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

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# Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No, it will take place in one location.**

**Current Protocol Length**

Number of Steps: 16
Number of Shots: 43

# Introduction

**1. Introductory Interview Statements**

NOTE: Authors used the draft for filming, while the videographer had the final script, so there may be some mismatched interview shot numbers.

**REQUIRED:**

1.1. **Lingyong Jiang:** Amounts of studies have shown that BMSCs derived from craniofacial bone exhibited superior capability than those from axial and appendicular bones. Therefore, mBMSCs are considered to be the preferred resources for future therapeutic applications of craniofacial diseases so it is essential to establish a method for rapidly isolating and culturing mBMSCs in vitro.

1.2. **Lingyong Jiang:** This protocol combined the advantages of whole bone marrow adherence and fluorescent cell sorting method and could obtain enough numbers of purified mBMSCs in a short time.

1.3. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

1.4. Enter author name**:** Click here if you choose this question. Please write in a style that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

**OPTIONAL:**

1.5. **Yueyang Hong:** If you are trying this protocol for the first time， I think it is very important to understand the anatomic structure and functional movement of the rat mandible, which can be achieved by watching this video or reading related literature.

**OPTIONAL:** Why is visual demonstration of this method critical?

**Ethics Title Card**

1.6. Procedures involving animal subjects have been approved by the Animal Care Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

# Protocol

## 2. Harvesting Rat Mandibles

2.1. After euthanizing the rat, place it in a clean fume hood in a supine position **[1]**. Incise the skin and buccinator muscles from the bilateral angulus oris to the posterior region of the mandible **[2]**.

2.1.1. WIDE: Establishing shot of talent placing the rat in the fume hood.

2.1.2. Talent incising the skins and buccinator muscles.

2.2. 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 **[1]**.

2.2.1. Talent opening the mouth to expose the mandibular teeth.

2.3. Completely disconnect the buccal muscles and the tendons attached to the coracoids and the inferior border of mandible **[1]**, then press the mandibular posterior teeth and rotate them back and downwards until the condyles on both sides are clearly exposed **[2]**.

2.3.1. Talent disconnecting the buccal muscles and tendons.

2.3.2. Talent rotating the teeth back and forth.

2.4. Separate the mandible body from the skull **[1]** and clean the adherent soft tissue on the bone surface using a wet gauze **[2]**. Place the bone in a 10-centimeter sterile glass dish filled with precooled minimum essential medium-alpha or PBS on ice to preserve the viability **[3]**.

2.4.1. Talent separating the mandible body from the skull.

2.4.2. Talent cleaning the soft tissue with a gauze.

2.4.3. Talent placing the bone in the dish with PBS.

## 3. Isolation and Cultivation of the mBMSCs

3.1. Cut off the anterior bone along the mesial edge of the first molar from the mandibular body **[1]**, then remove the mandibular ramus including coracoid and condyle along the distal edge of the third molar to expose the marrow cavity **[2]**.

3.1.1. Talent cutting off the anterior bone.

3.1.2. Talent removing the mandibular arms.

3.2. Fill a 10-centimeter sterile glass dish with 10 milliliters of alpla-MEM *(pronounce 'alpha-M-E-M')* with 10% FBS **[1]**. Aspirate the medium with a 10-milliliter syringe **[2]**,

then insert the needle into the bone marrow cavity and repeatedly flush the bone marrow into the dish **[3]**.

3.2.1. Talent filling the dish with medium, with the medium container in the shot.

3.2.2. Talent aspirating the medium.

3.2.3. Talent inserting the needle in the bone marrow cavity and flushing the bone marrow.

3.3. Flush the bone cavity at least 3 times from both mesial and distal sides of the bone, respectively, until the bone turns white **[1]**.

3.3.1. Talent flushing and bone turning white.

3.4. Transfer the media containing the flushed cells into a 15-milliliter centrifuge tube **[1]** and centrifuge it at 800 rpm and for 5 minutes **[2]**. Discard the supernatant **[3]** and resuspend the cells with 3 milliliters of alpha-MEM with 10% FBS **[4]**.

3.4.1. Talent transferring the media into the centrifuge tube.

3.4.2. Talent putting the tube in the centrifuge and closing the lid.

3.4.3. Talent discarding the supernatant.

3.4.4. Talent resuspending the cells.

3.5. Plate the cells in a new 10-centimeter culture dish **[1]** and incubate them at 37 degrees Celsius in a 5% carbon dioxide incubator **[2]**.

3.5.1. Talent plating the cells.

3.5.2. Talent putting the cells in the incubator and closing the door.

## 4. Flow Cell Sorting

4.1. Aspirate the culture medium **[1]** and wash the dish with PBS **[3]**. Add 2 milliliter of 0.25% trypsin with 0.02% EDTA in the dish and digest the cells at 37 degrees Celsius for 5 minutes **[2]**, then add 4 milliliters of alpha-MEM with 10% FBS to stop the reaction **[3]**.

4.1.1. Talent aspirating the culture medium.

4.1.2. Talent washing the dish with PBS.

4.1.3. Talent adding trypsin-EDTA to the cells, with the trypsin-EDTA container in the shot.

4.1.4. Talent adding medium to the cells.

4.2. Transfer the cells suspension into a 15-milliliter centrifuge tube **[1]** and centrifuge it at 800 rpm for 5 minutes **[2]**. Resuspend the cells in 120 microliters of PBS with 10% FBS after centrifugation **[3]**.

4.2.1. Talent transferring the cell suspension into the centrifuge tube.

4.2.2. Talent putting the tube in the centrifuge and closing the lid.

4.2.3. Talent resuspending the cells in PBS.

4.3. Transfer 100 microliters of the cell suspension into a new microcentrifuge tube **[4.4.1]**

4.3.1. Talent adding antibody against CD16-CD32 to the cells. NOTE: Author performed 4.4.1 before 4.3.1, VO was adjusted accordingly.

4.4. Block the cell suspensions with 1 microliter of antibody against CD16-CD32 at 4 degrees Celsius for 15 minutes **[4.3.1]** and stain the cells with PE-conjugated antibody against CD45, FITC-conjugated antibody against CD90, and APC-antibody against CD29 **[2]** at 4 degrees Celsius for 1 hour in the dark **[3]**. Use the other 20 microliters of cell suspension as unstained negative control **[4]**.

4.4.1. Talent transferring the cell suspension into a microcentrifuge tube.

4.4.2. Talent adding antibodies to the cells.

4.4.3. Talent putting the cells in the refrigerator.

4.4.4. Talent labeling the leftover cell suspension “control”.

4.5. Centrifuge the tubes at 800 rpm for 5 minutes **[1]**, discard the supernatant **[2]**, and resuspend the cells in 0.5 milliliters of PBS with 10% FBS **[3]**. Add 10 microliters of 0.01 milligrams per milliliter DAPI 10 minutes before analysis **[4]**.

4.5.1. Talent putting the tube in the centrifuge and closing the lid.

4.5.2. Talent discarding the supernatant.

4.5.3. Talent resuspending the cells.

4.5.4. Talent adding DAPI to the cells.

4.6. Use 40-nanometer filters placed on centrifuge tubes to filter the cells **[1]**, then analyze the cells on a fluorescence-activated cell sorter **[2]**. First, remove dead cells from total cell count by gating DAPI-negative cells, then gate for CD29-positive-CD90-positive-CD45-negative in the selected cells as targeted mBMSCs *(spell out ‘M-B-M-S-Cs’)* **[3]**.

4.6.1. Talent filtering the cells.

4.6.2. Talent bringing the cells to the FACS machine.

4.6.3. Talent programming the gates.

4.7. Collect the sorted mBMSCs into a 15-milliliter centrifuge tube with 5 milliliters of alpha-MEM with 10% FBS **[1]**. Centrifuge the tubes at 800 rpm for 5 minutes **[2]** and remove the collection buffer **[3]**. Add 1 milliliter of fresh medium to resuspend the cells, then plate them in a 6-centimeter culture dish **[4]**.

4.7.1. Cells collecting in the tube.

4.7.2. Talent putting the tube in the centrifuge and closing the lid.

4.7.3. Talent removing the collection buffer.

4.7.4. Talent plating resuspended cells.

# Results

**5. Results: Osteogenic, Adipogenic, and Chondrogenic Differentiation of mBMSCs**

5.1. Using this protocol, a large proportion of cells adhered to the plate on the third day after the initial culture. After an additional 3 to 4 days of culture, the cell confluence reached 70 to 80% **[1]**. Fluorescent cell sorting was used to purify mBMSCs, which accounted for about 81.1% in the P0 cells **[2]**.

5.1.1. LAB MEDIA: Figure 1 B.

5.1.2. LAB MEDIA: Figure 1 C.

5.2. After culturing P2 mBMSCs in a 6-well plate for a week, a significant amount of colony forming units were observed **[1]**.

5.2.1. LAB MEDIA: Figure 1 D.

5.3. To assess the multi-lineage differentiation ability of the mBMSCs, they were induced into osteo-, chondro- and adipo-lineages **[1]**. Increased activity of ALP **[2]**, red calcific nodules under alizarin red staining **[3]**, and increased expression of osteogenic specific genes *Runx2 (pronounce 'runks-2')*, *Alp (spell out "A-L-P")*, *Bsp* and *Ocn* indicated osteogenic induction **[4]**.

5.3.1. LAB MEDIA: Figure 2 A.

5.3.2. LAB MEDIA: Figure 2 A, B, and C. *Video Editor: Emphasize the ALP images and B.*

5.3.3. LAB MEDIA: Figure 2 A, B, and C. *Video Editor: Emphasize the alizarin red images and C.*

5.3.4. LAB MEDIA: Figure 2 D.

5.4. Oil-red-O staining was used to identify adipogenesis **[1]**. Numerous lipid-rich vacuoles were evident after 9 days of induction **[2]**. Furthermore, expression of adipogenic specific genes *Pparγ1* and *Cebpa (spell out "P-P-A-R-Gamma" and "C-E-B-P-A")* was increased **[3]**.

5.4.1. LAB MEDIA: Figure 3 A.

5.4.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the Oil-red-O adipogenic induction image.*

5.4.3. LAB MEDIA: Figure 3 B and C.

5.5. The samples showed positive staining for Alcian blue during microscopic observation of chondrogenic differentiation **[1]**. In addition, immunostaining with anti-type 2 collagen antibody showed enhanced accumulation of cartilage matrix **[2]**.

5.5.1. LAB MEDIA: Figure 4 A.

5.5.2. LAB MEDIA: Figure 4 B.

# Conclusion

**6. Conclusion Interview Statements**

NOTE: Again, wrong draft used by author so there might be some slating mismatch.

6.1. **Yueyang Hong:** Ensuring the viability of mBMSCs is very importance in this protocol. This purpose could be achieved by choosing proper experimental animal， operating on ice and shortening the operating time.

*6.1.1. Suggested B-roll: 2.4.*

6.2. Enter author name**:** Click here to answer. Please use language that you will be comfortable memorizing and speaking aloud. Limit length to 30 or fewer words.

6.3. **Lingyong Jiang:**. 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.