bio-protocol

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Skeletal Stem Cell Isolation from Cranial Suture Mesenchyme and Maintenance of Stemness in Culture

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Abstract

Skeletal stem cells residing in the suture mesenchyme are responsible for calvarial development, homeostatic maintenance, and injury-induced repair. These naïve cells exhibit long-term self-renewal, clonal expansion, and multipotency. They possess osteogenic abilities to regenerate bones in a cell-autonomous manner and can directly replace the damaged skeleton. Therefore, the establishment of reliable isolation and culturing methods for skeletal stem cells capable of preserving their stemness promises to further explore their use in cell-based therapy. Our research team is the first to isolate and purify skeletal stem cells from the calvarial suture and demonstrate their potent ability to generate bone at a single-cell level. Here, we describe detailed protocols for suture stem cell (SuSC) isolation and stemness maintenance in culture. These methods are extremely valuable for advancing our knowledge base of skeletal stem cells in craniofacial development, congenital deformity, and tissue repair and regeneration.

Keywords: Bone regeneration, Calvaria, Cell-based therapy, Craniofacial, Skeletal stem cell, Sphere culture, Skeletogenic mesenchyme, Suture stem cell, Mesenchymal stem cell, Osteogenesis

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Background

Conventional methods have shown successful isolation of mesenchymal stromal cells (MSCs) from bone marrow and other tissues (Friedenstein *et al.*, 1974; da Silva Meirelles *et al.*, 2006). MSCs contain skeletal stem cells (SSCs) with self-renewing and skeletogenic differentiating abilities (Sacchetti *et al.*, 2007). However, only ~10–20% of them are genuine SSCs (Robey *et al.*, 2014). The difficulties in engraftment, survival, and differentiation of the transplanted MSCs have also been well documented (Caplan and Correa, 2011; Zeitouni *et al.*, 2012). Furthermore, the cellular source of the endogenous MSC remains unknown. Here, we describe a protocol for the isolation of SSCs from the calvarial suture mesenchyme. These SuSCs have been demonstrated to generate bone at a single cell level upon kidney capsule transplantation, which can faithfully assess SuSC properties *in vivo* (Maruyama *et al.*, 2016). It is also important to develop a protocol capable of maintaining their stemness *in vitro*. The successful establishment of the skeletal stem cell culture protocol permits further assessments of stem cell characteristics, *e.g.*, self-renewal, proliferation, fate determination, and differentiation in an *ex vivo* setting. The preservation of stem cell stemness in culture is also critical for bone tissue engineering and opens the possibility of exploring next-generation therapeutics. We also describe an *ex vivo* protocol to culture SuSCs for an extended period. The cultured SuSCs can generate bones upon implantation to the ectopic site (Maruyama *et al.*, 2021). We demonstrate that this culture method can determine the label-retaining ability and asymmetric division of SuSCs. It also provides an outstanding system to further our examination of additional stem cell characteristics, *e.g.*, cell fate determination, generation of skeletal progenitors, and skeletogenic differentiation, as well as studies at the transcriptome (RNA-seq), epigenetics (ATAC-seq and ChIP-seq), and single-cell levels (scRNA-seq). Using these new tools, we can reconstruct lineage relationships between cells within craniofacial and skeletal tissues—a long-standing challenge in biology. These are extremely important advancements in stem cell-based therapy for bone regeneration and repair.

Materials and Reagents

1. Cell strainer 40 µm Nylon (Falcon, catalog number: 352340)
2. Ultra-Low Attachment Surface 24-well plate (Corning, catalog number: 3473)
3. Dulbecco's Phosphate-Buffered Saline without calcium & magnesium (DPBS; Corning, catalog number: 21-031-CV), 2 years shelf life at room temperature
4. 0.2% collagenase A (Roche, catalog number: 11088793001), freshly reconstituted from lyophilized stock in PBS
5. 10 mM HEPES (1 M buffer solution; Gibco, catalog number: 15630-080), 24 months shelf-life at 4°C
6. Penicillin-Streptomycin (Gibco, catalog number: 15140-122), 12 months shelf life at -20°C
7. 100 µg/mL transferrin (Sigma, catalog number: T8158) at -20°C
8. 20 nM progesterone (Sigma, catalog number: P8783) at -20°C
9. 30 nM Sodium selenite (Sigma, catalog number: S8295) at -20°C
10. 60 nM Putrescine dihydrochloride (Sigma, catalog number: P5780), 4 years shelf life at -20°C
11. EGF (Mouse EGF Recombinant Protein; Gibco, catalog number: PMG8041), 1 year shelf life at -20°C
12. bFGF (mouse FGF-basic Recombinant Protein; Gibco, catalog number: PMG0035), 1 year shelf life at -20°C
13. B27 supplement (Gibco, catalog number: 17504044,), 1 year shelf life at -20°C
14. Insulin (Sigma, catalog number: I1882) at -20°C
15. 0.25% trypsin (Gibco, catalog number: 25200-056), 2 years shelf life at -20°C
16. Soybean trypsin inhibitor (SBTI; Sigma, catalog number: T9128) at -20°C
17. Digestion Buffer (see Recipes)
18. Sphere Culture Media (see Recipes)
19. 2× SBTI Solution (see Recipes)

Equipment

1. Shaker (Thermo Scientific, MaxQtm 4000 Benchtop Orbital Shakers)
2. Centrifuge (Thermo Scientific, Sorvall ST 16R Centrifuge)
3. Incubator (Thermo Forma, Series II Water Jacketed CO_2 Incubator)

Procedure

A. Isolation of mesenchymal cells from suture mesenchyme

1. Dissect a 2 mm wide portion of calvarial tissue containing the sagittal (SAG) suture and its adjacent parietal bones from mouse calvarium (Figure 1).

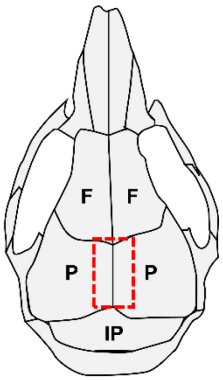

Figure 1. Schematic of mouse calvarial bones and sutures.
F, frontal bone; P, parietal bone; IP, interparietal bone.

2. Hint: It is easy to remove the calvarium from the meninges. The cutout size should be as small as possible, to limit the amounts of contaminant cells from the bone marrow and periosteum.
3. Grab both sides of the parietal bones with two forceps and gently pull them apart (Figure 2).

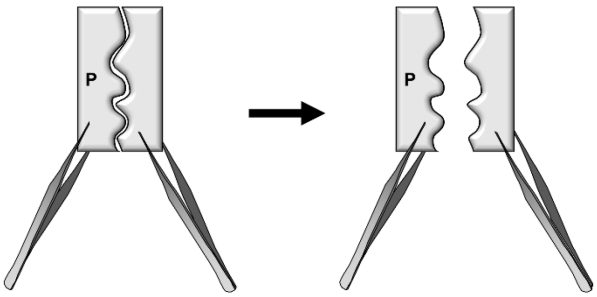

Figure 2. Schematic diagrams illustrating the separation of bone pieces to release the cells from the suture mesenchyme.

4. Attention: The dissected calvarial tissue containing two small parietal bone pieces must be separated in the midline to expose the suture mesenchyme. Otherwise, the number of skeletal stem cells would be significantly reduced.
5. Hint: To avoid contamination of stem cells from the bone marrow, do not cut the separated calvarial tissue

into pieces (this process is performed for calvarial cell isolation). Do not keep the dissected tissue on ice, rather quickly incubate it with Digestion Buffer as described in step 6.

6. Submerge the separated pieces in 40 mL of Digestion Buffer and incubate in a shaker at 200 × *g* (MaxQtm 4000 Benchtop Orbital Shakers) and 37°C for 1 h.
7. Filter the dissociated cells through a 40 µm strainer.
8. Spindown the cells by centrifugation at 400 × *g* and 4°C for 7 min.
9. Remove the supernatant to collect the cell pellets.
10. Hint: About 5 × 10^4 cells can be obtained from one sagittal suture
11. Hint: Suture mesenchymal cells have been successfully isolated from the calvaria of mice at postnatal day 10 (P10) and P28.

B. Sphere culture

1. Resuspend the cell pellets with Sphere Culture Media and count the cell number.
2. Seed the cells in 2 mL of sphere culture media at 10^4–10^5 cells per well on an Ultra-Low Attachment Surface 24-well plate (Figure 3A).
3. Carefully change half the amount of the culture media in the culture every other day, without disturbance of cell settling.
4. Hint: Sphere formation will be visible within 10 to 14 days (Figure 3B and Maruyama *et al*., 2021).

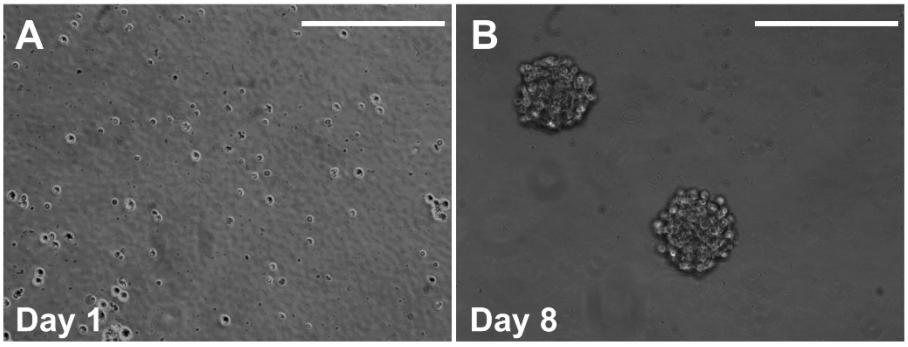

Figure 3. Representative images showing cells after 1 day (A) and 8 days (B) of the sphere culture. Scale bars, 200 µm.

5. Hint: Most of the sphere is derived from a single stem cell (Maruyama *et al.*, 2021).
6. Hint: Stem cell stemness, *e.g*., bone-forming ability and quiescence, has been demonstrated to be maintained in culture using the 1st and 3rd passage of the sphere by kidney capsule transplantation and label-retaining analyses (Maruyama *et al.*, 2021).
7. Hint: Bone-forming ability is assessed by the kidney capsule transplantation assay, followed by von Kossa staining and immunostaining of osteoblast markers. Quiescence is examined by the pulse-chase assay in sphere culture.

C. Passaging spheres

1. Some spheres may be weakly attached to the bottom of the well. To detach the sphere, gently pipette the culture media up and down.
2. The cell suspension is collected by centrifugation at 300 × *g* and 4°C for 7 min.
3. Remove the supernatant and tap the tube to gently break up the pellets.
4. Add 1 mL of 0.25% Trypsin to resuspend the pellet, followed by incubation at 37°C for 5 min.
5. Add 1 mL of 2× SBTI solution to stop the activity of the trypsin.
6. Centrifuge at 300 × *g* and 4°C for 7 min.

7. Remove supernatant and tap the tube to gently break up the pellets.
8. Resuspend the cells in the Sphere Culture Media and count cell numbers.
9. Seed the cells at 10^4–10^5 cells per well.
10. Hint: This culture method has been limited to five passages.

Data analysis

A. Sphere measurement

1. Gently pipette the medium to detach the spheres from the culture plate.
2. Hint: No special pipet tips are required, only general ones that generate a little media flow.
3. Take images using a camera under a microscope, *e.g.*, NIKON CB-115 (Figures 5, 6, Supplemental Figures S9, S10 of Maruyama *et al.*, 2021).
4. Measure the sphere number and size by ImageJ (Figures 6, Supplemental Figures S9, S10 of Maruyama *et al.*, 2021).
5. Hint: For the basic function of ImageJ: "ImageJ Basics (PDF file)" on the official page (https://imagej.nih.gov/ij/index.html) under "Documentation"-"Tutorials and Examples".
6. Count spheres that are >20 μm in diameter.
7. Perform at least three independent experiments for statistical evaluation.
8. Examine the statistical significance of sphere number and size using a two-sided Student's *t*-test.

B. Evaluation of bone formation after kidney capsule transplantation

1. Perform whole-mount von Kossa staining of bone in the transplanted kidney capsule (Figures 4, 5, 6, Supplemental Figures S9, S10 of Maruyama *et al.*, 2021).
2. Image using a microscope, *e.g.*, NIKON CB-115.
3. Measure the size of the stained area by ImageJ.
4. Perform at least three independent experiments for statistical evaluation.
5. Examine the statistical significance of sphere number and size using a two-sided Student's *t*-test (Supplemental Figure S10 of Maruyama *et al.*, 2021).

Recipes

1. Digestion Buffer (40 mL)

DPBS containing 0.2% collagenase A, 1% Penicillin-Streptomycin, and 10 mM HEPES

2. Sphere Culture Media

25 μg/mL insulin
100 μg/mL transferrin
20 nM progesterone
30 nM Sodium selenite
60 nM putrescine
20 ng/mL EGF
20 ng/ml bFGF
1× B27 supplement
1% Penicillin-Streptomycin

3. 2× SBTI Solution

2 mg SBTI in 1 mL of DPBS

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Competing interests

The authors declare no competing financial interests.

Ethics

Care and use of experimental animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester and IACUC at the Forsyth Institute.

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