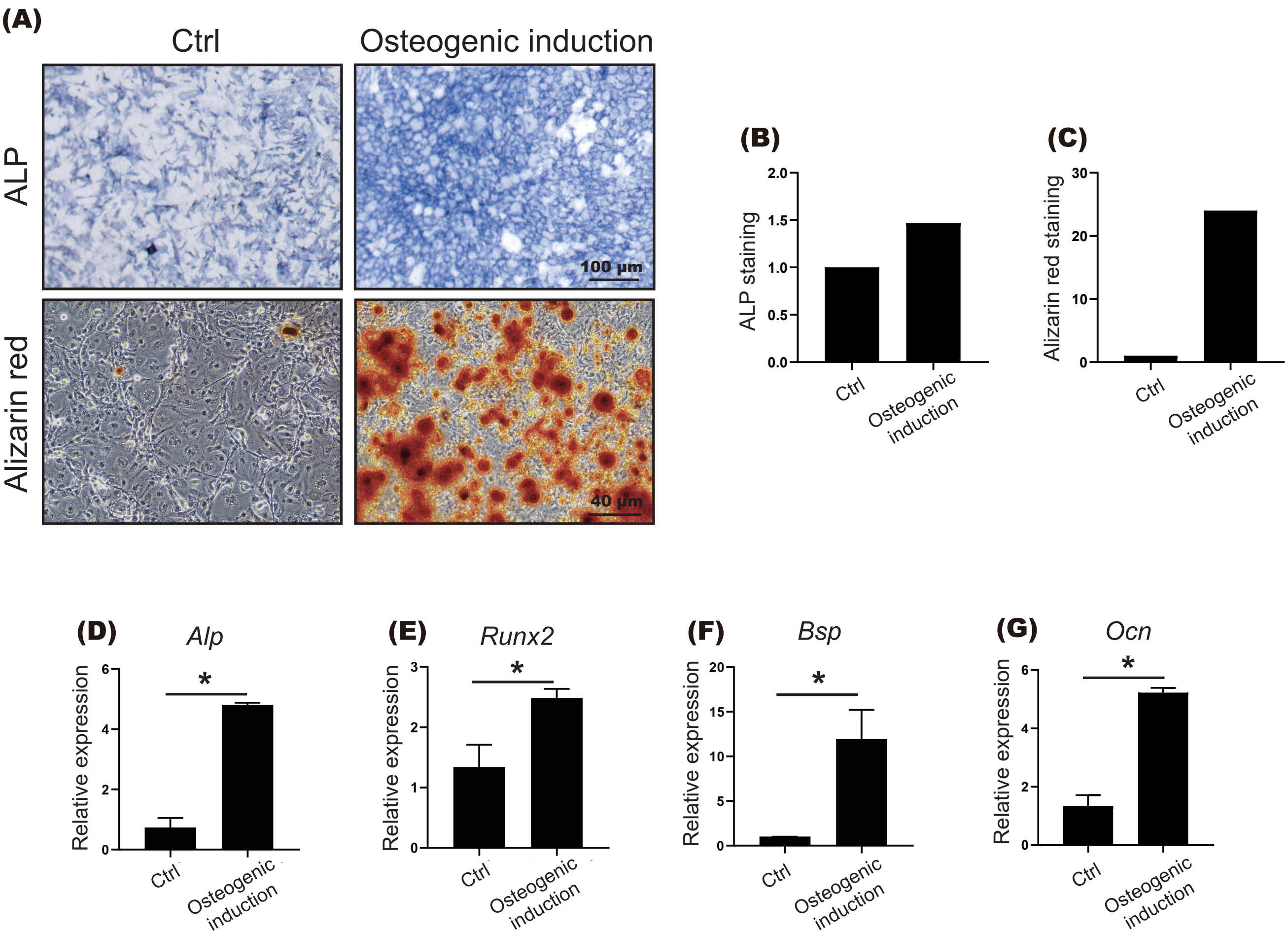
22 Li, X., Zhang, Y., Qi, G. Evaluation of isolation methods and culture conditions for rat bone marrow mesenchymal stem cells. *Cytotechnology.* **65** (3), 323-334 (2013).

23 Kagami, H., Agata, H., Tojo, A. Bone marrow stromal cells (bone marrow-derived multipotent mesenchymal stromal cells) for bone tissue engineering: basic science to clinical translation. *International Journal of Biochemistry and Cell Biology.* **43** (3), 286-289 (2011).

24 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.* **25** (11), 2739-2749 (2007).

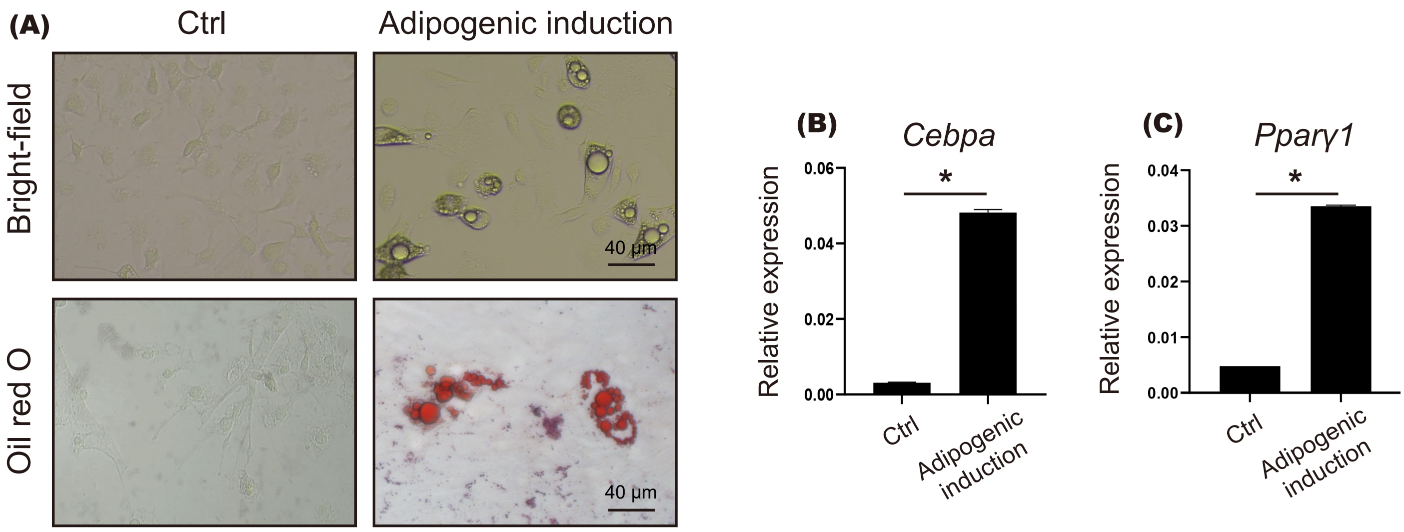
25 Abdallah, B. M., Alzahrani, A. M., Abdel-Moneim, A. M., Ditzel, N., Kassem, M. A simple and reliable protocol for long-term culture of murine bone marrow stromal(mesenchymal) stem cells that retained their in vitro and in vivo stemness in long-term culture. *Biology Proceedings Online.* **21**, 3 (2019).

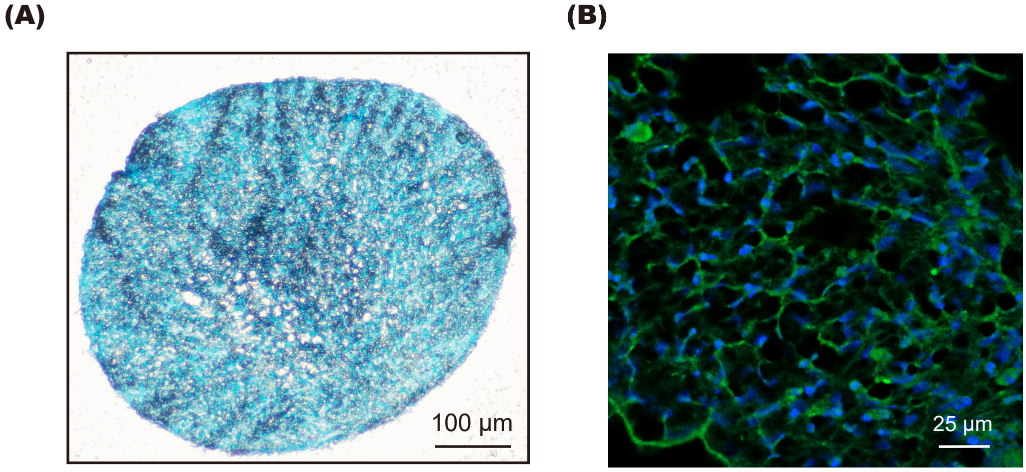

(A)
P0 mBMSCs
P1 mBMSCs
P2 mBMSCs
Isolation & plating
Change media
Fluorescent cell sorting & plating
plating
Osteogenesis
Adipogenesis
Chondrogenesis
Day0
Day3
Day7
Day14
(B)
Sprague-Dawley rat
1 cm
5 mm
20 μm
(C)
(D)
96.9%
85.1%
98.4%
SSC
DAPI
PE-CD45
FITC-CD90
APC-CD29
20 μm


(A)
Ctrl
Osteogenic induction
ALP
Alizarin red
100 μm
40 μm
(B)
ALP staining
2.0
1.5
1.0
0.5
0.0
Ctrl
Osteogenic induction
(C)
Alizarin red staining
30
20
10
0
Ctrl
Osteogenic induction
(D)
Alp
Relative expression
*
Ctrl
Osteogenic induction
(E)
Runx2
Relative expression
*
Ctrl
Osteogenic induction
(F)
Bsp
Relative expression
*
Ctrl
Osteogenic induction
(G)
Ocn
Relative expression
*
Ctrl
Osteogenic induction

Figure

Click here to access/download;Figure;figure3 (1).pdf




(A)
100 μm
(B)
25 μm

culture medium

1.α-MEM culture medium（with 10%FBS）

2.Osteogenic induction medium

3.Osteogenic differentiation medium

4.Adipogenic differentiation medium A

5.Adipogenic differentiation medium B ：

6.Chondrogenic differentiation medium:

component	final concentration
α-minimum essential medium	
Fetal bovine serum	10%
Penicillin and streptomycin	1%
α-MEM culture medium（with 10%FBS）	70%
Osteogenic differentiation differentiation medium	30%
Osteogenic differentiation basal medium	
Fetal bovine serum	10%
Glutamine	1%
Penicillin-Streptomycin	1%
Ascorbic acid	0.20%
β-Glycerophosphate	1%
Dexamethasone	0.01%
Adipogenic differentiation basal medium	
Fetal bovine serum	10%
Glutamine	1%
Penicillin-Streptomycin	1%
Insulin	0.20%
IBMX	0.10%
Rosiglitazone	0.10%
Dexamethasone	0.01%
Adipogenic differentiation basal medium	
Fetal bovine serum	10%
Glutamine	1%
Penicillin-Streptomycin	1%
Insulin	0.20%
Chondrogenesis differentiation basial medium	
Dexamethasone	0.01%
Ascorbic acid	0.30%
ITS	1%
Sodium pyruvate	0.10%
Proline	0.10%
TGF-β3	1%

antibody	concentration
CD90.1 (Thy-1.1) Monoclonal Antibody	0.5mg/mL
CD45 Monoclonal Antibody	0.2mg/mL
CD29 Antibody	0.2mg/mL
CollagenII rabbit polyclonal antibody	5mg/mL
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	1mg/mL

primer	sequence（5' to 3'）
GAPDH	Foward: CGGCAAGTTCAACGGCACAGTCAAGG
	Reverse: ACGACATACTCAGCACCAGCATCACC
Runx2	Foward: GCCTTCAAGGTTGTAGCCCT
	Reverse: TGAACCTGGCCACTTGGTTT
ALP	Foward: AAACTCGCTTATGGTCCCCG
	Reverse: TGGGTTTGAATTCCTGCGGT
BPS	Foward: GCACGGTTGAGTATGGGGAA
	Reverse: ATCCTGACCCTCGTAGCCTT
Ocn	Forward:CAACCCCAATTGTGACGAGC
	Reverse:GGCAACACATGCCCTAAACG
Cebpa	Foward: AGTCGGTGGATAAGAACAGCAACG
	Reverse: CGGTCATTGTCACTGGTCAACTCC
Pparγ1	Foward: CCATCGAGGACATCCAAGACAACC
	Reverse: GTGCTCTGTGACAATCTGCCTGAG

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials list .xls

Name of Material/ Equipment
0.25% Trypsin-EDTA (1X)
10cm culture dish
acutenaculum
Adipogenic differentiation medium
Alcian Blue
Alizarin red
Alkaline Phosphatase Color Development Kit
alpha-Minimum essential medium
Anti -CollagenII Rabbit pAb
Antibodies against CD16/CD32
Antifade Mounting Medium with DAPI
APC anti-mouse/rat CD29 Antibody
Biosafety cabinet
CD45 Monoclonal Antibody (OX1), PE, eBioscience
CD90.1 (Thy-1.1) Monoclonal Antibody (HIS51), FITC, eBioscience
Centrifuge
Chondrogenesis differentiation medium
Confocal laser scanning microscope
Countess II FL Automated Cell Counter
Crystal Violet Staining Solution
Fetal Bovine Serum
Goat Anti-Rabbit IgG H&L (Alexa Fluor 488)
Incubator
Inverted microscope
Magzol reagent(Trizol reagent)
micropipettor
Oil Red O
Osteogenic differentiation medium
Penicillin-Streptomycin
Phosphate-buffered saline (1X)
PrimeScript RT Master Kit
Proteinase K
QuickBlock Blocking Buffer
scissor
SYBR1 Premix
Toluidine Blue
Trypan Blue Solution, 0.4%

Company	Catalog Number	Comments/Description
Gibco	25200072	
Corning		
Cyagen biosciences inc.	MUBMX-90031	
Beyotime Biotechnology		
Sigma-Aldrich	A5533	
Beyotime Biotechnology	C3206	
GE Healthcare HyClone Cell Culture	SH30265.01B	
Abcam	ab34712	
Beyotime Biotechnology	P0131	
biolegend inc	102215	
Esco	AC2-4S8-CN	
Invitrogen	12-0461-82	
Invitrogen	11-0900-85	
cence	L500	
cyagen biosciences inc.		
Zeiss	LSM880	
Invitrogen	AMQAF1000	
Beyotime Biotechnology	C0121	
GE Healthcare HyClone Cell Culture	SH30084.03	
abcam	ab150077	
Esco	CCL-170B-8	
olympus	CKX53	
Magen		
Eppendorf		
cyagen biosciences inc.	MUBMX-90021	
Gibco	15070063	
Gibco	20012027	
TakaRa Bio Inc	RR036A	
Sigma-Aldrich	P6556	
Beyotime Biotechnology	P0260	
TakaRa Bio Inc		
Beyotime Biotechnology		
Gibco	15250061	

Dear editor,

Thank you for giving us the opportunity to submit a revised draft of the manuscript "Isolation and Cultivation of Mandibular Bone Marrow Mesenchymal Stem Cells in Rats" for publication in JOVE. We appreciate the time and effort that you and the reviewers dedicated to providing insight comments on our manuscript.

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Thank you for the feedback, we have retained the format of the manuscript and revised the manuscript in revisions mode.

2. Please address all the specific comments marked in the manuscript.

Thank you for reminding us. We have revised the manuscript according to the marker comments in it point by point.

3. Once done please ensure that the highlight is 2.75 page including heading and spacings (no less than 1 page).

Thank you for the feedback. We have highlighted the part to be filmed including headings.

Thank you for the feedback. We believe that the revised manuscript has been greatly strengthened by the additional experiments performed. We would be delighted to have this work published in JOVE.

Sincerely yours,

Yueyang,Hong