Regenerative Therapy 28 (2025) 402–412

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

3D dynamic culture of muse cells on a porous gelatin microsphere after magnetic sorting: Achieving high purity proliferation

Zhe Lu [1], Shifeng Ren [1], Bingjie Wang, Yajun Zhang, Xiaodong Mu*, Zhihui Wang**

School of Pharmaceutical Sciences, State Key Laboratory of Advanced Drug Delivery and Release Systems, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, Shandong, China

ARTICLE INFO

Article history:
Received 11 October 2024
Received in revised form
16 December 2024
Accepted 4 January 2025

Keywords:
Stem cell therapy
Muse cell
Multipotency
3D cell culture
Microsphere

ABSTRACT

Muse cell has become a promising source of cells for disease treatment due to its remarkable characteristics, including stress tolerance, low tumorigenicity, effective homing ability, and differentiation into histocompatibility cells after transplantation. However, there are some obvious obstacles that need to be overcome in the efficient expansion of Muse cells. We extracted mesenchymal stem cells (MSCs) from human umbilical cord and their MSCs phenotypes were verified by flow cytometry. Then, immune magnetic sorting was performed to obtain Muse cells, and the expression of pluripotency related factors and the ability to differentiate into three germ layers were verified with sorted Muse cells. We then tested a new 3D culture method with dynamic microsphere carrier to possibly expand Muse cells more efficiently. Finally, in vivo experiments were conducted to check the homing ability of Muse cells to muscle injury. Our results showed that, the cultivation and expansion of Muse cells can be more effectively achieved through dynamic microsphere carrier; compared to non-Muse cells, Muse cells have stronger pluripotency and differentiation ability, and their homing ability in the muscle injury mice model is superior to that of non-Muse cells. Therefore, with the method of immune magnetic sorting and dynamic microsphere carrier, highly regenerative Muse cells can be more effectively sorted and expanded from MSCs.

1. Introduction

Cell therapy is expected to become an effective intervention method for treatment of many diseases, and various types of cells are being considered as potential candidate cell sources [1,2]. In particular, stem cells have aroused great interest in research; their abilities of self-renewal and to differentiate into mature adult cells make them promising for human tissue regeneration [3,4]. In the early 1980s, human embryonic stem cells (ESCs) were first discovered, which greatly changed the development direction of regenerative medicine [5,6]. However, some studies have shown that there is immune rejection in the process of transplantation of ESCs, and the unrestricted proliferation of ESCs is more likely to form abnormal fetuses. These adverse phenomena and bioethical problems caused by the use of ESCs make it unable to be used in regenerative medicine [7]. Induced pluripotent stem cells (iPSCs) also face problems such as potential tumorigenicity and genetic variation, which restrict their clinical application [8,9]. Mesenchymal stem cells (MSCs) can secrete a large number of cytokines and regulate inflammatory reactions, which can protect injured tissues [10], and mesenchymal stem cells have great differentiation potential and can transform across the boundary of mesoderm, ectoderm and endoderm germ layers. In addition, MSCs will not form teratoma in animal models, and cause minimal immune rejection. MSCs have the advantages of wide sources and non tumorigenicity, and are a potential source of stem cells. Therefore, MSCs have attracted much attention in the filed of stem cell therapy. However, compared with ESCs and iPSCs, MSCs have weak cellular pluripotency, and their survival time in vivo is usually shorter after transplantation.

Muse cells are adult stem cells accidently discovered by Japanese scientist Mari dezawa in 2007 and first reported in 2010 [11].

* Corresponding author.
** Corresponding author.
E-mail addresses: muxiaodong@sdfmu.edu.cn (X. Mu), wangzhihui@sdfmu.edu.cn (Z. Wang).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

[1] Authors contributed equally to this work.

https://doi.org/10.1016/j.reth.2025.01.003

Due to their unique properties, these cells are called multilineage differentiation stress tolerance (Muse) cells [12]. Because these stem cells are natural cells without genetic modification, they are not prone to develop into cancer cells and are safer than induced multifunctional stem cells (i.e., induced pluripotent cells/iPS cells) that need to be genetically modified during culture. While embryonic stem cells are obtained from blastocysts or embryos, mesenchymal stem cells are derived from donated umbilical cords (which all have informed consent); muse cells are isolated from mesenchymal stem cells, and there are no ethical issues [13,14]. It can migrate to the defect tissue in vivo and spontaneously differentiate into defect cells with low-end granzyme activity. It does not have tumorigenicity and does not form teratoma in immunodeficient mouse [15,16]. Muse cells are endogenous pluripotent stem cells, which can be effectively isolated from adult mesenchymal tissues (such as bone marrow, adipose tissue and dermis) and from commercially obtained cultured fibroblasts [11,17–20]. SSEA-3 is a surface marker of pluripotent stem cells and the most commonly used marker of Muse cells. It can be isolated from a variety of tissues by double positive markers of CD105 and SSEA-3 [11]. Muse cell is able to express marker genes of embryonic stem cells (Nanog, OCT4, SOX2, SSEA-3), and can be induced to differentiate into three germ cell lines.

Muse cells has been found to play a positive role in treating a variety of diseases, such as stroke, liver cirrhosis, myocardial infarction, ischemia-reperfusion lung injury, diabetic skin lesions, spinal cord injury, cartilage injury, dermatitis, severe pancreatitis, enteritis [20–27].

One of the challenges for the research and medical application of Muse cells is that the number of Muse cells in tissues is very low. Muse cells constitute approximately 0.01–0.03 % of bone marrow monocytes[11,28], 0.01–0.2 % of peripheral blood cells [29], and 1–6% of mesenchymal stem cells from various sources [30]. To date, bone marrow derived mesenchymal stem cells (BM-MSCs) and umbilical cord derived mesenchymal stem cells (UC-MSCs) are the most commonly used sources of Muse cells [11,18]. Also, the proliferation rate of Muse cells is generally similar to or slightly slower than that of fibroblasts [11]. Therefore, how to obtain sufficient Muse cells has become the key challenge to further researches and potential medical applications.

2. Materials and methods

2.1. Cell source

This experiment used mesenchymal stem cells from umbilical cord sources. The experiment is divided into two methods for extracting mesenchymal stem cells to explore the content of Muse cells and determine the extraction method of MSCs (Fig. 1).

Cell self crawling extraction method: The umbilical cord was cleaned with 10 % penicillin streptomycin physiological saline, and then a 1 cm small piece was constructed. 25 mL of mesenchymal stem cell serum-free complete culture medium was added, and placed in a 37 °C, 5 % CO_2 constant temperature incubator. The medium was changed seven days later, and cells crawled out three days later. When the cell density reaches 70 %–80 %, cell passage is carried out. When passing to the third generation, the phenotype of the cells is identified through flow cytometry.

Collagenase method: the umbilical cord was cleaned with 10 % penicillin streptomycin physiological saline, and then a 1 cm small piece was constructed. Then add collagenase for digestion for 30 min, and then pass through 100 μm's filter screen for filtering, 25 mL of mesenchymal stem cell serum-free complete culture medium was added, and placed in a 37 °C, 5 % CO_2 constant temperature incubator. When the cell density reaches 70 %–80 %, cell passage is carried out. When passing to the third generation, the phenotype of the cells is identified through flow cytometry.

LTT experiment: P3 mesenchymal stem cells (MSCs) were digested with LTT trypsin for 1 h, 4 h, 8 h, and 16 h, followed by cell viability (trypan blue staining), and the SSEA3 cell positivity rate of MSCs after LTT was detected by flow cytometry.

2.2. Immunomagnetic sorting

Incubate cells with SSEA3(thermo, Cat No MA1-020) for 60 min, removes unbound primary antibody by centrifugation, and incubates with immune magnetic beads (Miltenyi Biotec Cat No 130-047-401) for 15 min. SSEA3 positive cells are sorted on a magnetic rack. SSEA3 and CD105 positive rates are detected by flow cytometry.

2.3. Immunofluorescence

Soak the slides of cells that have already crawled onto the culture plate with PBS three times, each time for 5 min. Fix the slide with 4 % paraformaldehyde for 15 min, and soak the slide with PBS three times for 5 min each time. Add strong cell permeability solution and let it penetrate for 10 min. Soak the glass slides with PBS three times, each time for 3 min. Add sealing solution and seal at room temperature for 30 min. Remove the sealing solution and add diluted SOX2 (cell signaling, Cat No 3579S), OCT4 (cell signaling, Cat No 2890S), Nanog (cell signaling, Cat No 4903S), and SSEA3 (Novus, Cat No NB100-1832) primary antibodies to each slide and incubate overnight at 4 °C. Dilute the fluorescein coupled secondary antibody with antibody dilution buffer and incubate the sample in dark for 1–2 h. Rinse three times with 1x PBS for 5 min each time. Subsequently, dapi staining was performed. Finally, imaging was performed using a fluorescence microscope.

2.4. Western blot

Immunoblotting was used to detect OCT3/4 (cell signaling, Cat No 2890S) in Muse cells and non-Muse cells; SOX2 (cell signaling, Cat No 3579S); The expression of Nanog (cell signaling, Cat No 4903S) is used to study the pluripotency of Muse cells and non-Muse cells.

2.5. Induced differentiation of Muse cells

Induce lipogenesis: Add 1 mL of 0.1 % gelatin to a six well plate, shake well to evenly cover the bottom of each well, and place on an ultra clean table for 30 min. After 30 min, remove the gelatin and idnoculate 2×10^4 cm^2 Muse cells and no-Muse cells into a 6-well plate with gelatin, and add 2 mL of fat induced differentiation medium (Oricell, Cat No HUXUC-90031) to each well. After induction, oil red O staining was used.

Induce liver formation: Add 1 mL of 0.1 % gelatin to a six well plate, shake well, so that it can evenly cover the bottom of each well, and place it on a super clean table for 30 min. After 30 min, remove the gelatin and inoculate Muse cells and no-Muse cells into a 6-well plate with gelatin (2×10^4 cells/cm^2), and add 2 mL of Hepatogenic differentiation medium (Oricell, Cat No HUXMX-90101) to each well. During this period, fresh mature liver cell culture medium was replaced every 3 days. After the maturation stage of liver cells, identification testing can be conducted.

Induction of osteogenesis: Add 1 mL of 0.1 % gelatin to a six well plate, shake well, and allow it to evenly cover the bottom of each well. Place it on an ultra clean table for 30 min. Inoculate 2×10^4 cm^2 Muse cells and no-Muse cells into a 6-well plate with gelatin, and add 2 mL of bone induction differentiation medium (Oricell,

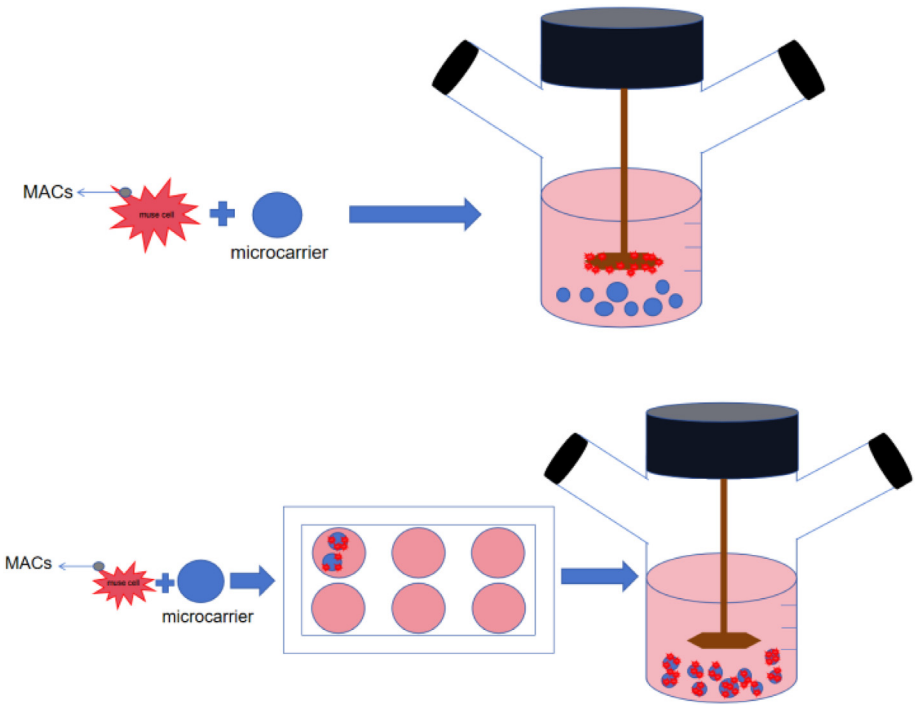

Fig. 1. 3D dynamic cultivation system. After magnetic sorting, the sorted Muse cells will carry magnetic beads. If both the microcarrier and Muse cells are placed in a stirring culture bottle at the same time, they will be sucked away by the device's magnet, causing cell apoptosis.Due to the large volume and weight of the microcarriers, it is difficult for magnets to remove the microcarriers containing cells.Therefore, Muse cells and microcarriers were cultured in a low adsorption cell culture plate for 4 days, and then transferred to a stirring bottle for cultivation.

Cat No HUXUC-90021) to each well. Change the culture medium every 3 days. After 2 weeks of induction, the morphology and growth of the cells were observed and stained with alizarin red.

Induce into nerves: Add 1 mL of 0.1 % gelatin to a six well plate, shake well to evenly cover the bottom of each well, and place on a super clean table for 30 min. Inoculate 2 × 10^4cm2 Muse cells and no-Muse cells into a 6-well plate with gelatin, and add 2 mL of nerve induced differentiation medium (Table 1) to each well. Change the culture medium every 3 days, and finally perform Nestin immunofluorescence staining.

2.6. Muse cell culturing

Grouping of cultivation methods: Divide the cultivation methods into four groups: Muse-2D, Muse-3D static, MSC LTT classical, MSC no-LTT classic, Muse-3 dynamic, Muse- LTT classic.

1 Muse-2D: After magnetic separation, The initial number of Muse cells after magnetic separation is 6 × 10^5, Muse cells are directly inoculated into a regular culture bottle for wall attachment culture. When fully grown, they are subcultured at a rate of 10000 cells/cm^2.

Table 1
Induce neural differentiation reagents.

™Neurobasal	thermo	21103049
N-2 (100X)	Thermo	17502048
B-27™ (50X)	Thermo	17504044
L-glutamine (200 mM)	Thermo	A2916801
2-Mercaptoethanol	Thermo	21985023
Human EGF Recombinant Protein	Cell signing	72528S
Human FGF2 Recombinant Protein	Abcam	ab9596

2 Muse-3D static: After magnetic separation, Muse cells were added to a low suction plate for culture. The initial number of Muse cells after magnetic separation is 6 × 10^5, The initial concentration of Muse cells was 10000 cells/mL, and were digested and passaged with pancreatic enzyme or protease every 3–4 days. The culture medium was increased proportionally.

3 MSC LTT classic: Determine the initial number of MSCs based on the positive ratio of SSEA3 and CD105 detected by the third generation MSC flow cytometry, with an initial number of MSCs = 6 × 10^5/1.9 %.After digesting MSC cells with trypsin for 4 h (LTT), they are added to a low adsorption culture bottle for 7 days. The cells aggregate into small balls, which are then inoculated into a regular culture bottle for adherent culture for 2 days. Then, the cells are digested with trypsin for 4 h, low adsorption culture plate for 7 days, adherent culture for 2 days, repeated multiple cycles.

4 MSC no-LTT classic: Determine the initial number of MSCs based on the positive ratio of SSEA3 and CD105 detected by the third generation MSC flow cytometry, with an initial number of MSCs = 6 × 10^5/1.9 %. After digesting MSC cells with trypsin for 4 h, they are added to a low adsorption culture bottle for 7 days. The cells aggregate into small balls, which are then inoculated into a regular culture bottle for adherent culture for 2 days (Only the first loop performs LTT), low adsorption culture plate for 7 days, adherent culture for 2 days, repeated multiple cycles.

5 Muse-3D dynamic: After magnetic separation, the initial number of Muse cells is 6 × 10^5. The Muse cells were added to a low adsorption culture plate (30000 cells/mg) with 20 mg microcarrier (Cytoniche, SF125; specific degradation microcarrier materials with the particle size being 50–500 μm in diameter, and the effective growth area is about 9000 cm^2/g), and an appropriate amount of culture medium was added. Four days

later, the cells were added to a low adsorption culture bottle and stirred for cultivation. The culture medium and microcarrier were increased proportionally for passage (Fig. 1).

6 Muse-LTT classic:After magnetic separation, Muse cells were added to a low adsorption culture bottle and cultured for 6 days. The cells aggregated into small balls, which were then inoculated into a regular culture bottle for 3 days of wall adhering culture. Then, LTT was performed, followed by ball forming and wall adhering, repeating this process for multiple cycles.

Note: Each cycle or generation of cells is sampled for trypan blue cell count and flow cytometry detection (SSEA3; CD105).

2.7. Staining of live and dead cells

This assay was performed with Calcein-AM/PI kit (Solarbio, CA1630). The cell precipitate obtained by centrifugation is treated with 1X Resuspend Assay Buffer, add 1-2μL of Calcein AM (stock solution) to every 1 mL of cell volume, blow and mix well, and incubate at 37 °C in the dark for 20–25 min. Take 3–5 μL of the original PI solution from the reagent kit and add it to the stained cells mentioned above. Stain at room temperature in dark for 5 min. Incubate fluorescent cells at 450g for 5 min, centrifuge to remove staining solution. Wash 450g of cells with 1X PBS for 5 min. After centrifugation, resuspend cells with 1X PBS. Take 3–5 μL and drop it onto a clean slide. After pressing the slide with a clean cover glass, please perform fluorescence microscopy in a timely manner.

2.8. Apoptosis and proliferation assays

Prepare cells for Muse-3D static and Muse-3 dynamic cultures, and detect their apoptosis and proliferation at day 1, 3, and 7.

Calcein AM/PI live/dead cell dual staining experiment: This assay was performed with Calcein-AM/PI kit (Solarbio, CA1630). The cell precipitate obtained by centrifugation is treated with 1 × Resuspend Assay Buffer, add 1-2μL of Calcein AM (stock solution) to every 1 mL of cell volume, blow and mix well, and incubate at 37 °C in the dark for 20–25 min. Take 3–5 μL of the original PI solution from the reagent kit and add it to the stained cells mentioned above. Stain at room temperature in dark for 5 min. Incubate fluorescent cells at 450g for 5 min, centrifuge to remove staining solution. Wash cells by centrifugation with 1x PBS, resuspend cells with 1X PBS after centrifugation, take 3–5 μL and drop onto a clean glass slide. Press the slide onto a clean cover glass and detect under a fluorescence microscope.

EdU-488 cell proliferation detection: Add an equal volume of 2X EdU working solution from the EdU-488 cell proliferation kit (Beyotime, C0071S) and culture medium to the cultured cells, and the final concentration of EdU will be reduced to 1X. Then continue to incubate the cells for 2 h. After EdU labeling of the cells, remove the culture medium and add 1 mL of paraformaldehyde fixative. Fix at room temperature for 15 min. Remove the fixative and wash the cells three times with 1 mL of detergent per well, each time for 3–5 min. Remove the detergent, use 1 mL of permeate per well, then prepare and add the corresponding reagents according to the instructions of the reagent kit, and finally observe the results under a fluorescence microscope.

2.9. Cell localization in soft tissue injury models

Animal Handling: The experimental animals were used NOD-Prkdc^{scid}IL2rg^{em9} Mice animals, then the hind thighs of the animals were forcefully clamped with hemostatic forceps to break the thighs, creating musculoskeletal injury models.

Immunomagnetic sorted Muse cells and non Muse cells were stained with DIR dye (MCE, 100068-60-8) and incubated for 30 min.

After 24 h of skeletal muscle injury in animals, stained and non stained Muse cells were injected into the tail vein, and the distribution of injected cells was observed through imaging of small animals 24 h later.

2.10. Statistics

For cell culture experiments, the sample size was determined to be at least n = 3 independent biological repeats, while in each experiment every sample had three technical repeats. Data are presented as means ± standard error of the mean (s.e.m.), unless otherwise noted. Histological scoring was analyzed by the Kruskal-Wallis and Steel-Dwass tests, with a 95 % confidence interval. For consistency in comparisons, significance in all figures is denoted as follows: $*P < 0.05$, $**P < 0.01$.

3. Results

3.1. Acquisition of Muse cells

Muse cells are isolated from the human umbilical cord and obtained by culturing human umbilical cord mesenchymal stem cells, followed by immunomagnetic sorting. Firstly, the phenotype of umbilical cord mesenchymal stem cells was analyzed by flow cytometry, and the results showed CD90; CD73; CD105 is positive, CD34 is negative (Fig. 2).

As the time of trypsin digestion (LTT) increases, the cell viability of MSCs shows a downward trend, dropping to below 50 % at 8 h. Therefore, in order to ensure normal cell viability, this experiment used 4-h trypsin digestion (4h LTT). Then, flow cytometry was used to detect the positive rates of SSEA3 and CD105 in MSCs of 4-h LTT, with a positive rate of 9.58 % (Fig. 3-A), The double positive ratio of SSEA3 and CD105 is 6.62 % (Collagenase method), and the double positive ratio of SSEA3 and CD105 is only 3.58 (Cell self crawling extraction method). Therefore, mesenchymal stem cells cultured using collagenase digestion method were used to extract Muse cells (Fig. 3-B). Subsequently, Muse and non Muse cells were separated using immunomagnetic sorting method, and the flow cytometry analysis of positive cells showed SSEA3; The proportion of CD105 positivity was 98.7 % (Fig. 3-C). Immunofluorescence staining showed that both Muse and non Muse cells expressed CD105, and SSEA3 was highly expressed in Muse cells, while SSEA3 was almost not expressed in non Muse cells (Fig. 3-D).

3.2. Characterization of Muse cells

Immunofluorescence staining revealed that OCT3/4, SOX2, and NANOG markers were highly expressed in Muse cells, while non Muse cells showed almost no or low expression of OCT3/4, SOX2, and NANOG markers (Fig. 4 A-C). The results of Western blot showed that the expression of OCT3/4, SOX2, and NANOG in Muse cells was significantly higher than that in the non Muse group ($p < 0.01$) (Fig. 4-D).

3.3. Triple embryonic differentiation of Muse cells

In the induced differentiation experiment, after 3 weeks of osteogenic induction, Alizarin Red staining (with a scale of 200 μ m) was performed, and red and black calcium deposits appeared in the cytoplasm of Muse cells and non Muse cells, with Muse cells having more calcium deposits; After 3 weeks of induction of adipogenesis, oil red O staining (with a scale of 50 μm) was performed, and the

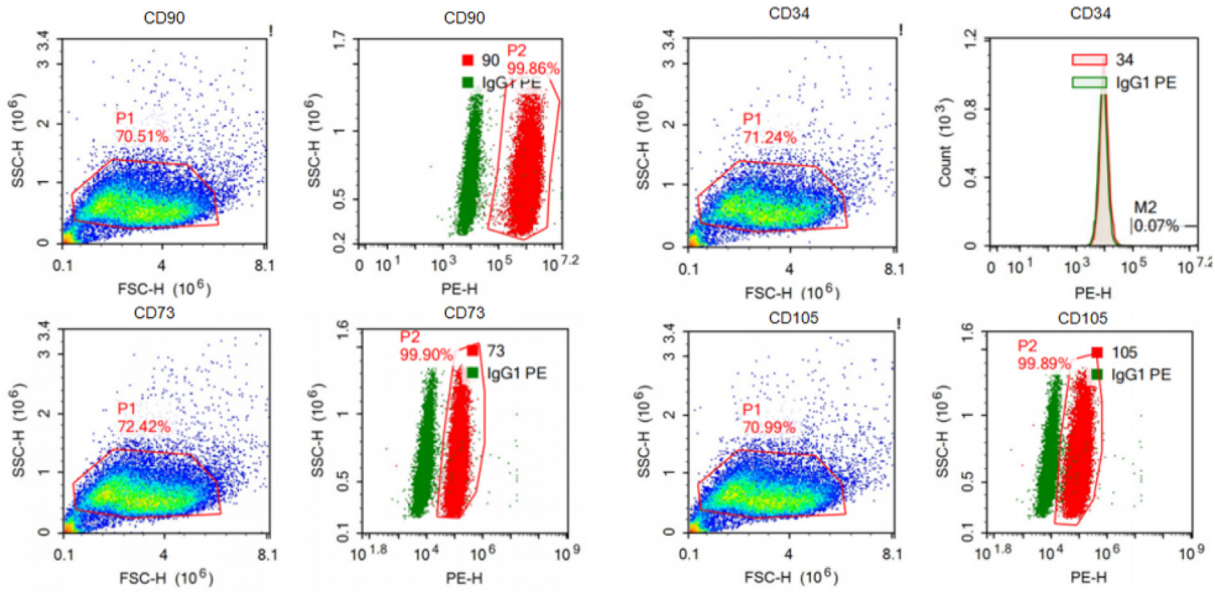

Fig. 2. Identification of mesenchymal stem cells. The flow detection result of the third generation MSCs is: CD90 (PE); CD73 (PE); CD105 (PE) is positive, while CD34 (PE) is negative.

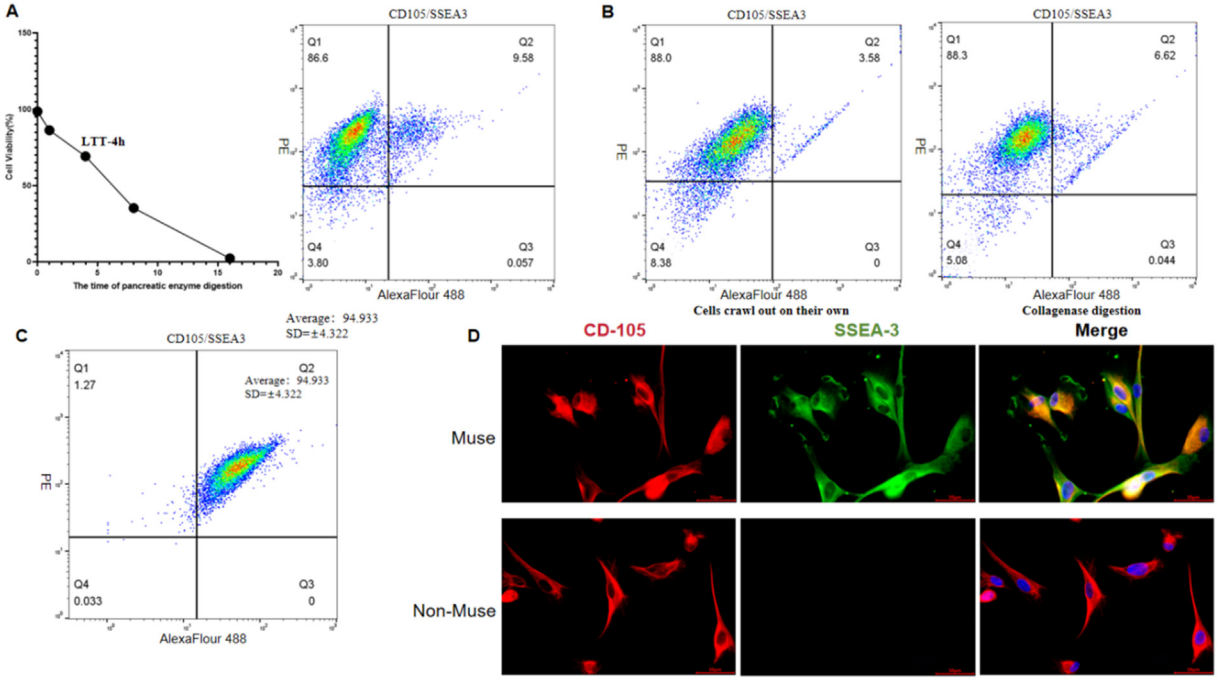

Fig. 3. Extraction of Muse cells. **A:** Cell viability curves of trypsin digestion (LTT) at 1h, 4h, 8h, 16h. The positivity rate of SSEA3 and CD105 in MSCs detected by flow cytometry for 4-h LTT was 9.58 %. **B:** SSEA3 (Alexa Flour 488) was found in the third-generation mesenchymal stem cells cultured using the cell self crawling method; The proportion of CD105 (PE) positive cells was 3.58 %, and SSEA3 (Alexa Flour 488) was found in the third-generation mesenchymal stem cells cultured using collagenase digestion method; The proportion of CD105 (PE) positive cells is 6.62 %. **C:** Cell SSEA3 after immunomagnetic sorting (Alexa Flour 488); The proportion of CD105 (PE) positive cells is 94.933 % (n = 3, average 94.933 %, SD = ±4.322). **D**: Immunofluorescence staining of Muse cells and non-Muse cells SSEA3, CD105 staining situation (n = 3).

lipid droplets in the cells were red. Muse cells induced more and larger oil droplets than non Muse cells. Induction of liver differentiation revealed that both Muse and no use glycogen PAS staining showed a purple red color, with Muse cells showing a deeper and more intense purple red color. Neurogenic differentiation was induced and it was found that Muse expressed Nestin ectodermal markers, while non-Muse cells almost did not express ectodermal markers (Fig. 5).

3.4. Exploration of Muse cell expansion methods

Divide the cultivation methods into five groups, namely Muse-2D, Muse-3D static, Muse classical, Muse-3D dynamic, MSC LTT

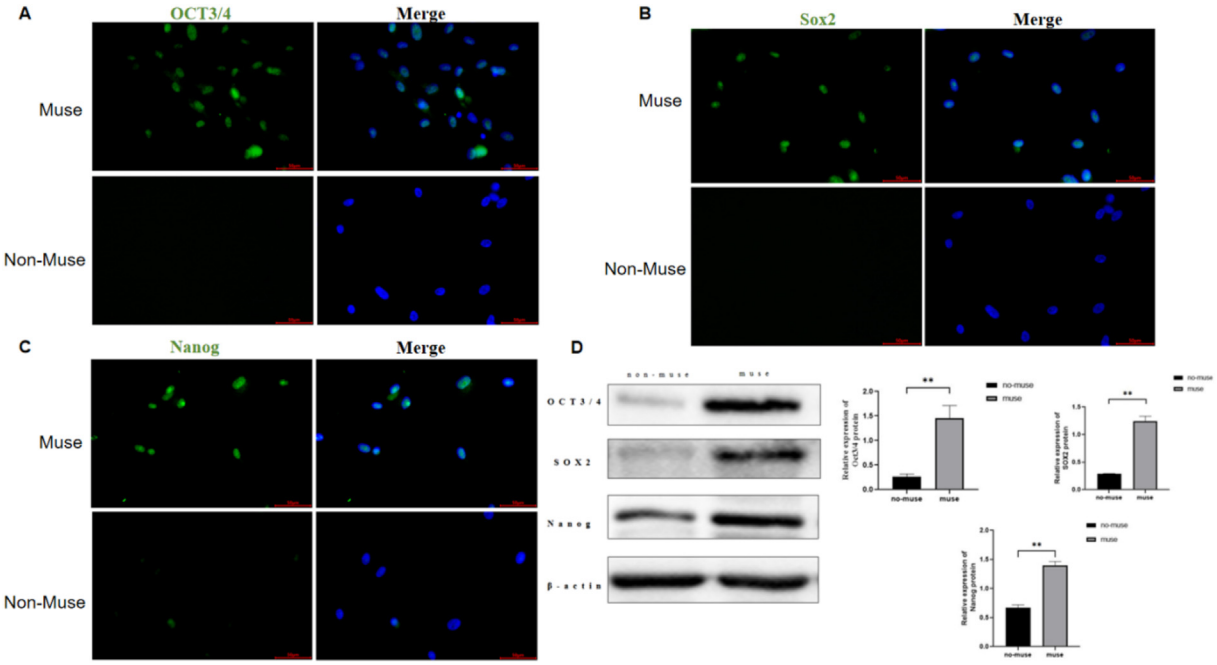

Fig. 4. Characterization of Muse cells. **A-C**: Immunofluorescence staining of Muse cells and non-Muse cells OCT3/4, SOX2, and Nanog staining (n = 3); **D**: OCT3/4, SOX2, Nanog immunoblotting analysis of Muse cells and non-Muse cells (n = 3). The expression of OCT3/4, SOX2, and Nanog markers is higher in the Muse cell SSEA3, while the expression of OCT3/4, SOX2, and Nanog markers is lower in the non-Muse cell SSEA3. The expression of OCT3/4, SOX2, and Nanog in the Muse cell group is significantly higher than that in the non Muse group. (**$P < 0.01$).

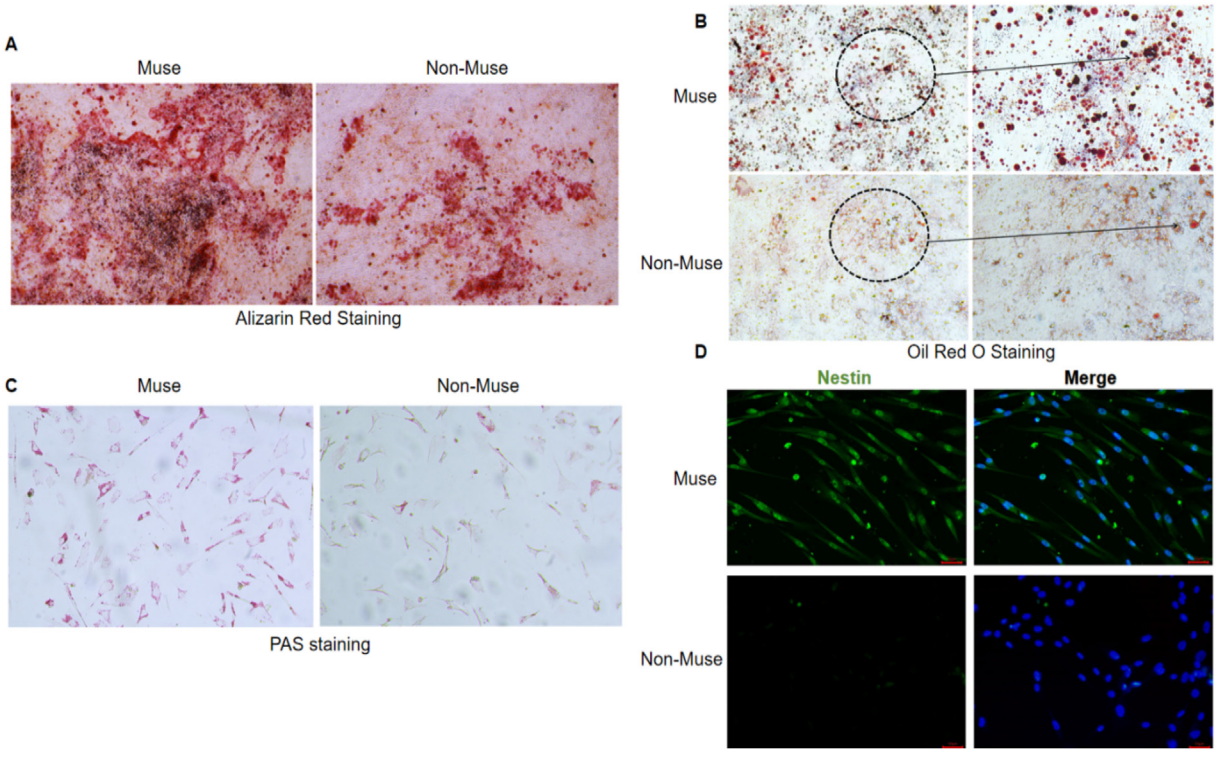

Fig. 5. Triple embryonic differentiation of Muse cells. **A:** Alizarin Red Staining of Muse Cells and non-Muse Cells (scale; 100 μm). **B:** Oil Red O staining of Muse cells and non-Muse cells (scale: 200 μm, 100 μm). **C:** PAS staining of glycogen in Muse cells and non-Muse cells (scale; 100 μm). **D:** Nestin immunostaining of Muse cells and non-Muse cells (n = 3, scale; 100 μm).

classical, and MSC no LTT classical, to investigate the proliferation of Muse cells. It was found that the positive rate of SSEA3 in Muse-2D group cells showed a decreasing trend, and the proliferation of Muse cells was slow. In the Muse-3D static group, the positive rate of SSEA3 remained at a high level, but it did not increase in value, and instead, there was cell apoptosis. The positive rate of SSEA3 is increasing in the MSC LTT classic group, but the time period is longer. In the MSC no LTT classical group, the positive rate of SSEA3 decreased and the increase was slow. The SSEA3 positivity rate in the Muse LTT classical group remained around 60 %–70 %, and the purity of Muse cells was lower than that in the Muse-3D dynamic group. Muse-3D dynamic group cells can maintain a high positive rate of SSEA3 and significantly increase proliferation (Fig. 6).

3.5. Apoptosis and proliferation assay of Muse cells during 3D culture process

We conducted live and dead cell staining and EDU enrichment experiments on Muse-3D static and Muse-3D dynamic 3D culture methods, and found that Muse cells without static cell scaffolds showed apoptosis. With the prolongation of time, the number of apoptotic cells increased, and the proliferation of Muse cells decreased, mainly concentrated on the outer side of the cell cluster. Muse cells with dynamic cell scaffolds exhibit less apoptosis and stronger proliferation ability (Fig. 7).

3.6. Cell localization in soft tissue injury

Through tail vein injection of Muse and no use cells, it was found that there was a significant fluorescence intensity at the injury site of the Muse cell group animal model, while the fluorescence intensity at the injury site of the no use cell group animal model was weaker. Therefore, in animal models of skeletal muscle injury, there are more Muse cells and fewer non-Muse cells at the site of injury. It can be seen that Muse cells have better targeting ability than non-Muse cells (Fig. 8).

4. Discussion

At present, Muse cells have been found to exist in bone marrow, fat, skin, spleen, pancreas, umbilical cord and other mesenchymal tissues, and the commonly used screening sources are bone marrow mesenchymal, fat mesenchymal tissue, umbilical cord mesenchymal tissue, but the content of Muse cells in these tissues is relatively small [31]. Muse cells account for about 0.01–0.03 % of BM monocytes [11,28],0.01–0.2 % of peripheral blood cells [29],0.01–0.2 % of peripheral blood cells, and 1–6% of various MSCs [30]. Muse cells are a subset of MSCs that can be distinguished from other cells by SSEA-3. As a result, Muse cells were positive for both pluripotent markers (SSEA3) and mesenchymal markers (CD105), while non-Muse cells were only positive for mesenchymal markers (CD105) [32]. At present, the commonly used screening method is trypsinization combined with adherent-suspension culture method [11], Flow cytometry screening and magnetic sorting methods, but FACS screened cells cannot be directly used in animal experiments, the screening process is expensive, and flow sorting is more damaging to cells. Two studies documented the use of MACS to screen Muse cells from bone marrow mesenchymal cells and adipose mesenchymal cells, but only 77.1 % and 71.3 % of cells were screened with SSEA3+ [33],However, trypsinization combined with adherent-suspension culture method for the extraction of Muse has a longer cycle. This study was the first time to screen Muse cells with MACS method from umbilical cord mesenchymal cells, and the percentage of SSEA3+ reached 94.933 %.

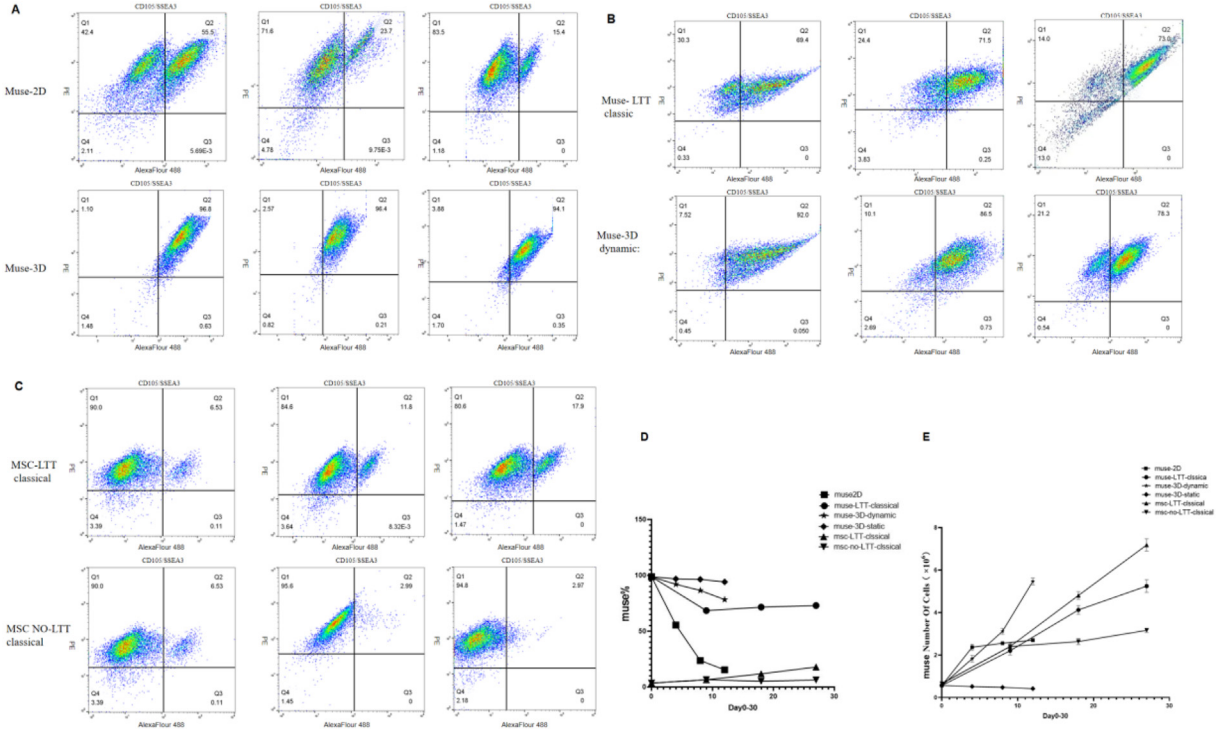

Fig. 6. Cultivation method of Muse cells. **A-C:** Muse-2D, Muse-3D static, Muse classic, Muse-3D dynamic, MSC LTT classic, and MSC no LTT classic. The positive ratio of SSEA3 and CD105 in each group. **D:** Trend curve of Muse content in each group (SSEA3, CD105 double positive). **E:** Value curve of Muse cells in each group.

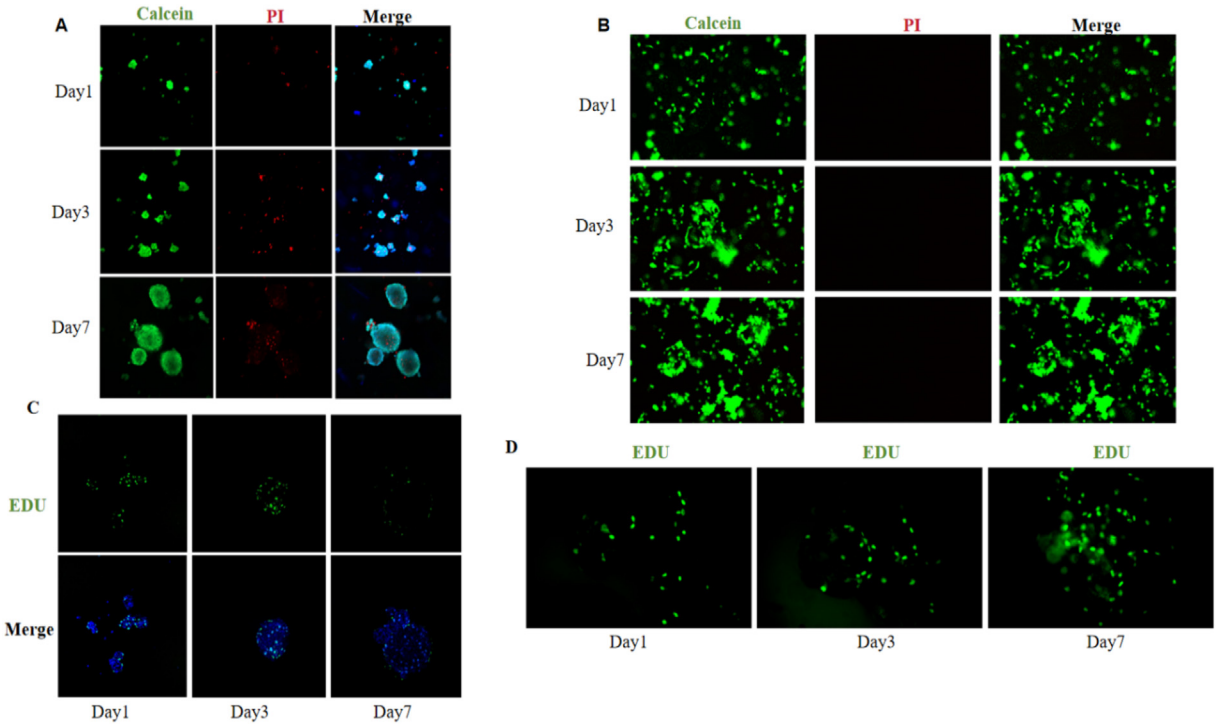

Fig. 7. Apoptosis and proliferation assay of Muse cells during 3D culture process. **A:** Muse-3D static for 1 day, 3 days, 7 days, culture Muse cells, Calcein AM/PI staining. **B:** Muse-3D dynamic for 1 day, 3 days, 7 days, culture Muse cells, Calcein AM/PI staining. **C:** Muse-3D static for 1 day, 3 days, 7 days, culture Muse cells, EDU staining. **D:** Muse-3D dynamic for 1 day, 3 days, 7 days, culture Muse cells for EDU staining.

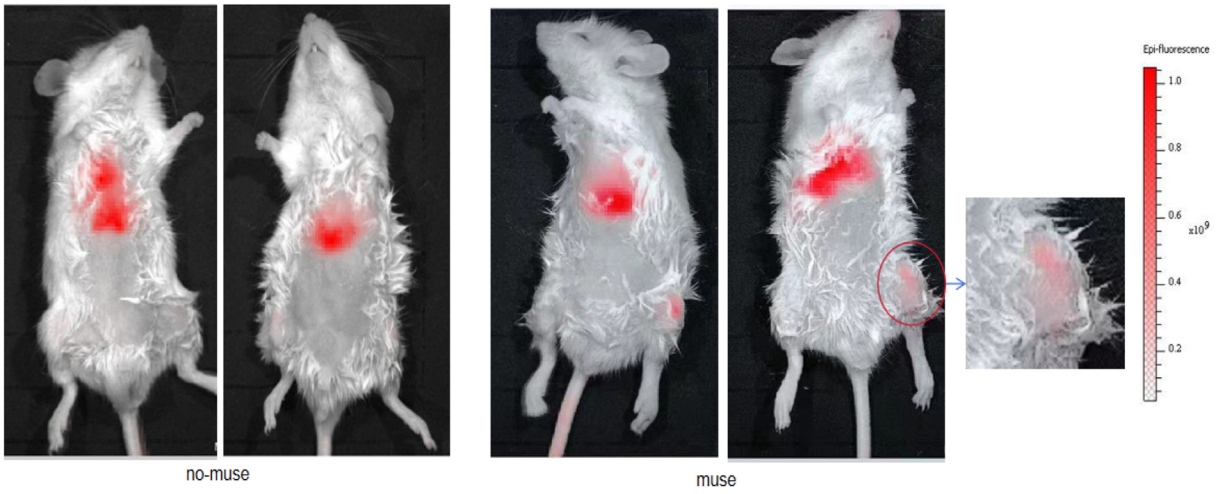

Fig. 8. Localization map of Muse cells and non-Muse cells in small animal live imaging.

Studies have shown that Muse cells express pluripotency-related genes, such as OCT3/4, SOX2, Nanog, while no-Muse cells hardly express them [13]. In this study, it was found that Muse cells expressed high pluripotency markers such as SSEA3, OCT3/4, SOX2, and Nanog, while no-Muse cells expressed almost no or low expression of cell pluripotency markers, which was similar to the above results. In addition, individual Muse cells can form ESCs-like clusters in suspension and exhibit trigerm differentiation potential, whereas single non-Muse cells cannot survive in suspension [11]. Muse cells are capable of differentiating into mesodermal lineage cells [23], endodermal lineage cells [34] and ectodermal lineage cells [35]. In contrast, the ability of mesenchymal stem cells to differentiate is limited to adipocytes, osteocytes, chondrocytes, and hepatocytes [36]. The differentiation ratio is lower than that of Muse cells. After inducing differentiation between Muse and no-Muse, it was found that the calcium nodules (red) appeared in alizarin red staining of Muse cells were significantly higher than those in no-Muse group, and the oil droplets in Muse group were significantly larger than those in no-Muse after adipogenic induced differentiation, and the purple-red Muse group was deeper than that in no-Muse group after periodic acid (glycogen PAS) staining, and the fluorescence intensity of Muse group was higher than that

in no-Muse group after fluorescence staining of Nestin. Therefore, in summary, the trigger layer differentiation ability of Muse was significantly higher than that of the no-Muse group, which may be related to the high expression of SSEA3 in Muse. OCT3/4; SOX2; The cause of Nanog is related because of the pluripotency factor Nanog; OCT4 and SOX2 play a key role in maintaining cell pluripotency (KRGG database).Studies have shown that in the absence of SOX2, human embryonic stem cells are unable to retain their pluripotency and neural progenitor cell properties [37], and high levels of OCT4 lead to the opening of differentiation-related enhancers [38]. Therefore, OCT4 is an enhancer of stem cell differentiation.

In the adherent culture state, Muse cells proliferate by symmetrical and asymmetric cell division, which means that one Muse cell will produce a non-Muse cell and a new Muse cell [11], As a result, the percentage of Muse cells in the total cell population gradually decreases. In this study, it was found that the positive rate of SSEA3 in adherent cultured Muse cells (Muse-2D) continued to decrease, and there was no significant proliferation of Muse cells. And studies have shown that the expression levels of OCT3/4, Nanog, and SOX2 genes in Muse cells were significantly increased in suspension compared to adherent cells [23], and the expression of OCT3/4, Nanog, and SOX2 was significantly increased when Muse cells in adherent culture were transferred to suspension, which was 50 to hundreds of times higher than that in adherent culture. It may be that 2D culture only supports stem cell growth in one plane, and cannot reproduce the real 3D stereoscopic microenvironment of cells in an organism. The 2D culture environment is far inferior to the 3D culture in many aspects such as biological activity, medium structure, and nutrient release, so that stem cells gradually lose their original characteristics, morphology, structure and function, resulting in their research results are often inconsistent with the results of in vivo tests, and the accuracy is low, and the culture environment provided is very different from the microenvironment in vivo, which will inevitably have a negative effect on the proliferation and differentiation of stem cells. According to the above speculation, 2D culture of Muse is a type of stem cell, and there is also a certain effect on the loss of stemness of Muse cells. Studies have shown that traditional 3D cell culture slows down cell proliferation [39]. Ki67 expression or BrdU expression is reduced and shows reduced proliferative potential [40–42], Cell death inside the spheroid may be due to insufficient oxygen and nutrient permeation. In addition, this study showed that a large number of dead cells were observed in the spheroids by live/dead staining, which may also be due to poor nutrient availability and limited oxygen flow through the internal structures of the spheroids [43,44]. Interestingly, although the medium and oxygen concentrations of spheroid cores may be reduced leading to apoptosis, their stem cell-associated transcription factors appear to be elevated, which has been confirmed in many studies [43,45,46]. Therefore, it is crucial to find a culture method that can maintain the characteristics of stem cells while maintaining the proliferative potential of cells.

One study found [11], After trypsinizing (LTT), the unsorted MSC cells were added to a low-adsorption culture flask for 7 days, and the cells were aggregated into small balls, and then seeded into ordinary culture flasks for adherent culture, and then LTT, then spherical, and then adherent, so that the method of repeating multiple cycles can finally obtain high-purity Muse and can also be amplified by Muse, and this experiment confirmed that LTT can improve the purity of Muse. Therefore, trypsinization is the most critical step in this method, and the purity of Muse cells cannot be guaranteed without LTT digestion, so the proliferation and purity of Muse cells in MSC LTT classic in this experiment are higher than those of MSC no-LTT classic. Although this method can finally obtain high-purity Muse cells and can also allow Muse cells to be expanded, the procedure is more complex and the cycle time is longer. Although the Muse cells were proliferated by this method, the expected effect was not as good as that of the Muse-3D dynamic method. Bioreactor cultured stem cells can facilitate clinical-scale expansion and maintain differentiation potential, which is critical for cell manufacturing [47]. Therefore, a series of explorations were carried out by using the method of microcarrier combined with bioreactor to culture Muse.

Muse cells are usually dormant and only become active when the cells are subjected to extreme stress [11]. Intravenously injected Muse cells can all recognize the location of the injured site and migrate specifically to the damaged site. Sphingosine-1-phosphate (S1P) is an alarm signal that attracts Muse cells to the site of injury, primarily through the expression of sphingosine-1-phosphate receptor 2 (S1PR) on the surface of Muse cells, causing them to return preferentially to the site of injury [48]. In this study, the homing ability of Muse cells and no-Muse cells was explored through animal models of musculoskeletal muscle injury, and it was found that Muse had stronger homing ability.

The above results indicate that Muse cells have stronger pluripotency and triple germ differentiation ability compared to non-Muse cells, and their homing and targeting abilities are better than those of non-Muse cells in animal skeletal muscle injury models. Moreover, through comparison of various Muse cell culture methods, it was found that the microcarrier stirring method can achieve the amplification and cultivation of Muse cells.

Muse cells have also been studied for clinical trials. Japan's Mitsubishi Chemical Holdings' Life Sciences Research Institute LTD is conducting clinical studies of a Muse cell product called CL2020. Six clinical trials using the intravenous donor CL2020 are currently underway. Their studies include acute myocardial infarction (beginning in February 2018), ischemic stroke (September 2018), epidermolysis bullosa (December 2018), spinal cord injury (July 2019), neonatal hypoxic encephalopathy (January 2020), and amyotrophic lateral sclerosis (January 2021) [49–54]. The preliminary results of the above six clinical trials showed that Muse cells infusion did not cause serious adverse reactions, and the efficacy of subacute ischemic stroke and acute myocardial infarction was more prominent. However, in general, the number of enrolled patients was small, and more accurate efficacy judgement needs to be verified by more patients enrolled in phase II and III clinical trials.

Author contributions

ZL, SR, XM and ZW performed literature search, study design, and paper writing; ZL, SR, BW and YZ performed experiments and data collection; ZL, SR and YZ performed data analysis.

Ethics statement

Healthy pregnant women voluntarily donate their umbilical cord at Qilu Hospital of Shandong province after signing an informed consent form for the isolation and cultivation of umbilical cord derived mesenchymal stem cells (MSCs). Written informed consent was obtained from these mothers before the study. All procedures were approved by the Ethics Committee of Fangzi District People's Hospital of Shandong Province (ethical approval number: YCU20220019).

Declaration of competing interest

The authors declare no competing interests.

Acknowledgement

This study was supported by research fundings from Shandong First medical University and Shandong Academy of Medical Science, and funding from Shandong Provincial Natural Science Foundation to XM (ZR2022MC025).

References

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

[2] Kuroda Y, Wakao S, Kitada M, Murakami T, Nojima M, Isolation Dezawa M. Culture and evaluation of multilineage-differentiating stress-enduring (muse) cells. Nat Protoc 2013;8:1391–415. https://doi.org/10.1038/nprot.2013.076.

[3] Fus-Kujawa A, Mendrek B, Trybus A, Bajdak-Rusinek K, Stepien KL, Sieron AL. Potential of induced pluripotent stem cells for use in gene therapy: history, molecular bases, and medical perspectives. Biomolecules 2021;11:699. https://doi.org/10.3390/biom11050699.

[4] Simerman AA, Perone MJ, Gimeno ML, Dumesic DA, Chazenbalk GD. A mystery unraveled:nontumorigenic pluripotent stem cells in human adult tissues. Expet Opin Biol Ther 2014;14:917–29. https://doi.org/10.1517/14712598.2014.900538.

[5] Damdimopoulou P, Rodin S, Stenfelt S, Antonsson L, Tryggvason K, Hovatta O. Human embryonic stem cells. Best Pract Res Clin Obstet Gynaecol 2016 Feb;31:2–12. https://doi.org/10.1016/j.bpobgyn.2015.08.010. Epub 2015 Sep 11. PMID: 26602389.

[6] Wu DC, Boyd AS, Wood KJ. Embryonic stem cell transplantation: potential applicability in cell replacement therapy and regenerative medicine. Front Biosci 2007 May 1;12:4525–35. https://doi.org/10.2741/2407. PMID: 17485394.

[7] Lee AS, Tang C, Cao F, Xie X, van der Bogt K, Hwang A, et al. Effects of cell number on teratoma formation by human embryonic stem cells. Cell Cycle 2009 Aug 15;8(16):2608–12. https://doi.org/10.4161/cc.8.16.9353. Epub 2009 Aug 24. PMID: 19597339; PMCID: PMC2866168.

[8] Malik N, Rao MS. A review of the methods for human iPSC derivation. Methods Mol Biol 2013;997:23–33. https://doi.org/10.1007/978-1-62703-348-0_3. PMID: 23546745; PMCID: PMC4176696.

[9] Olgasi C, Cucci A, Follenzi A. iPSC-derived liver organoids: a journey from Drug screening, to disease modeling, arriving to regenerative medicine. Int J Mol Sci 2020 Aug 27;21(17):6215. https://doi.org/10.3390/ijms21176215. PMID: 32867371; PMCID: PMC7503935.

[10] Bertolo A, Mehr M, Janner-Jametti T, Graumann U, Aebli N, Baur M, et al. An in vitro expansion score for tissue-engineering applications with human bone marrow-derived mesenchymal stem cells. J Tissue Eng Regen Med 2016 Feb;10(2):149–61. https://doi.org/10.1002/term.1734. Epub 2013 Apr 10. PMID: 23576360.

[11] Kuroda Y, Kitada M, Wakao S, Nishikawa K, Tanimura Y, Makinoshima H, et al. Unique multipotent cells in adult human mesenchymal cell populations. Proc Natl Acad Sci U S A 2010;107(19):8639–43.

[12] Leng ZK, Gao ZC, He XJ, Zhao YJ, Sun LJ, Zhai JJ, et al. Cultivation, screening, identification and transplantation of Muse cell from human umbilical cord-derived for spinal cord injury in rats. China J Orthop Traumatol 2019;32:327~334.

[13] Dezawa M, Wakao S, Kitada 14M, Kuroda Y, Shigemoto T, Matsuse D, et al. Muse cells provide the pluripotency of mesenchymal stem cells: direct contribution of Muse cells to tissue regeneration. Cell Transplant 2016;25(5):849–61. https://doi.org/10.3727/096368916X690881.

[14] Velasco MG, Satué K, Chicharro D, Martins E, Torres-Torrillas M, Peláez P, et al. Multilineage-differentiating stress-enduring cells (muse cells): the future of human and veterinary regenerative medicine. Biomedicines 2023;11(2):63. https://doi.org/10.3390/biomedicines11020636. Published 2023 Feb 20.

[15] Ogura F, Wakao S, Kuroda Y, Tsuchiyama K, Bagheri M, Heneidi S, et al. Human adipose tissue possesses a unique population of pluripotent stem cells with nontumorigenic and low telomerase activities: potential implications in regenerative medicine. Stem Cell Dev 2014;23(7):717–28. https://doi.org/10.1089/scd.2013.0473.

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

[17] Fei W, Wu J, Gao M, Wang Q, Zhao YY, Shan C, et al. Multilineage-differentiating stress-enduring cells alleviate atopic dermatitis-associated behaviors in mice. Stem Cell Res Ther 2021 Dec 20;12(1):606. https://doi.org/10.1186/s13287-021-02671-5. PMID: 34930455; PMCID: PMC8686553.

[18] Kuroda Y, Wakao S, Kitada M, Murakami T, Nojima M, Dezawa M. Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. Nat Protoc 2013;8(7):1391–415. https://doi.org/10.1038/nprot.2013.076.

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

[20] Leng Z, Sun D, Huang Z, Tadmori I, Chiang N, Kethidi N, et al. Quantitative analysis of SSEA3+cells from human umbilical cord after magnetic sorting. Cell Transplant 2019;28(7):907–23. https://doi.org/10.1177/0963689719844260.

[21] Yabuki H, Wakao S, Kushida Y, Dezawa M, Okada Y. Human multilineage-differentiating stress-enduring cells exert pleiotropic effects to ameliorate acute lung I schemia–reperfusion injury in a rat model. Cell Transplant 2018;27(6):979–93. https://doi.org/10.1177/0963689718761657.

[22] Fouad AM, Gabr MM, Abdelhady EK, Zakaria MM, Khater SM, Ismail AM, et al. In vitro differentiation of human multilineage differentiating stress-enduring (Muse) cells into insulin producing cells[J]. J Genet Eng Biotechnol 2018;16(2):433–40.

[23] Iseki M, Kushida Y, Wakao S, Akimoto T, Mizuma M, Motoi F, et al. Muse cells, nontumorigenic plu ripotent-like stem cells, have liver regeneration capacity through speci fic homing and cell replacement in a mouse model of liver fibrosis. Cell Transplant 2017;26(5):821–40.

[24] Abe T, Aburakawa D, Niizuma K, Iwabuchi N, Kajitani T, Wakao S, et al. Intravenously transplanted human multilineage-differentiating stress-enduring cells afford brain rep air in a mouse lacunar stroke model. Stroke 2019;51(2). STROKEAH A119026589.

[25] Mahmoud EE, Kamei N, Wakao S, Dezawa M. Adachi N.,et al. Therapeutic potential of multilineage-differe ntiating stress-enduring cells for osteochondral repair in a Rat model. St em Cells Int 2017;8154569. https://doi.org/10.1155/2017.

[26] Fei W, Wu J, Gao M, Wang Q, Zhao YY, Shan C, et al. Multilineage-differentiating stress-enduring cells alleviate atopic dermatitis-as sociated behaviors in mice. Stem Cell Res Ther 2021:12.

[27] Fukase M, Sakata N, Wakao S, Unno M, Dezawa M. Intravenous injection of human multilineage-differe ntiating stress-enduring cells alleviates mouse severe acute pancreatitis with out immunosuppressants. Surg Today 2022 Apr;52(4):603–15. doi:10.1007.

[28] Wakao S, Akashi H, Kushida Y, Dezawa M. Muse cells, newly found non-tumorigenic pluripotent stem cells, reside in human mesenchymal tissues. Pathol Int 2014;64(1):1–9. https://doi.org/10.1111/pin.12129.

[29] Tanaka T, Nishigaki K, Minatoguchi S, Nawa T, Yamada Y, Kanamori H, et al. Mobilized Muse cells after acute myocardial infarction predict cardiac function and remodeling in the chronic phase.

[30] Nicola A, Tiziana S, Servet Ö, Di Giovanni B, Massimo V, Mariarosa M, et al. Stress and stem cells: adult Muse cells tolerate extensive genotoxic stimuli better than mesenchymal stromal cells. Oncotarget 2018;9(27):19328–41. https://doi.org/10.18632/oncotarget.25039.

[31] Nicola A, Tiziana S, Servet zcan, Di Bernardo G, Venditti M, Melone M, et al. Stress and stem cells: adult Muse cells tolerate extensive genotoxic stimuli better than mesenchymal stromal cells. Oncotarget 2018;9(27):19328–41. https://doi.org/10.18632/oncotarget.25039.

[32] Fisch SC, Gimeno ML, Phan JD, Simerman AA, Dumesic DA, Perone MJ, et al. Pluripotent nontumorigenic multilineage differentiating stress enduring cells (Muse cells): a seven-year retrospective. Stem Cell Res Ther 2017 Oct 18;8(1):227. https://doi.org/10.1186/s13287-017-0674-3. PMID: 29041955; PMCID: PMC5646122.

[33] Uchida H, Niizuma K, Kushida Y, Wakao S, Tominaga T, Borlongan CV, et al. Human muse cells reconstruct neuronal circuitry in subacute Lacunar stroke model. Stroke 2017 Feb;48(2):428–35. https://doi.org/10.1161/STROKEAHA.116.014950. Epub 2016 Dec 20. PMID: 27999136; PMCID: PMC5262965.

[34] Amin M, Kushida Y, Wakao S, Kitada M, Tatsumi K, Dezawa M. Cardiotrophic growth factor-driven induction of human muse cells into cardiomyocyte-like phenotype. Cell Transplant 2018 Feb;27(2):285–98. https://doi.org/10.1177/0963689717721514. PMID: 29637816; PMCID: PMC5898685.

[35] Uchida H, Morita T, Niizuma K, Kushida Y, Kuroda Y, Wakao S, et al. Transplantation of unique subpopulation of fibroblasts, muse cells, ameliorates experimental stroke possibly via Robust neuronal differentiation. Stem Cell 2016 Jan;34(1):160–73. https://doi.org/10.1002/stem.2206. Epub 2015 Sep 28. PMID: 26388204.

[36] Zhu X, Yan T, Cheng C, Ma J, Xiang J, Lv Y, et al. Mesenchymal stem cells (MSCs) in targeted Drug Delivery: literature review and exploratory data on migrating and differentiation capacities of bone MSCs into hepatic progenitor cells. Curr Top Med Chem 2021 Oct 5;21(14):1251–67. https://doi.org/10.2174/1568026621666210708092728. PMID: 34238160.

[37] Zhang S, Bell E, Zhi H, Brown S, Imran SAM, Azuara V, et al. OCT4 and PAX6 determine the dual function of SOX2 in human ESCs as a key pluripotent or neural factor. Stem Cell Res Ther 2019 Apr 18;10(1):122. https://doi.org/10.1186/s13287-019-1228-7. PMID: 30999923; PMCID: PMC6471829.

[38] Strebinger D, Deluz C, Friman ET, Govindan S, Alber AB, Suter DM. Endogenous fluctuations of OCT4 and SOX2 bias pluripotent cell fate decisions. Mol Syst Biol 2019 Sep;15(9):e9002. https://doi.org/10.15252/msb.20199002. PMID: 31556488; PMCID: PMC6759502.

[39] Rybkowska P, Radoszkiewicz K, Kawalec M, Dymkowska D, Zabłocka B, Zabłocki K, et al. The metabolic changes between monolayer (2D) and three-dimensional (3D) culture conditions in human mesenchymal stem/stromal cells derived from adipose tissue. Cells 2023 Jan 1;12(1):178. https://doi.org/10.3390/cells12010178. PMID: 36611971; PMCID: PMC9818744.

[40] Kaminska A, Wedzinska A, Kot M, Sarnowska A. Effect of long-term 3D spheroid culture on WJ-MSC. Cells 2021 Mar 24;10(4):719. https://doi.org/10.3390/cells10040719. PMID: 33804895; PMCID: PMC8063822.
[41] Baraniak PR, McDevitt TC. Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. Cell Tissue Res 2012 Mar;347(3):701–11. https://doi.org/10.1007/s00441-011-1215-5. Epub 2011 Aug 11. PMID: 21833761; PMCID: PMC4149251.
[42] Yang E, Liu N, Tang Y, Hu Y, Zhang P, Pan C, et al. Generation of neurospheres from human adipose-derived stem cells. BioMed Res Int 2015;2015:743714. https://doi.org/10.1155/2015/743714. Epub 2015 Feb 26. PMID: 25815334; PMCID: PMC4357140.
[43] Son YB, Bharti D, Kim SB, Jo CH, Bok EY, Lee SL, et al. Comparison of pluripotency, differentiation, and mitochondrial metabolism capacity in three-dimensional spheroid formation of dental Pulp-derived mesenchymal stem cells. BioMed Res Int 2021 Jul;13:5540877. https://doi.org/10.1155/2021/5540877. PMID: 34337022; PMCID: PMC8294966.
[44] Murphy KC, Hung BP, Browne-Bourne S, Zhou D, Yeung J, Genetos DC, et al. Measurement of oxygen tension within mesenchymal stem cell spheroids. J R Soc Interface 2017 Feb;14(127):20160851. https://doi.org/10.1098/rsif.2016.0851. PMID: 28179546; PMCID: PMC5332570.
[45] Liu Y, Muñoz N, Tsai AC, Logan TM, Ma T. Metabolic reconfiguration supports reacquisition of primitive phenotype in human mesenchymal stem cell aggregates. Stem Cell 2017 Feb;35(2):398–410. https://doi.org/10.1002/stem.2510. Epub 2016 Oct 26. PMID: 27726254.
[46] Cheng NC, Chen SY, Li JR, Young TH. Short-term spheroid formation enhances the regenerative capacity of adipose-derived stem cells by promoting stemness, angiogenesis, and chemotaxis. Stem Cells Transl Med 2013 Aug;2(8):584–94. https://doi.org/10.5966/sctm.2013-0007. Epub 2013 Jul 11. PMID: 23847001; PMCID: PMC3726138.
[47] Dos Santos F, Campbell A, Fernandes-Platzgummer A, Andrade PZ, Gimble JM, Wen Y, et al. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. Biotechnol Bioeng 2014 Jun;111(6):1116–27. https://doi.org/10.1002/bit.25187. Epub 2014 Feb 4. Erratum in: Biotechnol Bioeng. 2015 Mar;112(3):644. PMID: 24420557.
[48] Cao J, Yang Z, Xiao R, Pan B. Regenerative potential of pluripotent non-tumorgenetic stem cells: multilineage differentiating stress enduring cells (Muse cells). Regen Ther 2020 Jul 27;15:92–6. https://doi.org/10.1016/j.reth.2020.04.011. PMID: 33426206; PMCID: PMC7770368.
[49] Noda T, Nishigaki K, Minatoguchi S. Safety and efficacy of human Muse cell based product for acute myocardial infarction in a first-in-human trial. Circ J 2020;84:1189–1192.
[50] Niizuma K, Osawa SI, Endo H, Izumi SI, Ataka K, Hirakawa A, et al. Randomized placebo-controlled trial of CL2020, an allogenic Muse cell–based product, in subacute ischemic stroke. J Cerebr Blood Flow Metabol 2023;43:2029–39.
[51] Fujita Y, Nohara T, Takashia S, Natsuga K, Adachi M. Yoshida K.,et al. Intravenous allogeneic multilineage differentiating stress enduring cells in adults with dystrophic epidermolysis bullosa: a phase 1/2 open-label study. J Eur Acad Dermatol Venereol 2021;35:e528–31.
[52] Koda M, Imagama S, Nakashima H, Ito S, Segi N, Ouchida J, et al. Safety and feasibility of intravenous administration of a single dose of allogenic-Muse cells to treat human cervical traumatic spinal cord injury: a clinical trial. Stem Cell J Stem Cell Res 2024;15:1–9.
[53] Yamashita T, Nakano Y, Sasaki R, Tadokoro K, Omote Y, Yunoki T, et al. Safety and clinical effects of a Muse cell based product in patients with amyotrophic lateral sclerosis: results of a phase 2 clinical trial. Cell Transplant 2023;32:1–12.
[54] Matsuyama N, Shimizu S, Ueda K, et al. Safety and tolerability of a multilineage differentiating stress-enduring cell-based product in neonatal hypoxic-ischaemic encephalopathy with therapeutic hypothermia (SHIELD trial): a clinical trial protocol open-label, non-randomised, dose-escalation trial. BMJ Open 2022;12:e057073.