Isolation and characterization of putative mesenchymal stem cells from mammalian gut

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Abstract Here, we provide a protocol for reliable isolation and subculture of putative mesenchymal stem cells from mice colons. This method provides a good approach to cultivate and characterize putative colonic mesenchymal stem cells (cMSCs). A high purity of cMSCs can be obtained according to this protocol. The whole isolation processes of cMSCs take about 2 h with two important digestion steps involved. Only with common culture medium, maturation of cMSCs in culture proceeds approximately 2 weeks to allow relevant researches to be conducted. This protocol sheds light on better cultivation of MSCs in vitro from post-natal colon tissues. These putative cMSCs share common phenotypic property with those in vivo reported, and contain potential lineage differentiation capacity. The successful culture of cMSCs in vitro provides an ideal model for study of MSCs biology in the intestine.

Keywords Mesenchymal stem cells · Colon · Characterization · Primary culture

Introduction

The past decades have witnessed great efforts in stem cell research and application, among which mesenchymal stem cells (MSCs) have drawn wide attention and are thriving increasingly (Barker 2014; Guiducci et al. 2010; Kumar et al. 2014; Taran et al. 2014). MSCs, also called adult tissue stem cells, are multipotent stromal cells that, similar to stem cells, possess the capacity for self-renewal and differentiation into variety of cell types, including osteoblasts, chondrocytes, and adipocytes (da Silva Meirelles et al. 2008).

Cumulative literatures reported that MSCs, residing in the gastrointestinal tract, contribute substantially to maintaining epithelium homeostasis (Barker 2014; Quante et al. 2009) and promoting tissue regeneration following injury such as radiation enteropathy (Ciorba et al. 2012). Moreover, recent studies even gave new insights into the biological role of stem cells in cancer progression of the intestine, and highlighted intestinal stem cells as a promising therapeutic target for treatment of cancer in the intestine (Barker et al. 2009; Ong et al. 2014; Rizk et al. 2012). These properties make cMSCs highly attractive in the field of generative medicine and cancer therapeutics.

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To our knowledge, MSCs from various tissues are heterogeneous in phenotypical and functional profiles (da Silva Meirelles et al. 2008; da Silva Meirelles et al. 2006). However, these cell populations also exhibit common features with respect to plastic adhesion and fibroblast-like in shape (da Silva Meirelles et al. 2006; Meirelles et al. 2003; Walker et al. 2010). Despite the maturation of technologies in isolating mesenchymal stem cells from many stromal tissues, including bone marrow (Meirelles and Nardi 2003), umbilical cord (Kim et al. 2010), and adipose (Hwa Cho et al. 2006), it is yet not easy to isolate mesenchymal stem cells from colonic tissues due to the small populations existing in subepithelium near the crypt zone and the fact that relatively tight junction between enterocytes that impede the isolation processes (Barker 2014). Here, we present a detailed method for extraction and cultivation of mammalian colonic mesenchymal stem cells in common culture for medical studies. This paper also provides the current most advisable defining markers to characterize mesenchymal stem cells from colonic tissues.

Materials and methods

Reagent preparation

Before cell isolation, several important stock solutions should be prepared ahead of time. Table 1 lists some necessary stock solutions used for each preparation.

Isolation procedures

All procedures were conducted in conformity with the Administrative Committee of Experimental Animal

Care and Use at the Second Military Medical University. Up to three C57BL/6 mice (4–6 w) were euthanized, respectively, following abdomen sterilization with iodine solution and 75 % ethanol. The colon tissues were collected all the way from cecum to rectum, and stored in a 15 ml tube with 5 ml pre-cold irrigation buffer. The colons were then irrigated thoroughly with pre-cold buffer solution using a 10 ml syringe matched with a lavage needle. After irrigation, colons were opened longitudinally in a sterilized glass dish with surgical scissors, following stretching with forceps to make them as flatten as possible, and sectioned into ~2 mm large pieces using a sterile razor blade. These small colon fragments were subjected to *Step 1* digestion in a 50 ml tube with digestive solution containing DTT and EDTA (Ambion, Carlsbad, CA, USA) at 37 °C in a culture chamber for 30 min, during which shaking would be suggested. The *Step 1* digestion results were then passed through a 150 mesh sterile steel filter. The residue was rinsed with EDTA solution for three times, each time with filtering through a fresh 150 mesh filter. The rinsed colon fragments were then transferred to a sterile glass dish containing ~2 ml digestive solution with type I collagenase (Sigma, St. Louis, MO, USA) and DNase I (Roche, Mannheim, Germany) involved, cut into mush with dissecting scissors, and the mixture was transferred to a fresh 50 ml tube containing additional ~13 ml *Step 2* digestive solution for second digestion. After 1 h incubation, 15 ml of CCM were added to quench enzyme activity. The mixture was then filtered using a sterile cell strainer (70 µm), followed by centrifugation at 1500 rpm for 5 min. The obtained pellets were washed once by resuspension in 5 ml CCM, and spinned down

Table 1 Key solutions for isolation of mesenchymal stem cells from mice colons

Stock solutions	Volume needed/ml	Aim
L-DMEM/D-HBSS (1:1)/10 mM HEPES/Abx	50	Irrigation buffer
CCM/5 mM EDTA/1 mM DTT	20	Step 1 digestion
L-DMEM/10 mM HEPES/Abx/5 mM EDTA	40	Rinse buffer
L-DMEM/10 mM HEPES/Abx/15 µg/ml collagenase/200 µg/ml DNase I	15	Step 2 digestion
CCM/200 µg/ml DNase	9	Culture

L-DMEM (Gibco), low-glucose Dulbecco's Modified Eagle Medium (containing glucose 1.0 g/L); D-HBSS (Gibco, Life Technologies, Carlsbad, CA, USA), Hank's balanced salt solutions without calcium and magnesium; HEPES (Gibco), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (1 M buffer solution); Abx (Gibco), penicillin-streptomycin mixture; CCM, complete culture medium (L-DMEM/10 Mm HEPES/Abx/10 % fetal bovine serum); DTT (Sigma, St. Louis, MO, USA), dithiothreitol (1 M)

again. The supernatant was discarded and the remaining cells were suspended in 9 ml CCM containing DNase I, and seeded in a 6-well culture plate (tissue culture treated) for 3 wells. Cells were incubated in a humidified cell culture chamber under conditions of 5 % CO₂ and 21 % O₂, at 37 °C. After 12 h incubation, cells without adhesion were removed, and 2 ml fresh CCM were added. During primary culture, the medium was suggested to be changed at day 4. Usually, a confluence of ~90 % occurred at day 7. The confluent cells were then trypsinized and passaged at a rate of 1:2 for subculture. A flow chart in Fig. 1 outlines the whole procedures for primary isolation and culture of mesenchymal stem cells from mice colons.

Fig. 1 Flow chart of key steps in extracting and cultivating colonic mesenchymal stem cells from mice

Proliferation assay of cultured cells from mice colons

After subculture of the cells from colons for 3 passages, well grown cells were detached enzymatically using 1:1 D-HBSS/0.25 % trypsin-EDTA, and suspended in fresh CCM. A total of 5000 cells in 100 µl CCM were seeded to each well of 96-well plates, and then subjected to cultivation in a normal cell incubator. The medium were changed with fresh CCM every day to enrich for cMSCs. After aspiration of supernatant, 110 µl L-DMEM containing 10 µl CCK-8 (Dojindo, Kumamoto, Japan) were added to each well at the indicated time (1, 2, 4, 6, 8 days after cell plating), followed by incubation at 37 °C for ~2 h in a normal cell culture chamber, and O.D. values were then detected at 450 nm using a Microplate Reader. Proliferative capacity of cMSCs will be well indicated by O.D. values, which are in proportion to cell proliferation rate.

FACS detection of CD29 and CD44 expression in cells isolated from mice colons

Cells at passage 3 were used for flow cytometry analysis. After growing to ~90 % confluence, plastic-adherent cells were trypsinized with 0.25 % trypsin/EDTA, and pelleted at 1500 rpm for 5 min, followed by wash with pre-cold 1 % BSA/HBSS for at least once. Cell pellets were resuspended in appropriate 1 % BSA/HBSS containing phycoerythrin (PE)-labeled rat anti-mouse CD44 or fluorescein isothiocyanate (FITC)-labeled hamster anti-rat CD29 (BD Pharmingen, San Diego, CA, USA), and incubated at RT for 1 h protected from light. At the end of staining, cells were pelleted, rinsed once in cold 1 % BSA/HBSS, and resuspended in 500 µl cold HBSS for FACS analysis. The cells were analyzed using a BD FACScan flow cytometer (Heidelberg, Germany). Unstained samples were used to set up a threshold, and the isotype specific conjugated anti-IgG (BD Pharmingen) was used as a negative control. The FlowJo software was used for building histograms.

Immunofluorescence staining of intracellular expression of COX-2 in isolated cells

Detached cells at passage 3 were suspended in CCM at a concentration of 1×10^5 cells/ml. Single cell suspensions were then plated in a 6-well plate (tissue

culture treated), 1 ml for each well, and incubated in a normal cell culture chamber overnight. After cell adherence, the supernatant was removed, and cells were rinsed with D-HBSS for one or two times to remove excess medium. Then cells were fixed in 4 % paraformaldehyde (PFA) for ~30 min at room temperature (RT) and rinsed thoroughly with D-HBSS, following membrane permeabilization using 0.25 % Triton X-100/D-HBSS for 15 min or more time. Excess Triton X-100 was removed by rinsing with D-HBSS, and cells were incubated in 3 % BSA/D-HBSS at RT for 30 min to block unspecific protein binding. After blocking, a certain amount of 3 % BSA solution containing COX-2 antibody (Santa Cruz, Santa Cruz, CA, USA) was added to the 6-well plate, and incubated for 30 min at RT. At the end of incubation, excess COX-2 antibody was removed by rinsing with D-HBSS twice, followed by incubation with Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for another 30 min at RT (away from light). Excess second antibodies were removed, and fluorescent labeled COX-2 expression was observed and photographed under phase-contrast inverted microscope equipped with fluorescence monitors. Photomicrographs were taken with a computer-assisted microscope (Leica, Wetzlar, Germany).

Lineage differentiation induction procedures

For lineage differentiation analysis, plastic adherent cells at passage 3 were detached and suspended in CCM to make a concentration of 1×10^5 cells/ml single cell suspension. A total of 250 μ l cell suspension were added to each well of a 48-well plate (tissue culture treated), and incubated in a cell culture chamber (5 % CO₂, 21 % O₂ at 37 °C). After attachment, cells were rinsed with D-HBSS to wash out residual CCM, and 250 μ l osteogenic or adiogenic medium (STEMCELL Technologies, Vancouver, BC, Canada) were added to each well. The induction medium were changed every 3 days. Three or four weeks later, the induction medium were removed, cells were rinsed carefully with ultra-pure water (UPW), fixed with 4 % PFA for 30 min at RT, and washed once in UPW, then an appropriate volume of Alizarin Red S staining solution was added to each well for verification of Ca²⁺ deposition through chromogenic reaction

(stained for 30 min at RT). For lipid staining, a certain amount of Oil Red O solution was added. At the end of staining, excess dye was removed by rinsing with UPW. Both stained calcium nodules and lipid droplets were observed and photographed using phase-contrast inverted microscope.

Statistics

Data were expressed as the mean $\pm$ the standard deviation (SD).

Results

Morphological and self-renewal profiles of mesenchymal cells isolated from mice colons

During primary culture, increased fibroblast-like cells were observed in the plate (Fig. 2a). Subculture in CCM showed no difference in cell shape (Fig. 2b). We also found that the isolated cells from mice colons showed obvious proliferative ability (Fig. 2c). This

Fig. 2 Morphological property and proliferation ability of cells isolated from mice colons. **a** Representative images of primary cultured cells from colons in CCM at different times. (Scale bar represents 100 μ m, original magnification: $\times 100$). **b** Representative images of subcultured cells from colons in CCM at different passages. P, passage. (Scale bar represents 100 μ m, original magnification: $\times 100$). **c** Cell proliferation of cells isolated from mice colons as indicated by CCK-8 assay, $n = 5$ –6 for each assay

result well confirmed their intrinsic proliferative property similar to stem cells.

Phenotypic profiles of mesenchymal cells isolated from mice colons

To our knowledge, there is yet no specific marker for identification of mesenchymal stem cells. Interestingly, recent studies have found that both CD29 and CD44 are positive expressed in almost all adult tissue stem cells (da Silva Meirelles et al. 2006). In vitro and in vivo studies have indicated that colonic mesenchymal stem cells also co-expressed CD29 and CD44 extracellularly (Walker et al. 2010), with much more expression in CD29 than CD44, (97.48 ± 1.08 %) versus (91.18 ± 1.80 %). Flow cytometry analysis indicated that those cells isolated from mice colons through our established methods exhibited high levels of CD29 and CD44 (Fig. 3).

Another noticeable finding was that the cells we isolated from mice colons appeared to constitutively express cyclooxygenase-2 (COX-2) intracellularly (Fig. 4a), which is hardly or seldom expressed in gastrointestinal tract physically.

Fig. 3 FACS analysis of CD29 and CD44 expression in cultured cells from mice colons. **a** Representative flow-cytometry histograms indicate immunophenotypic molecules of CD29 and CD44 (right peaks) expressed by cells isolated from colons, compared with controls (left peaks). **b** Statistical histograms show percentage of CD29 and CD44 positive cells detected by flow-cytometry ($n = 6$). Data are expressed as the mean $\pm$ SD

Fig. 4 Intracellular COX-2 expression in cMSCs and differentiation profile of isolated cells from mice colons. **a** Immunofluorescence images show protein expression of COX-2 in isolated cells from colons. (original magnification: $\times 400$; Scale bar: 30 μ m). **b** Alizarin Red S staining shows Ca^{2+} deposition in extracellular matrix of isolated cells cultured in osteogenic medium (original magnification: $\times 200$; Scale bar: 50 μ m); Oil Red O staining shows lipid droplets in isolated cells cultured in adipogenic medium (original magnification: $\times 400$; Scale bar: 30 μ m)

These results together indicate that the cells isolated from mice colons are highly similar to colonic MSCs.

Differentiation capacity of mesenchymal cells isolated from mice colons

Differentiation potency, a principle determinant to distinguish stem cells from other cell types, is commonly used to characterize mesenchymal stem cells. With respect to mesenchymal stem cells, they have the capacity to differentiate into mesenchymal cell lineages, including osteoblasts and adipocytes when cultured under appropriate conditions (da Silva Meirelles et al. 2006; da Silva Meirelles et al. 2009). We found that the cells we isolated from mice colons were able to differentiate into both osteoblast cells and adipocytes. After culturing in osteogenic and adipogenic medium for approximately 1 month, significant calcium and lipid depositions were observed under microscope, as indicated by staining with Alizarin Red S and Oil Red O, respectively (Fig. 4b). This result well confirmed their stemness property as for pluripotent cells.

Altogether, our findings demonstrate that the cells isolated from mice colons are putative mesenchymal stem cells as previously reported (Walker et al. 2010).

Discussion

Mesenchymal stem cells have been reported to exist in almost all post-natal tissues, and have been isolated from many tissues (da Silva Meirelles et al. 2006). However, there have been no procedures in detail to isolate and characterize putative MSCs from mammalian intestinal tract.

In this paper, we aimed to establish a reliable and detailed method to better isolate tissue-specific mesenchymal stem cells from mammalian colons with relatively high purity. Our findings showed that those fibroblast-like cells we isolated co-expressed CD29, CD44, and COX-2, which were concordant with previous studies in both morphological and phenotypic profiles (Walker et al. 2010). We also found that these cells extracted from mice colons did not express CD31 and CD34 (data not shown). Moreover, these cells exhibited extensive differentiation capability in vitro. This protocol will help to better explore biological role of cMSCs in the gastrointestinal tract, as well as their effect on cancer progression and therapeutics.

Currently, it remains elusive to prospectively identify MSCs due to a lack of specific markers. It has been shown that several potential stem cell markers, including Lgr5 (Barker et al. 2007), Bmi1 (Sangiorgi and Capecchi 2008), and Lrig1 (Powell et al. 2012), are found expressed in the intestine and play essential parts in regulating intestinal stem cell functions. Thus, further investigation should focus on characterizing the isolated cells in more details.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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