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Stemness distinctions between the ectomesenchymal stem cells from neonatal and adult mice

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ABSTRACT

Ectomesenchymal stem cells (EMSCs), a type of adult stem cells derived from cranial neural crest, can be non-invasively harvested from respiratory mucosa and play vital roles in therapies based on their stemness. However, whether donor age has any impact on the stemness of EMSCs remains elusive and is essential for EMSCs-based therapies. To address this, we first cultivated EMSCs from neonatal mice aged 1 week and adult mice aged 3 months or 6 months, and then compared their morphology, proliferative capacity, and pluripotency through various induced differentiation assays. The results showed that neonatal EMSCs were fibroblast-like, more regular compared to adult EMSCs; the proliferative capacity of neonatal EMSCs was higher than that of adult EMSCs. More importantly, after neural, adipogenic, chondrogenic, and osteogenic differentiation, neonatal EMSCs differentiated into respective cell types significantly better than adult EMSCs. Notably, EMSCs from mice aged 3 months differentiated into mesodermal lineages better than those from 6 months old mice after induction. Collectively, these results suggest donor ages have significant impact on the EMSCs from respiratory mucosa.

1. Introduction

Stem cell transplantation and tissue engineering based on stem cells (SCs) are promising strategies to alleviate or even cure traumatic lesions and degenerative diseases such as spinal cord injury, Parkinson's disease, and diabetes. Stemness or pluripotency is one intrinsic and essential property of stem cells, which implies the capacity of stem cells to generate several types of terminal differentiated cells (Ho et al., 2017). Quite evidently, if cells with stronger stemness are chosen, better plasticity and adaptation to the application will ensue. The three major sources of SCs in vertebrates are embryonic, adult, and induced pluripotent stem cells. Recently, the adult SCs have attracted increasing attention because of the gradual appreciation of their advantages over the other two types of SCs, especially fewer ethical and safety concerns (Sugimura et al., 2017).

The cells residing in the lamina propria of cranial mucosa have been recognized as derivatives of cranial neural crest cells (CNCCs) and have been named as ectomesenchymal stem cells (EMSCs) (Lin et al., 2006). Even in adults, EMSCs still retain some essential characteristics of CNCCs. For instance, the EMSCs isolated from the lamina propria of respiratory mucosa still express nestin, human natural killer-1 (HNK-1), $p75^{NTR}$, all of which are markers for CNCCs (Chen et al., 2015). Moreover, EMSCs have the potential to differentiate into neural lineage, adipocytes, chondrocytes, and osteocytes, confirming their roles as precursors to the ectoderm and mesoderm derivatives (Zhang et al., 2015). Hence, the strong stemness of EMSCs has vast implications for both theoretical and practical researches (Liu et al., 2013).

Several lines of evidences indicate that the gradual decrease of the stemness of adult SCs intertwines with the senescence of the whole body (Asumda and Chase, 2011; Choumerianou et al., 2010; Gharibi et al., 2014; Bressan et al., 2012). Recently, Zhao et al. also discovered that nucleus pulposus mesenchymal stem cells (NPMSCs) from old rats displayed senescent features (Zhao et al., 2017). Thus, aging might have some impact on the stemness of adult SCs. Following this thoughts, we hypothesize that the stemness of the bulk population of EMSCs derived from lamina propria of cranial mucosa are different between neonatal and adult mice. Here, we cultured EMSCs from mice aged 1 week, 3 months, and 6 months and evaluated the expression levels of stemness markers. After that, the 3 different cell populations were subjected to various inducing culture media to differentiate into neurons, chondrocytes, adipocytes and osteocytes. The results demonstrated the distinction of stemness of EMSCs from neonatal and adult mice.

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2. Materials and methods

2.1. Cultures and characterization of EMSCs

The C57BL mice were purchased from Jiangsu experimental animal center, and all the animal procedures complied with the guidelines issued by the Jiangsu University Animal Care and Ethics Committee (protocol number 2016-08-07). The mouse respiratory mucosa was dissected and minced into pieces (about 1 mm^3) which were subjected to explant culture method in plastic flasks in an atmosphere of 5% CO_2 at 37 °C. After the cells migrated from the tissue and grew into confluency of more than 90%, the cells were passaged and designated as the first generation. After two consecutive passages, the cultured cells of the third generation gradually acquired universal morphology and were used to perform all of the following assays. During the growth of these cells, their morphology was recorded with an inverted microscope (Zeiss Axio Observer; GmbH, Germany).

Immunofluorescence staining was used to characterize the cells from mice of different ages. Briefly, cells were fixed with 4% paraformaldehyde at 4 °C for 12 h. After two times rinses with PBS, the cells were treated with 0.3% Triton X-100 dissolved in PBS and then blocked with bovine serum. The primary antibodies including CD133 (rabbit polyclonal, BA3993-2, Boster; Wuhan, China), HNK-1 (mouse monoclonal, BM0325, Boster), nestin (rabbit polyclonal, ab27952, Abcam; San Francisco, USA), vimentin (Rabbit polyclonal, PB0378, Boster), oct-4 (rabbit polyclonal, sc-9081, Santa Cruz; California, USA) were diluted in PBS containing 1% (w/v) BSA and 0.3% (v/v) Triton X-100 and then incubated the cells overnight. After two washes, cells were incubated with the corresponding Cy3-conjugated secondary antibodies at 37 °C for 1 h. The same procedure was followed to prepare the negative controls except with no use of primary antibody. Finally, the fluorescence images were captured using the Zeiss Axio Observer. To ensure the quantitative comparability of captured images, the variables including illumination intensity, apertures, exposure time and gain size were fixed. The illumination intensity was adjusted to maximum; the exposure time was fixed at 100 ms; the gain parameter in the software was set as one.

To quantitatively compare the expression of markers for EMSCs and stemness, Western blotting was used. First, the cells of third generation were cultured in six wells plates. When the cells became fully confluent, they were washed several times following the aspiration of culture medium. Then these cells were lysed with a RIPA buffer containing proteinase inhibitor cocktail, EDTA, and PMSF (Solarbio; Beijing, China). The solution were moved into a centrifuge tube and mixed with SDS-PAGE loading buffer containing 2-mercaptoethanol. The samples were subjected to electrophoresis and then transferred to PVDF membranes. After blocked with BSA, the membranes were incubated with primary antibodies. The membranes were washed several times with TBS-T solution and then were incubated with the corresponding HRP-conjugated secondary antibodies. Finally, the signals were detected using an ECL kit (WBLUR0500, Millipore; MA, USA), and the pictures were captured with an imaging system (Sage Imaging System; Beijing, China). The images were analyzed using ImageJ software (version 1.51k, NIH, USA) and the expression level of every marker was normalized with β-actin. Four replicates were performed to statistically analyze the effect of age.

2.2. Proliferation of EMSCs in vitro

Ki-67 was chosen as the proliferation index to evaluate the growth of EMSCs. The EMSCs derived from mice of different ages were cultured in a 24-well plate, and then were used to perform immunofluorescent staining for Ki-67 (rabbit polyclonal, PA5-19462, Pierce, IL, USA) following the procedures mentioned above. In addition, Hoechst 33342 (H1399, Invitrogen, USA) was used to counterstain the nuclei. The pictures were captured and then analyzed with ImageJ to calculate the percentage of Ki-67 positive cells. Three replicative wells were performed, and five visual fields were calculated in each well.

Cell Counting Kit-8 (CCK-8, water soluble tetrazolium salt; Dojindo; Shanghai, China) was adopted to quantify EMSCs proliferative capacity according to the manufacturer's instruction. In short, EMSCs were seeded in 96-well plates at the same density and were cultured for 3, 6, 9 or 12 days. An aliquot of CCK-8 reagent was added to each well followed by incubation for 4 h at 37 °C. Finally, the absorbance of each well was measured at 450 nm with a spectrophotometer (μQuant, Biotek, VT, USA). Five replicative wells were performed.

2.3. Neurogenic differentiation assay

The neuronal differentiation assay was performed following our previous work (Zhang et al., 2015). Concisely, EMSCs derived from mice of various ages were seeded in 24-well or 6-well plates to be cultured in neurogenic medium which was DMEM/F-12 supplemented with 10% FBS (10270-106, Gibco), 2% B27 (17504-044, Gibco), 1 μg/ml ATRA (Merck, Germany), 50 ng/ml sonic hedgehog (Peprotech, USA), and 50 ng/ml BDNF (Peprotech) for 2 weeks. During the induction period, the medium was replaced every 3 days. At the endpoint, the cells in 24-well plates were fixed with 4% paraformaldehyde and were subjected to immunofluorescent staining with antibodies to neurofilament-200 (NF-200, mouse monoclonal, BM0100, Boster) and Tuj-1 (rabbit polyclonal, A01857-1, Boster). Meanwhile, the cells in 6-well were used to perform Western blotting with the same antibodies. The results were analyzed with ImageJ. Four replicates were performed.

2.4. Adipogenic differentiation

EMSCs were induced to differentiate into adipocytes following the protocol as previously described (Zhang et al., 2013). In brief, EMSCs were cultured in induction medium for 3 days, the formula of which was DMEM, 10% FBS, 2 mM insulin (I5523, Sigma), 500 mM IBMX, 1 mM dexamethasone, and 200 mM indomethacin (S1723, Selleck, TX, USA). Then the medium was replaced by maintaining medium formed by DMEM, 10% FBS, and 2 mM insulin for 1 day. These procedures composed of 3 days of induction medium and 1 day of maintaining medium were regarded as one inducing cycle. After three repeats of inducing cycle, adipocyte-like cells were observed and recorded with a phase contrast microscope. Two extra inducing cycles were applied to get more prominent effects before the Oil Red O (Solarbio) staining which aimed to demonstrate lipid droplets in these cells.

As to Oil Red O staining, the cells were fixed with 4% paraformaldehyde for 1 h prior to their soaking in 3 mg/ml Oil-red O in 60% isopropanol for 20 min. After washed with 60% isopropanol and pure water, the cells were observed and pictured using a phase contrast microscope.

2.5. Chondrogenic differentiation

For chondrogenic differentiation, EMSCs were cultured in DMEM containing 10% FBS, 10 ng/ml TGF-β1 (Peprotech), 0.1 mM dexamethasone and 1% ITS-supplement (insulin, transferrin, and selenium acid) for 30 days. During this period, the culture media were replaced every 5 days. Finally, immunofluorescent staining and Western blotting were applied to detect the expression of chondrocytes' markers aggrecan (rabbit polyclonal, sc-25674,Santa Cruz) and collagen II (rabbit polyclonal, sc-28887, Santa Cruz) in the induced cells.

2.6. Osteogenic differentiation

EMSCs were cultivated in 24-well plates and were induced to osteocytes by osteogenic medium composed of DMEM, 10% FBS, 0.1 mM dexamethasone, 10 mM β-glycerophosphate disodium, and 0.2 mM L-ascorbic acid 2-phosphate (Solarbio). The medium was replaced every