Cell Biology International ISSN 1065-6995
doi: 10.1002/cbin.11255

RESEARCH ARTICLE

Study of the protective effect on damaged intestinal epithelial cells of rat multilineage-differentiating stress-enduring (Muse) cells

Dong Sun[1], Liu Yang[1,2], Huan Cao[1,3], Zhong-Yang Shen[2,4] and Hong-Li Song[2,5]*

1 Tianjin First Central Hospital Clinic Institute, Tianjin Medical University, Tianjin, 300070, P.R. China
2 Department of Organ Transplantation, Tianjin First Central Hospital, Tianjin, 300192, P.R. China
3 NHC Key Laboratory of Critical Care Medicine, Tianjin, 300192, P.R. China
4 Key Laboratory of Transplant Medicine, Chinese Academy of Medical Sciences, Tianjin, 300192, P.R. China
5 Tianjin Key Laboratory of Organ Transplantation, Tianjin, 300192, P.R. China

Abstract

In this study, we determined whether multilineage-differentiating stress-enduring (Muse) cells exist in rat bone marrow and elucidated their effects on protection against the injury of intestinal epithelial cells associated with inflammation. Rat Muse cells were separated from bone marrow mesenchymal stem cells (BMMSCs) by trypsin-incubation stress. The group of cells maintained the characteristics of BMMSCs; however, there were high positive expression levels of stage-specific embryonic antigen-3 (SSEA-3; 75.6 ± 2.8%) and stage-specific embryonic antigen-1 (SSEA-1; 74.8 ± 3.1%), as well as specific antigens including Nanog, POU class 5 homeobox 1 (OCT 3/4), and SRY-box 2 (SOX 2). After inducing differentiation, α-fetoprotein (endodermal), α-smooth muscle actin and neurofilament medium polypeptide (ectodermal) were positive in Muse cells. Injuries of intestinal epithelial crypt cell-6 (IEC-6) and colorectal adenocarcinoma 2 (Caco-2) cells as models were induced by tumor necrosis factor-α stimulation in vitro. Muse cells exhibited significant protective effects on the proliferation and intestinal barrier structure, the underlying mechanisms of which were related to reduced levels of interleukin-6 (IL-6) and interferon-γ (IFN-γ), and the restoration of transforming growth factor-β (TGF-β) and IL-10 in the inflammation microenvironment. In summary, there were minimal levels of pluripotent stem cells in rat bone marrow, which exhibit similar properties to human Muse cells. Rat Muse cells could provide protection against damage to intestinal epithelial cells depending on their anti-inflammatory and immune regulatory functionality. Their functional impact was more obvious than that of BMMSCs.

Keywords: anti-inflammation; BMMSCs; damaged intestinal epithelial cells; Muse cells; rat; zonula occludens-1

Introduction

In 2010, Dezawa (Wakao et al., 2011) found a unique group of stem cells in human mesenchymal stem cells (MSCs), which were originally identified to be cells resistant to stress following lengthy trypsin incubation, and were positive for both stage-specific embryonic antigen-3 (SSEA-3), the hallmark of human embryonic stem cells (ESCs) and CD105, a human MSCs marker. This group of cells was termed multilineage-differentiating stress-enduring (Muse) cells. Further studies have demonstrated that Muse cells are positive for core pluripotency transcription factors (e.g., Nanog homeobox [NANOG], POU class 5 homeobox 1

*Corresponding author: e-mail: hlsong26@163.com
Zhong-Yang Shen and Hong-Li Song contributed equally to this work.

Abbreviations: AFP, α-fetoprotein; BMMSCs, bone marrow mesenchymal stem cells; Caco-2, colorectal adenocarcinoma 2; IEC-6, intestinal epithelial crypt cell-6; IFN-γ, interferon-gamma; IL, interleukin; Muse cells, multilineage-differentiating stress-enduring cells; NANOG, Nanog homeobox; NEFM, neurofilament medium polypeptide; PCNA, proliferating cell nuclear antigen; POU5F1 or OCT 3/4, POU class 5 homeobox 1; SOX 2, SRY-box 2; SSEA-3, stage-specific embryonic antigen-3; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor α; ZO-1, zonula occludens-1; α-SMA, α-smooth muscle actin

[POU5F1, OCT 3/4], and SRY-box 2 [SOX 2]) and could differentiate from single cells into endoderm, ectoderm, and mesoderm cells (Iseki et al., 2017; Wakao et al., 2018; Wang et al., 2018). Differing from ESCs and induced pluripotent stem cells, Muse cells exhibit safe telomerase activity as they did not form teratoma in immunodeficient mice, thereby eliminating this ethical concern in clinical applications (Dezawa, 2016; Yamauchi et al., 2017; Wakao et al., 2018). The study of Muse cells burgeoned into a new hotspot of stem cell research because of their excellent protective and reparative functions (Perone et al., 2018). Several basic research studies and clinical trials have indicated that Muse cells exhibit better protective and reparative effects than MSCs in studies on various diseases and models (e.g., liver disease [Iseki et al., 2017], nervous system diseases [Uchida et al., 2016], diabetes [Perone et al., 2018], skin regeneration [Hu & Longaker, 2017; Yamauchi et al., 2017], and myocardial infarction [Tanaka et al., 2018]).

Muse cells have been isolated from various tissues of the human body, including the bone marrow (Iseki et al., 2017), umbilical cord (Leng et al., 2019), skin fibroblasts (Uchida et al., 2016), and adipose tissue (Yamauchi et al., 2017). In different species, the presence of Muse cells has also been confirmed in the body of goats (Yang et al., 2013). However, there have been no reports regarding the existence of Muse cells in rats to date. Thus, in the present study, we sought to explore the presence of pluripotent stem cells similar to human Muse cells in rat bone marrow, and identify the associated cellular characteristics.

Studies regarding the immunoregulatory function of Muse cells would also be important to systematically evaluate the clinical application potential and prospects of Muse cells (Gimeno et al., 2017; Perone et al., 2018). Clinically, immune factors have been found to play a critical role in inflammatory bowel disease (IBD) (i.e., ulcerative colitis and Crohn's disease), and both the anti-inflammatory response and immune regulation are vital for the treatment schedule (Shouval & Rufo, 2017). Intestinal damage is often accompanied by the aberrant excretion of diversified inflammatory factors, including tumor necrosis factor-α (TNF-α) (Knipe et al., 2016). The hyperactivated inflammatory environment has been found to be involved in the reduction of intestinal epithelial cell proliferation activity and the destruction of the intestinal barrier structure (Gezginci-Oktayoglu et al., 2016). In this study, the rat small intestinal crypt epithelial cell line, IEC-6, and human colorectal adenocarcinoma cell line, Caco-2, which possess an ideal tight junction barrier structure, were selected as in vitro models of intestinal epithelial cells. Using proinflammatory recombinant TNF-α, we established models of inflammatory intestinal epithelial cell injury (Cao et al., 2017; Yin et al., 2019). The protective effects of Muse cells and bone marrow mesenchymal stem cells (BMMSCs) on damaged intestinal epithelial cells were evaluated.

Materials and methods

Isolation and culture of rat BMMSCs and Muse cells

BMMSCs were sterilely obtained from the bone marrow of the femur and tibia of Sprague–Dawley rats (male; weight range: 60–80 g). Sprague–Dawley rats were provided by the China National Institutes for Food and Drug Control (Beijing, China). Animal feeding and experimental procedures strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and were approved by the Ethics Committee of Tianjin First Central Hospital (Tianjin, China). BMMSCs were cultured in DMEM/F12 (Gibco, Grand Island, USA) until the third generation and then trypsinized in Trypsin-EDTA Solution (Gibco) over an 8-h time period. Fetal bovine serum (FBS; Biowest, Nuaillé, France) was added to neutralize the trypsin. The obtained suspended cells were centrifuged for 15 min at 740*g*. Rat Muse cells were resuspended in wells coated with poly-2-hydroxyethyl methacrylate (poly-HEMA; Sigma, USA; 12 mg/mL in 90% ethanol). Alternate suspended and adherent cultivation was used to obtain stable passages.

Flow cytometry (FCM)

Rat BMMSCs and Muse cells were incubated with the desired antibodies (e.g., anti-CD29, anti-CD90, anti-RT1A, anti-CD34, anti-CD45, anti-RT1B, anti-SSEA-3 [BioLegend, San Diego, USA], and anti-SSEA-1, [STEMCELL Technologies, Vancouver, BC, Canada]). Edu proliferation (RiboBio, Guangzhou, China) and Annexin V-FITC/PI apoptosis (KeyGen BioTECH, Nanjing, China) detection kits were used to determine the cellular proliferation and apoptosis rates following the manufacturer's guidelines.

Induction of adipogenic and osteogenic differentiation of Muse cells in vitro

The formula of the induction medium and the induction protocol are based on the manuals documented in our previous study (Liu et al., 2014). Orange-red lipid droplets were observed after oil red O staining in the cells, and deposited calcium salt was observed as a black substance after Von Kossa staining.

Immunofluorescence (IF)

The cells on plates were immobilized by acetone, permeabilized in phosphate-buffered saline (PBS)-Triton X buffer, and

Table 1 Polymerase chain reaction (PCR) primer sequences.

Genes	Primer sequence (5′–3′)	Product size (bp)
NANOG (F)	AGGACGAGACAGAAGGATCACC	216
NANOG (R)	AGAAGCCTCTTGGCGGAAC	
OCT 3/4 (F)	CAGGCAGGAGCACGAGTGG	265
OCT 3/4 (R)	CGAAGCGGCAGATGGTTGTC	
SOX 2 (F)	GTTACCTCTTCCTCCCACTCC	255
SOX 2 (R)	CCTCCCAATTCCCTTGTATC	
AFP (F)	TGAAACGCCATCGAAATGC	148
AFP (R)	CGTGTAGCCAATGAGGAACAG	
α-SMA (F)	AAGTATCCGATAGAACACG	296
α-SMA (R)	TAGATAGGCACGTTGTGAG	
NEFM (F)	AAAGCCCGCAAGAATCCAAG	240
NEFM (R)	TAGCACCATCGCCTCCCTC	
IL-6 (F)	GCGATGGAGTCAGAGGAAAC	204
IL-6 (R)	TGAGGCTAGCGCTAAGAAGC	
IFN-γ (F)	ACAACCCACAGATCCAGC	283
IFN-γ (R)	TCAGCACCGACTCCTTTT	
TGF-β (F)	CCAAGGAGACGGAATACAGG	107
TGF-β (R)	CTCGACGTTTGGGACTGATC	
IL-10 (F)	CACTGCTATGTTGCCTGCTC	105
IL-10 (R)	TGTGGGTCTGGCTGACTGG	
β-Actin (F)	GCGTGACATTAAAGAGAAGCTG	500
β-Actin (R)	AGAAGCATTTGCGGTGCAC	

contained a 5% goat serum protein solution. The cells were then incubated in dilution buffers containing different primary antibodies at 4°C as recommended, after which they were observed and photographed (Cao et al., 2017).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total messenger RNA (mRNA) was isolated from the cells using TRIzol (Thermo Fisher Scientific, MA, USA). Reverse-transcription and amplification were performed strictly following the product manuals (Yin et al., 2019). The parameters for reverse-transcription were established as 37°C for 20 min and 85°C for 5 s; and for amplification as 95°C for 10 s, 58°C for 20 s, and 72°C for 30 s for 45 cycles. The primers were synthesized by AuGCT Inc. (Beijing, China) with sequences shown in Table 1.

Western blotting

The total cellular proteins were extracted using RIPA Lysis Buffer (Solarbio), electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), labeled with target antibodies, including anti-NANOG (Novus Biologicals, CO, USA), anti-OCT 3/4, anti-AFP, anti-ZO-1 (Invitrogen Antibodies, CA, USA), anti-SOX 2, anti-α-SMA, anti-NEFM, anti-PCNA, anti-Occluding, and anti-β-actin (Proteintech Group, IL, USA) that were detected with ECL Prime Western Blotting Detection Reagent. The relative level of protein expression was calculated based on that of β-actin as internal control.

Damaged intestinal epithelial cell injury model caused by TNF-α and protection models using stem cells

The intestinal epithelial cell lines IEC-6 and Caco-2 were obtained from the Cell Bank of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and cultured in 10% FBS + 90% RPMI1640 (Gibco). The cells were seeded into a six-well plate at a density of 1×10^6 cells/well, and 100 ng/mL recombinant TNF-α (PeproTech, Rocky Hill, NJ, USA) were added for 48-h to simulate an in vitro model of intestinal epithelial cell damage. When detecting the protective ability of the stem cells, 5×10^6/well lymphocytes and 5×10^5/well stem cells were added and co-cultured with IEC-6 or Caco-2 for 24 h (Yin et al., 2017). According to the experimental design, the cells were divided into four groups (i) control group; (ii) TNF-α group; (iii) TNF-α/BMMSCs group; and (iv) TNF-α/Muse cell group. The co-culture system preventing cell-to-cell contact used a 0.8-μm Transwell assay (Corning, NY, USA). Experiments were performed three or more times independently.

Cell counting kit-8 (CCK-8)

CCK-8 detection reagent (BestBio, Shanghai, China) was added following the manufacturer's protocol. The optical density (OD) values were detected using a Multilabel Plate Reader (Yin et al., 2019).

Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatant of each group was collected after 24 h. The concentration of cytokines, including interleukin-6 (IL-6), interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), and IL-10 (R&D Systems, MN, USA) were measured using a commercial ELISA kit according to the manufacturer's protocol.

Statistical analysis

Quantitative data are expressed as the mean ± standard deviation (SD). Categorical variables were tested with a χ^2 test or Fisher's exact probability test, and the quantitative data were statistically analyzed using Student's t test. Statistical tests were performed with the SPSS statistical software package (version 21.0; SPSS Inc., USA) and Graphpad Prism statistical software package (version 5.01; Graphpad Software Inc, USA), with $P < 0.05$ representing statistically significant differences.

Results

Isolation and morphological observations of rat BMMSCs and Muse cells

Rat BMMSCs were observed as the long-shuttle type and displayed the ability to differentiate into adipogenic and osteogenic cells, indicating BMMSCs were successfully isolated and passaged (Figure 1A). After an 8-h trypsin incubation, ~16.50 ± 2.01% rat BMMSCs maintained normal morphologies and intact cell membranes. The wells with single-cell culture displaying characteristics presented by M-cluster accounted for ~12.50 ± 2.43% of the total wells (2.03 ± 0.14% of total BMMSCs). After passaging to the second-generation culture, the rat Muse cells were adherent and presented as the long-shuttle type (Figure 1B).

Identification of the basic characteristics of rat Muse cells

FCM results showed the presence of CD29, CD90, and RT1A as positive markers, while the absence of CD34, CD45, and RT1B negative markers was observed in both rat Muse cells and BMMSCs (Figures 1A and 1B). However, in contrast to BMMSCs, the level of SSEA-3 and SSEA-1 expression was significantly increased and the rates reached 75.6 ± 2.8% and 74.8 ± 3.1%, compared with 2.3 ± 0.3% and 2.1 ± 0.2% in BMMSCs ($P < 0.001$) (Figure 1 and Figure S1). In addition, 77.62 ± 5.3% of SSEA-1(+) cells expressed SSEA-3 (Figure 1B b9).

Ability for pluripotent differentiation and differentiation into three germ layers of Rat Muse cells

The IF assay showed that pluripotent stem cell markers including NANOG, OCT 3/4 and SOX 2 were expressed and detected as positive signals in the rat Muse cells (Figure 2A a1-3). The qRT-PCR and western blotting results showed that the level of mRNA and protein expression was significantly higher in Muse cells than in BMMSCs ($P < 0.05$) (Figure 2A a4-6). Gene markers of the capacity for germ layer differentiation, including α-fetoprotein (AFP, for the endoderm), α-smooth muscle actin (α-SMA, for the mesoderm), and neurofilament medium polypeptide (NEFM, for the ectoderm) similarly manifested ($P < 0.05$) (Figure 2B) after inducing differentiation using a specific medium.

Protective effect of Muse cells on IEC-6 and Caco-2 in direct contact co-culture

The CCK-8 and Edu assay showed that the cell viability and proliferation rates of IEC-6 and Caco-2 cells significantly decreased after the addition of exogenous TNF-α ($P < 0.001$) (Figures 3A and 3B), and the level of proteins, including proliferating cell nuclear antigen

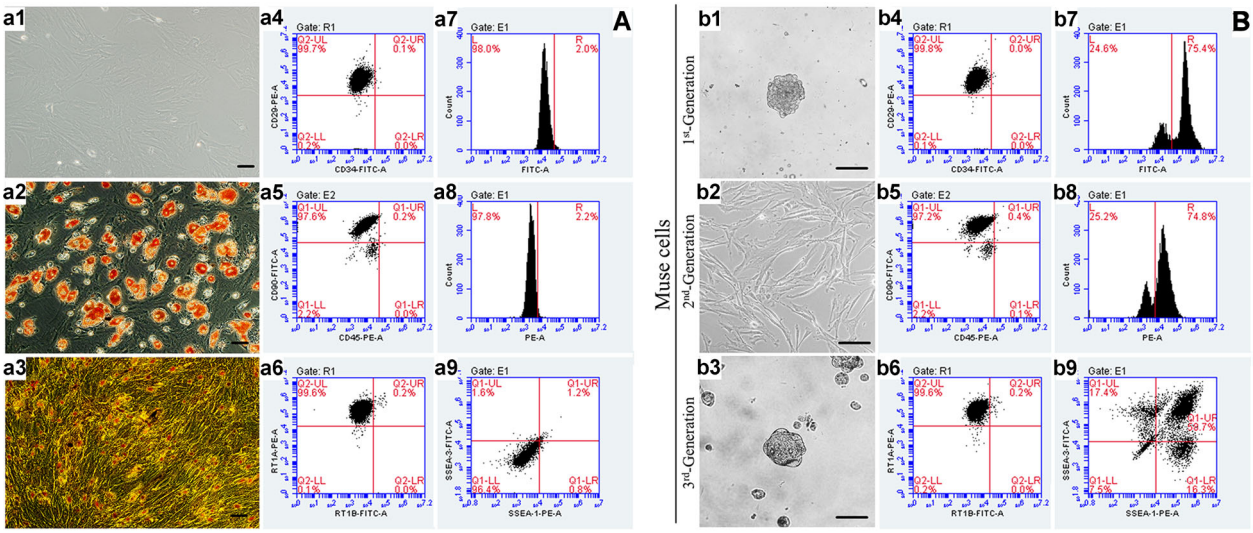

Figure 1 The morphologies and characteristics of rat bone marrow mesenchymal stem cells (BMMSCs) and multilineage-differentiating stress-enduring (Muse cells). (A) The morphology (a1) of BMMSCs lipoblasts (a2) and osteoblasts (a3) differentiated from BMMSCs were observed by microscopy. BMMSCs were positive for CD29 (a4), CD90 (a5), and RT1A (a6) and negative for CD34 (a4), CD45 (a5), RT1B (a6), SSEA-3 (a7) and SSEA-1 (a8) detected by flow cytometry (FCM). (B) Muse cells formed the Muse-cell-clusters (M-clusters) in suspension cultivation and long-shuttle types in adherent cultivation (b1–b3). Rat Muse cells were positive for CD29 (b4), CD90 (b5), and RT1A (b6) and negative for CD34 (b4), CD45 (b5), RT1B (b6) similar to BMMSCs, but positively expressed SSEA-3 (75.6 ± 2.8% vs. 2.3 ± 0.3%, b7) SSEA-1 (74.8 ± 3.1% vs. 2.1 ± 0.2%). In addition, 77.62 ± 5.3% of SSEA-1 (+) cells expressed SSEA-3 (b9). Scale bars = 50 μm.

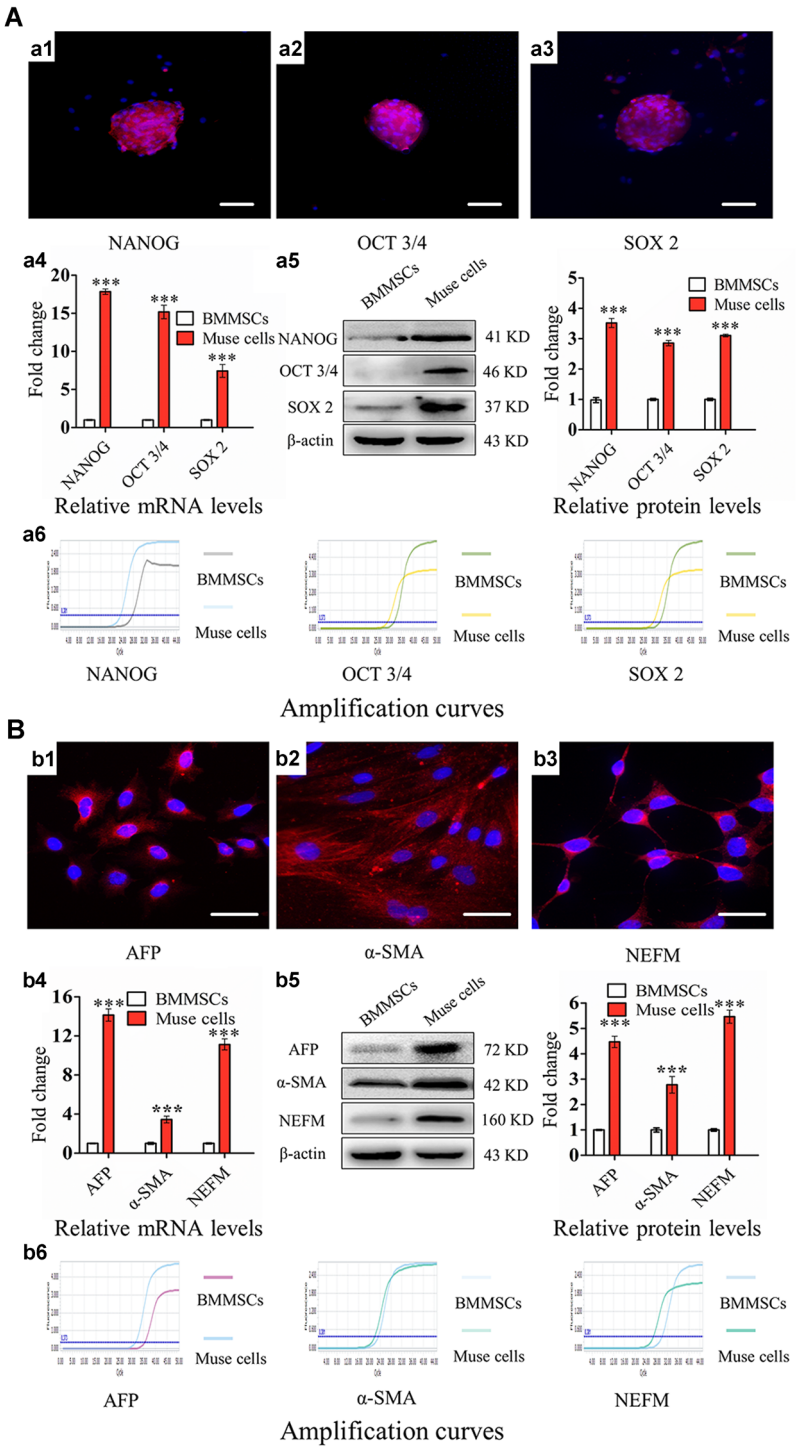

Figure 2 Rat multilineage-differentiating stress-enduring (Muse) cells were positive for pluripotent differentiation markers and differentiated into three lineages. (A) Muse cells were positive for NANOG, OCT 3/4, and SOX 2 determined by immunofluorescence (a1-a3), quantitative real-time polymerase chain reaction (qRT-PCR) (a4), and western blotting (a5). (B) After inducing differentiation, the Muse cells were positive for α-fetoprotein (AFP) (endodermal, b1), α-smooth muscle actin (α-SMA) (mesodermal, b2), and neurofilament medium polypeptide (NEFM) (ectodermal, b3). The amplification plots (a6, b6) are shown in the LightCycler Application (Roche, Zug, Switzerland). Mean ± standard deviation (SD); $***P < 0.001$; Scale bars = 50 μm.

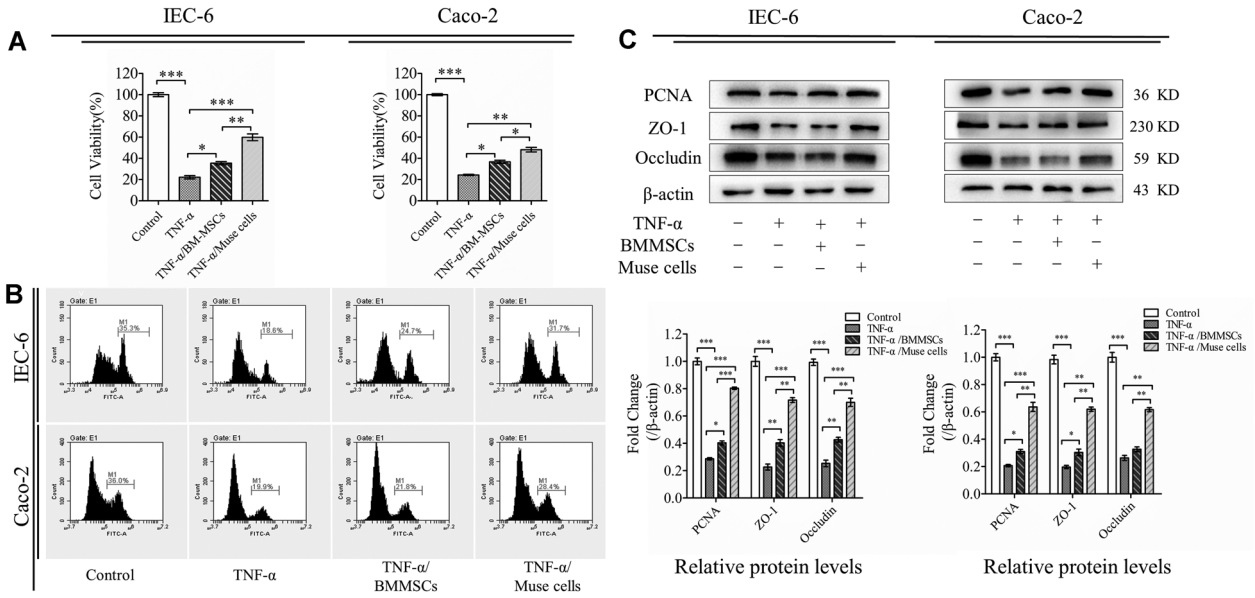

Figure 3 Rat multilineage-differentiating stress-enduring (Muse) cells protected the tumor necrosis factor α (TNF-α)-damaged intestinal epithelial crypt cell-6 (IEC-6) and colorectal adenocarcinoma 2 (Caco-2) intestinal epithelial cells. (A-B) Bone marrow mesenchymal stem cell (BMMSCs) and Muse cells were co-cultured with TNF-α-damaged IEC-6 and Caco-2 cells for 24 h to measure the protective effect of the stem cells, respectively. The cell viability of IEC and Caco-2 cells was determined with a cell counting kit-8 (CCK-8)-based cell viability assay (A), and proliferation was determined by an EdU Kit (B). (C) Western blotting analysis and quantitative analysis were used to detect the expression of proliferating cell nuclear antigen (PCNA), zonula occludens-1 (ZO-1), and Occludin. β-Actin served as an internal control. Mean ± standard deviation (SD). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

(PCNA), zonula occludens-1 (ZO-1), and Occludin were significantly decreased ($P < 0.05$) (Figure 3C), which demonstrated that an intestinal epithelial cell injury model had been well-established. After the supplementation of BMMSCs, the proliferation rates of the cells recovered ($P < 0.05$), and the molecular expression levels were rescued. In the Muse cell group, the protective effect was more apparent than that in the BMMSCs group ($P < 0.05$) (Figure 3).

Reparative effect of Muse cells on IEC-6 and Caco-2 in co-culture in Transwell chambers

When Transwell chambers were used, we found that BMMSCs and Muse cells displayed similar protective effects (Figures 4A–C). The levels of PCNA proteins in IEC-6 and Caco-2 cells were significantly decreased in the injured group. In the BMMSCs and Muse cells groups, PCNA expression was restored and the PCNA proteins were transported from the cytoplasm to the nucleus (Figure 4D). When labeling ZO-1 and determining the expression levels in IF assays, Muse cells were found to restore the level of ZO-1 expression in the cells and protect the broken structure (Figure 4D), indicating that the intestinal barrier structure can be protected by Muse cells; the effect was greater than that of BMMSCs.

Muse cells improved the inflammatory microenvironment

The ELISA assay showed that levels of inflammatory cytokines (e.g., IL-6 and IFN-γ) were reduced while the levels of anti-inflammatory cytokines (TGF-β and IL-10) ($P < 0.01$) were increased in Muse cells ($P < 0.01$) compared with BMMSCs ($P < 0.05$) (Figure 5A). Further studies showed that the rate of Muse cell apoptosis in the inflammatory environment was significantly lower than that of the BMMSCs (23.6 ± 2.3% vs. 43.3 ± 3.9%; $P < 0.01$) (Figure 5B). In addition, qRT-PCR results showed that the level of TGF-β and IL-10 mRNA in Muse cells within the inflammatory environment was significantly higher than that in BMMSCs (3.75- and 2.61-fold changes, respectively; $P < 0.01$) (Figure 5C).

Discussion

The discovery of Muse cells is perceived as a milestone in the field of stem cell research, providing a broad perspective for cell therapy (Young, 2018). In referring to the protocol for the collection of human Muse cells (Wakao et al., 2011), rat Muse cells were separated from BMMSCs via applied stress stimulation, which is dependent on the anti-stress response of Muse cells to ensure that this group of cells survives whereas other cells are lysed. Rat BMMSCs

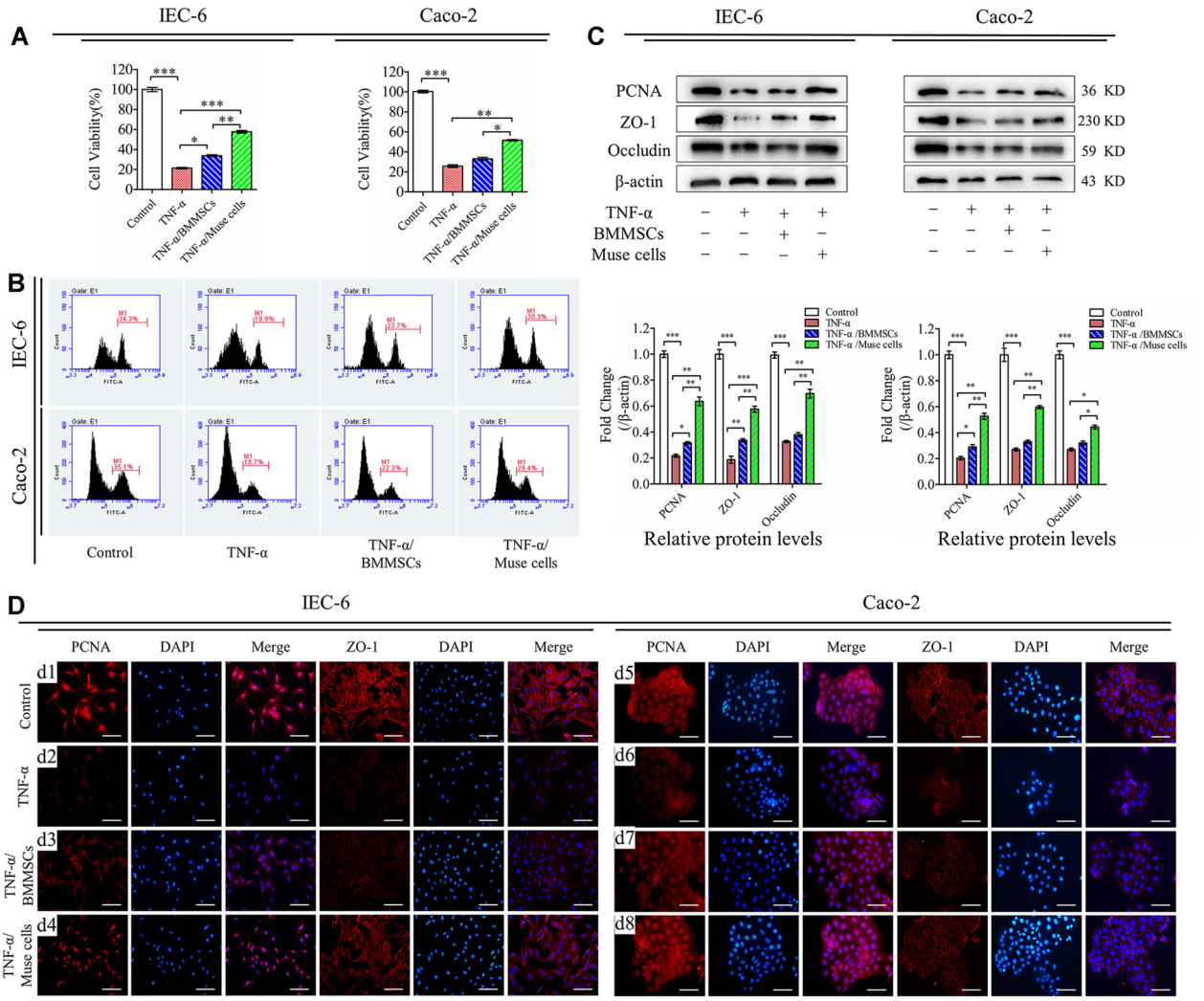

Figure 4 Rat multilineage-differentiating stress-enduring (Muse) cells protected the tumor necrosis factor α (TNF-α)-damaged intestinal epithelial cells in a Transwell chamber, intestinal epithelial crypt cell-6 (IEC-6) and colorectal adenocarcinoma 2 (Caco-2). (A-B) Bone marrow mesenchymal stem cells (BMMSCs) and Muse cells were co-cultured with TNF-α-damaged IEC-6 and Caco-2 cells for 24 h to measure the reparative effect of stem cells with a Transwell chamber, respectively. Cell viability of IEC and Caco-2 cells was determined by a cell counting kit-8 (CCK-8)-based cell viability assay (A), and proliferation was determined using an EdU Kit (B). (C) Western blotting analysis and quantitative analysis were used to detect the level of proliferating cell nuclear antigen (PCNA), zonula occludens-1 (ZO-1), and Occludin expression. β-Actin served as an internal control. (D) PCNA was labeled for cell proliferation and ZO-1 labeled for intestinal barrier structure by immunofluorescence. The groups were presented as follows: Control (d1, d5), TNF-α (d2, d6), TNF-α/BMMSCs (d3, d7), and TNF-α/Muse cells (d4, d8). DAPI: Dihydrochloride. Mean ± standard deviation (SD). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Scale bars = 50 μm.

were incubated in trypsin for 8-h to eliminate as many other cellular components as possible while maintaining the health of the target Muse cells. Under stress and undergoing single-cell culture, ~2.03% of rat BMMSCs could form Muse cell clusters and be stably passaged, a value close to that for human BMMSCs (1.9%) (Wakao et al., 2014). Further study showed that the Muse cells retained the classical molecular markers of BMMSCs, as well as the capacity for differentiation into osteogenic and adipogenic cells (Yin et al., 2017). Dezawa (Wakao et al., 2011) was the first to discover that the ratio of SSEA-3-positive cells was increased after long-term incubation with trypsin, and described this as the characteristic marker of Muse cells. Liu et al. (2016) identified SSEA-3 (+) in Muse cells following long-term incubation with trypsin by IF. The proportion of SSEA-3 (+) positive cells in Muse cells was reported to be ~90% (Heneidi et al., 2013) and 57.7 ± 11.8% (Gimeno et al., 2017). In the present study, the proportion was ~75.6%, which further proved that the cells we isolated could be considered Muse cells. Interestingly,

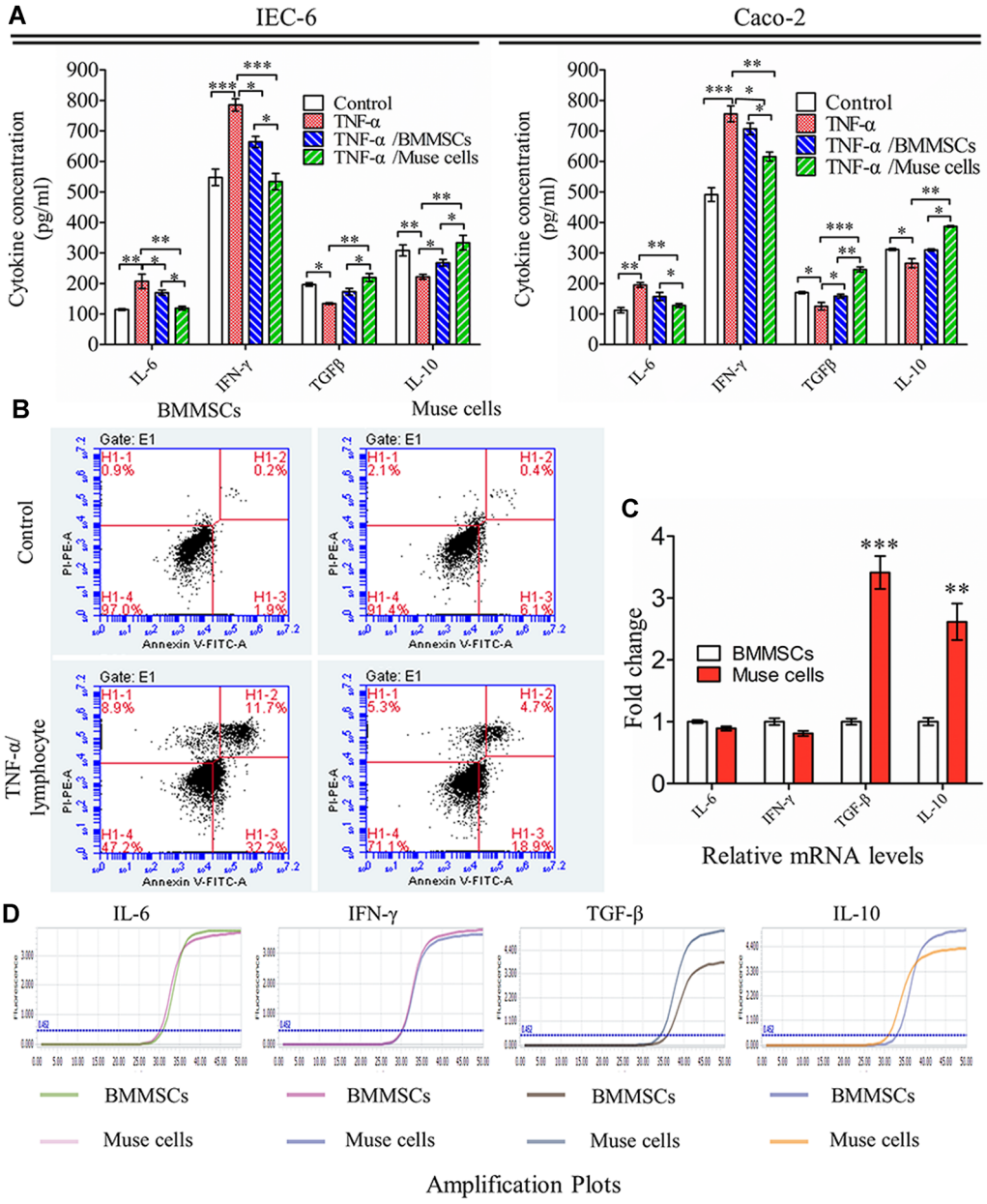

Figure 5 Rat multilineage-differentiating stress-enduring (Muse) cells demonstrated the anti-inflammatory ability for protecting damaged intestinal epithelial crypt cell-6 (IEC-6) and colorectal adenocarcinoma 2 (Caco-2) intestinal epithelial cells. (A) Bone marrow mesenchymal stem cell (BMMSCs) and Muse cells were co-cultured with tumor necrosis factor α (TNF-α)-damaged IEC-6 and Caco-2 cells for 24 h. The concentration of the proinflammatory cytokines (interleukin-6 [IL-6] and interferon-γ [IFN-γ]) and anti-inflammatory cytokines (e.g., transforming growth factor-β (TGF-β) and IL-10) were determined by enzyme-linked immunosorbent assay (ELISA). (B) Apoptosis of BMMSCs and Muse cells was tested with an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection Kit. (C) The level of IL-6, IFN-γ, TGF-β, IL-10 messenger RNA (mRNA) expression of BMMSCs and Muse cells was measured and normalized to that of β-actin. (D) The amplification plots are shown in the LightCycler Application. Mean ± standard deviation (SD). $*P < 0.05$, $**P < 0.01$; $***P < 0.001$.

our study also indicated that the ratio of SSEA-1 (+) cells was increased after long-term incubation in trypsin. Li et al. (2017) termed SSEA-1 (+) cells in pigs as SSEA-1-expressing enhanced reprogramming (SSER) cells, and such SSER cells were considered to be different from Muse cells as they were negative for SSEA-3. However, in our study, ~78% of rat SSEA-1 (+) cells expressed SSEA-3. The difference between the two types of cellular patterns has not been studied systematically and will be a future research direction in our laboratory. In addition, the pluripotent

differentiation ability of Muse cells is the decisive factor for distinguishing Muse cells from MSCs (Dezawa, 2016; Toyoda et al., 2019). Our results showed that rat Muse cells were positive for pluripotent stem cell markers. Similar results were observed in the determination of AFP, α-SMA, and NEFM, which are used for the identification of Muse cells (Gimeno et al., 2017; Wakao et al., 2018). In summary, our study found the presence of cells in the bone marrow of rats that have similar characteristics to human Muse cells, and this portion of cells expressed SSEA-3 and SSEA-1 at high levels, as well as other pluripotent stem cell markers. However, we have currently only completed preliminary studies on rat Muse cells. Thus, it appears to be more appropriate to temporarily use the term "rat Muse-like cells". Advanced sorting methods and complete identification evidence will be the focus of our work in further studies.

IEC-6 and Caco-2 injured by TNF-α represent classic in vitro cell models for damaged intestinal epithelial cells in an inflammatory environment (Cao et al., 2017; Yin et al., 2019). We found that the proliferation of intestinal epithelial cells was restored after co-culturing with Muse cells, and the levels of expression of the tight junction proteins, ZO-1 and Occludin were upregulated. The effects in intestinal epithelial cells co-cultured with Muse cells were more obvious than those in epithelial cells co-cultured with BMMSCs (Mahmood et al., 2019). These results suggested that Muse cells have more positive effects on the protection of epithelial cells. The release of inflammatory factors during intestinal injury is a serious role obstacle to the repair of intestinal epithelial cells (Knipe et al., 2016). To further clarify the role of the immune microenvironment during damage, Transwell chambers were used to inhibit cell-to-cell contact between the stem cells and intestinal epithelial cells during co-culture (Skottke et al., 2019). The results of these experiments showed that the protective effects associated with the Muse cell co-culture remained significant. ZO-1 labeling showed that the tight junction structure among intestinal epithelial cells was also restored. This indicates that Muse cells may play a protective role by improving immune microenvironment.

Previous studies have confirmed that Muse cells have immunoregulatory functions by the secretion of anti-inflammatory cytokines (e.g., TGF-β, IL-10, and complement C3) and antagonizing macrophages or T lymphocytes to secrete proinflammatory factors, which was more pronounced than that of MSCs and other component cells (Gimeno et al., 2017; Yabuki et al., 2018). This mechanism plays a fundamental role in the effective treatment of various diseases, including ischemia-reperfusion injury post lung transplantation (Yabuki et al., 2018), type I diabetes (Perone et al., 2018), and aortic aneurysm expansion (Hosoyama & Saiki, 2018). In our study, the level of some classical inflammatory-related cytokines was measured in the medium of the co-culture system comprised of Muse cells, BMMSCs, and intestinal epithelial cells (Gimeno et al., 2017; Yin et al., 2017). By detecting the levels of IL-6, IFN-γ, TGF-β, and IL-10, we found that the in the supernatant, the concentration of proinflammatory cytokines IL-6 and IFN-γ was lower, while the level of the anti-inflammatory cytokines TGF-β and IL-6, was significantly increased. In addition, the effect of co-culture with Muse cells was significantly greater than that for BMMSCs. This indicates that Muse cells could provide a more favorable immune microenvironment during the process of protecting intestinal epithelial cells.

The survival and colonization rate of BMMSCs is poor in complex inflammatory conditions, which is a critical limitation of their clinical application (Shen et al., 2017). In contrast, the antiapoptotic capacity of Muse cells is significantly improved, which is crucial for their stable anti-inflammatory and immunomodulatory functions. In addition, qRT-PCR results demonstrated that Muse cells exhibited a greater capacity to secrete TGF-β and IL-10 compared with BMMSCs, which can directly improve the intestinal inflammatory environment. The reason for such decreased IL-6 and IFN-γ expression may be that Muse cells inhibit lymphocyte secretion (Yabuki et al., 2018). This may provide a new perspective for revealing the mechanisms of immune regulation of MSCs. In short, various aspects of Muse cell mechanism, including greater antiapoptotic ability, a stronger ability to secrete anti-inflammatory factors and more ideal immune regulatory function, could synergistically protect intestinal epithelial cells. Our studies indicate that Muse cells have great potential in the treatment of intestinal inflammatory diseases (e.g., IBD).

Conclusions

To our knowledge, this is the first time that adult pluripotent stem cells presented features similar to human Muse cells in rats; these cells maintain the basic characteristics of BMMSCs and positively expressed SSEA-3 and SSEA-1, as well as other pluripotent molecular markers. On the basis of the signaling pathways associated with anti-inflammatory and immunomodulatory molecules, rat Muse cells have an ideal protective effect on damaged intestinal epithelial cells in vitro.

Acknowledgments and funding

The authors thank the relevant teachers at the NHC Key Laboratory of Critical Care Medicine and the Tianjin Key Laboratory of Organ Transplantation for their technical

support. This work was supported by the National Natural Science Foundation of China (Grant nos. 81670574, 81441022, and 81270528); Natural Science Foundation of Tianjin, China (Grant nos. 08JCYBJC08400, 11JCZDJC27800, and 12JCZDJC25200).

Conflicts of interest

The authors declare that they have no conflicts of interest.

References

Cao Y, Wu BJ, Zheng WP, Yin ML, Liu T, Song HL (2017) Effect of heme oxygenase-1 transduced bone marrow mesenchymal stem cells on damaged intestinal epithelial cells in vitro. Cell Biol Int 41(7): 726–38. https://doi.org/10.1002/cbin.10749

Dezawa M (2016) Muse cells provide the pluripotency of mesenchymal stem cells: direct contribution of muse cells to tissue regeneration. Cell Transplant 25(5): 849–61. https://doi.org/10.3727/096368916X690881

Gezginci-Oktayoglu S, Orhan N, Bolkent S (2016) Prostaglandin-E1 has a protective effect on renal ischemia/reperfusion-induced oxidative stress and inflammation mediated gastric damage in rats. Int Immunopharmacol 36: 142–50. https://doi.org/10.1016/j.intimp.2016.04.021

Gimeno ML, Fuertes F, Barcala Tabarrozzi AE, Attorressi AI, Cucchiani R, Corrales L, Perone MJ (2017) Pluripotent nontumorigenic adipose tissue-derived muse cells have immunomodulatory capacity mediated by transforming growth factor-beta1. Stem Cells Transl Med 6(1): 161–73. https://doi.org/10.5966/sctm.2016-0014

Heneidi S, Simerman AA, Keller E, Singh P, Li X, Dumesic DA, Chazenbalk G (2013) Awakened by cellular stress: isolation and characterization of a novel population of pluripotent stem cells derived from human adipose tissue. PLoS One 8(6): e64752. https://doi.org/10.1371/journal.pone.0064752

Hosoyama K, Saiki Y (2018) Muse cells and aortic aneurysm. Adv Exp Med Biol 1103: 273–91. https://doi.org/10.1007/978-4-431-56847-6_15

Hu MS, Longaker MT (2017) A MUSE for skin regeneration. J Invest Dermatol 137(12): 2471–72. https://doi.org/10.1016/j.jid.2017.07.825

Iseki M, Kushida Y, Wakao S, Akimoto T, Mizuma M, Motoi F, Dezawa M (2017) Muse cells, nontumorigenic pluripotent-like stem cells, have liver regeneration capacity through specific homing and cell replacement in a mouse model of liver fibrosis. Cell Transplant 26(5): 821–40. https://doi.org/10.3727/096368916X693662

Knipe JM, Strong LE, Peppas NA (2016) Enzyme- and pH-responsive microencapsulated nanogels for oral delivery of siRNA to induce TNF-α knockdown in the intestine. Biomacromolecules 17(3): 788–97. https://doi.org/10.1021/acs.biomac.5b01518

Leng Z, Sun D, Huang Z, Tadmori I, Chiang N, Kethidi N, Sabra A, Kushida Y, Fu YS, Dezawa M, He X, Young W (2019) Quantitative analysis of SSEA3+ cells from human umbilical cord after magnetic sorting. Cell Transplantation 28(7): 907–923, https://doi.org/10.1177/0963689719844260

Li D, Secher JO, Juhl M, Mashayekhi K, Nielsen TT, Holst B, Hall VJ (2017) Identification of SSEA-1 expressing enhanced reprogramming (SEER) cells in porcine embryonic fibroblasts. Cell Cycle 16(11): 1070–84. https://doi.org/10.1080/15384101.2017.1315490

Liu Q, Zhang RZ, Li D, Cheng S, Yang YH, Tian T, Pan XR (2016) Muse cells, a new type of pluripotent stem cell derived from human fibroblasts. Cell Reprogram 18(2): 67–77. https://doi.org/10.1371/journal.pone.0064752

Liu T, Fu NN, Song HL, Wang YL, Wu BJ, Shen ZY (2014) Suppression of microRNA-203 improves survival of rat bone marrow mesenchymal stem cells through enhancing PI3K-induced cellular activation. IUBMB Life 66(3): 220–27. https://doi.org/10.1002/iub.1259

Mahmood R, Mehmood A, Choudhery MS, Awan SJ, Khan SN, Riazuddin S (2019) Human neonatal stem cell-derived skin substitute improves healing of severe burn wounds in a rat model. Cell Biol Int 43(2): 147–57. https://doi.org/10.1002/cbin.11072

Perone MJ, Gimeno ML, Fuertes F (2018) Immunomodulatory properties and potential therapeutic benefits of muse cells administration in diabetes. Adv Exp Med Biol 1103: 115–29. https://doi.org/10.1007/978-4-431-56847-6_6

Shen ZY, Wu B, Liu T, Yang Y, Yin ML, Zheng WP, Song HL (2017) Immunomodulatory effects of bone marrow mesenchymal stem cells overexpressing heme oxygenase-1: protective effects on acute rejection following reduced-size liver transplantation in a rat model. Cell Immunol 313: 10–24. https://doi.org/10.1016/j.cellimm.2016.12.006

Shouval DS, Rufo PA (2017) The role of environmental factors in the pathogenesis of inflammatory bowel diseases: a review. JAMA Pediatr 171(10): 999–1005. https://doi.org/10.1001/jamapediatrics.2017.2571

Skottke J, Gelinsky M, Bernhardt A (2019) In vitro co-culture model of primary human osteoblasts and osteocytes in collagen gels. Int J Mol Sci 20(8): E1998. https://doi.org/10.3390/ijms20081998

Tanaka T, Nishigaki K, Minatoguchi S, Nawa T, Yamada Y, Kanamori H, Minatoguchi S (2018) Mobilized muse cells after acute myocardial infarction predict cardiac function and remodeling in the chronic phase. Circ J 82(2): 561–71. https://doi.org/10.1253/circj.CJ-17-0552

Toyoda E, Sato M, Takahashi T, Maehara M, Nakamura Y, Mitani G, Watanabe M (2019) Multilineage-differentiating stress-enduring (Muse)-like cells exist in synovial tissue. Regen Ther 10: 17–26. https://doi.org/10.1016/j.reth.2018.10.005

Uchida H, Morita T, Niizuma K, Kushida Y, Kuroda Y, Wakao S, Dezawa M (2016) Transplantation of unique subpopulation of fibroblasts, muse cells, ameliorates experimental stroke possibly via robust neuronal differentiation. Stem Cells 34(1): 160–73. https://doi.org/10.1002/stem.2206

Wakao S, Akashi H, Kushida Y, Dezawa M (2014) Muse cells, newly found non-tumorigenic pluripotent stem cells, reside in

human mesenchymal tissues. Pathol Int 64(1): 1–9. https://doi.org/10.1111/pin.12129

Wakao S, Kitada M, Kuroda Y, Shigemoto T, Matsuse D, Akashi H, Dezawa M (2011) Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. Proc Natl Acad Sci USA 108(24): 9875–80. https://doi.org/10.1073/pnas.1100816108

Wakao S, Kushida Y, Dezawa M (2018) Basic characteristics of muse cells. Adv Exp Med Biol 1103: 13–41. https://doi.org/10.1007/978-4-431-56847-6_2

Wang L, Xiao L, Zhang RZ, Qiu LZ, Zhang R, Shi HX (2018) Effects of acrylate/acrylamide polymers on the adhesion, growth and differentiation of Muse cells. Biomed Mater 14(1): 015003. https://doi.org/10.1088/1748-605X/aae5cb

Yabuki H, Watanabe T, Oishi H, Katahira M, Kanehira M, Okada Y (2018) Muse cells and ischemia-reperfusion lung injury. Adv Exp Med Biol 1103: 293–303. https://doi.org/10.1007/978-4-431-56847-6_16

Yamauchi T, Yamasaki K, Tsuchiyama K, Koike S, Aiba S (2017) The potential of muse cells for regenerative medicine of skin: procedures to reconstitute skin with muse cell-derived keratinocytes, fibroblasts, and melanocytes. J Invest Dermatol 137(12): 2639–42. https://doi.org/10.1016/j.jid.2017.06.021

Yang Z, Liu J, Liu H, Qiu M, Liu Q, Zheng L, Zhang Y (2013) Isolation and characterization of SSEA3(+) stem cells derived from goat skin fibroblasts. Cell Reprogram 15(3): 195–205. https://doi.org/10.1089/cell.2012.0080

Yin M, Shen Z, Yang L, Zheng W, Song H (2019) Protective effects of CXCR3/HO1 genemodified BMMSCs on damaged intestinal epithelial cells: role of the p38MAPK signaling pathway. Int J Mol Med 43(5): 2086–102. https://doi.org/10.3892/ijmm.2019.4120

Yin ML, Song HL, Yang Y, Zheng WP, Liu T, Shen ZY (2017) Effect of CXCR3/HO-1 genes modified bone marrow mesenchymal stem cells on small bowel transplant rejection. World J Gastroenterol 23(22): 4016–38. https://doi.org/10.3748/wjg.v23.i22.4016

Young W (2018) Future of Muse cells. Adv Exp Med Biol 1103: 309–15. https://doi.org/10.1007/978-4-431-56847-6_18

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Received 8 June 2019; accepted 19 October 2019.
Final version published online 6 November 2019.