A modified preplate technique for efficient isolation and proliferation of mice muscle-derived stem cells

Zhuqiu Xu · Lu Yu · Haibin Lu · Weifeng Feng · Lulu Chen · Jing Zhou · Xiaonan Yang · Zuoliang Qi

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Abstract We modified an existing protocol to develop a more efficient method to acquire and culture muscle-derived stem cells (MDSCs) and compared the characteristics of cells obtained from the two methods. This method is based on currently used multistep enzymatic digestion and preplate technique. During the replating process, we replaced the traditional medium with isolation medium to promote fibroblast-like cell adherence at initial replating step, which shortened the purifying duration by up to 4 days. Moreover, we modified the culture container to provide a stable microenvironment that promotes MDSC adherence. We compared the cell morphology, growth curve and the expression of specific markers (Sca-1, CD34, PAX7 and Desmin) between the two cell groups separately obtained from the two methods. Afterwards, we compared the neural differentiation capacity of MDSCs with other muscle-derived cell lineages. The protocol developed here is a fast and effective method to harvest and purify MDSCs from mice limb skeletal muscle.

Keywords Regenerative medicine · Cell and tissue culture · Myogenic stem cells · In vitro culture

Introduction

Myogenic cells are categorized into two types, satellite cells and muscle-derived stem cells (MDSCs), according to morphology, the expression of surface markers, and the ability to differentiate. In the past, satellite cells, which are capable of constant proliferation, self-renewal, and myogenesis, have been considered to be the only myogenic stem cells (Morgan and Partridge 2003; Collins et al. 2005). Subsequently, slowly adhering cells were isolated and identified from interstitial spaces of rodent skeletal muscles through specific markers, such as CD34, Sca-1, Desmin, and Pax7, and were characterized by a strong capacity for self-renewal and multipotent differentiation (Lee et al. 2000; Tamaki et al. 2002; Cottle et al. 2017). This finding led to the concept of MDSCs. MDSCs are able to differentiate into myogenic (Qu-Petersen et al. 2002), neural (Arsic et al. 2008), osteogenic (Lee et al. 2000), and hematopoietic lineages (Jackson et al. 1999). MDSCs, with their impressive ability to differentiate and self-renew and immune-privileged behavior in transplantation, are promising stem cells for clinical treatment (Chen et al. 2016; Han et al. 2017; Vojnits et al. 2017). In previous studies, MDSCs have been demonstrated to have the ability to promote

Z. Xu · L. Yu · H. Lu · W. Feng · L. Chen ·
J. Zhou · X. Yang (✉) · Z. Qi (✉)
Chinese Academy of Medical Science, Peking Union
Medical College, Plastic Surgery Hospital,
Beijing 100041, China
e-mail: yxnan@aliyun.com

Z. Qi
e-mail: public_qi@163.com

damaged skeletal muscle recovery (Lee et al. 2000; Tamaki et al. 2005), dysfunctional smooth muscle recovery (Zamboni et al. 2014; Nakajima et al. 2017) and peripheral nerve regeneration (Lavasani et al. 2014) through myogenesis and neurogenesis. In some cases, researchers grafted human MDSCs into mice which still presented therapeutic capacity (Lee et al. 2012; Lavasani et al. 2014). Even though muscle tissue is abundant and accessible, the intricate isolation techniques and lengthy culture times limit the application of MDSCs. On the basis of surface markers and physiological properties, various methods have been established to obtain and purify MDSCs, including fluorescence-activated cell sorting (FACS) (Tamaki et al. 2011) magnetic cell sorting (MCS) (Agley et al. 2015), density-gradient centrifugation (Che et al. 2011), and modified preplate techniques (Lavasani et al. 2013). Methods based on marker expression could be used to purify MDSCs in a short time; however, the surface markers vary and change during the culture, restricting the application of marker-profile-dependent isolation methods (Wu et al. 2010). At present, the most prevalent method used to isolate MDSCs is the preplate technique based on adhesion characteristics (Gharaibeh et al. 2008; Li et al. 2010). The preplate technique commonly requires 4 days to prepare collagen-coated flasks, 8 days to preplate, and an additional 2 weeks for cells to expand, which is complicated and time-consuming. Our own use of the method has failed to generate adequate samples for analysis. Hence, we attempted to modify the preplate technique by combining it with multistep enzymatic digestion according to the localization of MDSCs (Li et al. 2010; Meng et al. 2014). Moreover, we improved the medium and culture container (Table 1). We compared the two methods through morphological observation, growth curves, surface marker detection and by determining

differentiation capacity. The results indicated that our procedure takes less time to purify MDSCs and to obtain sufficient cells, generating $5.0\text{--}5.3 \times 10^4$ cells/cm² after a 1-week proliferation period. Additionally, we contrasted cells acquired from the two different methods at the same plating stage (PP2). The results showed that our method obtains cells with a greater differentiation capacity and expression of specific markers. Here, we describe the protocol of our technique and our analytical results.

Materials and methods

Solution preparation

Reagents and modified technique for cell isolation and culture

Collagenase-type XI: 0.2% (wt/vol) (Cat. #C7657, Sigma-Aldrich, St. Louis, MO, USA)

Dispase: 2.4 Unit/ml (Cat. #17105-041, Invitrogen, Carlsbad, CA, USA)

Trypsin–EDTA: 0.1% (wt/vol) trypsin solution (Cat. #59418c, Sigma-Aldrich)

Phosphate Buffered Saline: (PBS, #sc-362182, Santa Cruz Biotechnology, Santa Cruz, CA, USA)

Iscove's Modified Dulbecco's Medium (IMDM, Cat. #12440061, Gibco Laboratories, Gaithersburg, MD, USA)

Fetal Bovine Serum (FBS; Cat. #10099141, Gibco Laboratories)

Chick embryo extract (CEE; Cat. #100-163P, Gemini, Victoria, Australia)

Horse Serum (HS; Cat. #16050122, Gibco Laboratories)

Basic-Fibroblast Growth Factor (b-FGF, Cat. #450-33, PeproTech, Rocky Hill, NJ, USA)

Table 1 The table presents the improvements and differences in several aspects between the traditional preplate method and the modified method

Culture procedure	Traditional method	Modified method
Biopsy	Skeleton muscle	Skeleton muscle
Digestion	Enzymatic digestion	Multi-step enzymatic digestion
Replating medium	MDSC proliferation medium	Isolation medium
Culture medium	MDSC proliferation medium	MDSC proliferation medium
Collagen coat	Yes	No
Culture container	Flask	96-well culture cluster
Time for purifying	7 days	3 days

Epidermal Growth Factor (EGF, Cat. #315-09-100, PeproTech)

Platelet-derived Growth Factor (PDGF-BB, Cat. #315-18, PeproTech)

Penicillin/streptomycin (P/S; Cat. #15140122, Gibco Laboratories)

Tissue digestion solution: The working concentrations are 0.2% (wt/vol) collagenase Type-XI, 2.4 U/mL dispase, and 0.1% (wt/vol) Trypsin–EDTA. All enzyme solutions were diluted in PBS and stored at -20°C . Solutions were pre-warmed to 37°C before use.

Isolation medium (IM) This medium applied in the preplate process promotes the adhesion of fibroblasts and satellite cells. It contains IMDM, 20% FBS, 10 ng/ml b-FGF, 0.4 ng/ml EGF, 20 ng/ml IGF, and 1% P/S.

Proliferation medium (PM) This medium is used for the expansion of MDSCs. It contains IMDM, 10% FBS, 10% HS, 0.5% CEE, 10 ng/ml b-FGF, 10 ng/ml PDGF-BB, 20 ng/ml EGF, and 1% P/S.

Neural induction medium This medium is used to induce MDSCs to differentiate into neural lineage cells. It contains IMDM, 20 ng/ml b-FGF, 20 ng/ml EGF, 0.2 ng/ml IGF, 0.5 ng/ml NT-3, 20 μM forskolin, 2% B27, and 1% P/S.

We filtered all solutions through disposable 0.22- μm sterile filters (Corning, New York, NY, USA). Solutions can be stored at 4°C up to 1 month.

Biopsies

We performed all animal experiments at the Animal Laboratory of the Plastic Surgery Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China. Wild-type mice (2 weeks old, C57BL/6J, female) and GFP mice (2 weeks old, female) were provided by the Biomedical Research Institute of Nanjing University, Nanjing, China. The animal was cervically dislocated and limb muscle were exposed. Gastrocnemius and soleus from the hindlimbs and biceps and triceps from forelimbs were freshly dissected, the muscles were kept intact and connective tissue was carefully removed. In this comparison test, we normally used 10 wild-type mice and obtained in total 4.5–5 g skeletal muscle for each group which could be adjusted according to the research demand. Ten GFP mice were used for GFP-MDSCs culture. We combined all biopsies in

centrifuge tubes with IMDM and 1% P/S and performed all procedures under a sterile culture hood. The muscle harvesting took no longer than 3 h and after isolation of all biopsies, we started the digestion procedures instantly.

Digestion

After harvesting, we removed IMDM and washed skeletal muscle pieces with D-PBS; then we centrifuged the samples at $1000\times g$ at 4°C for 5 min.

We removed the supernatant, re-suspended biopsies in 10 ml of pre-warmed 0.2% collagenase-type XI, and then shaken on a tube rocker for 1 h at 37°C .

We centrifuged the tissue-enzyme solution at $1000\times g$ at 4°C for 5 min, and then removed the supernatant. Slurries were re-suspended in 10 ml dispase (2.4 U/ml) solution, and then the slurries were rotated on a tube rocker for 45 min at 37°C .

The tissue-enzyme solution was centrifuged at $1000\times g$ at 4°C for 5 min and the supernatant was removed. Slurries were re-suspended in 0.1% trypsin solution, and then the slurries were rotated on a tube rocker for 30 min at 37°C .

10 ml IMDM with 20% FBS was added to terminate digestion; then we centrifuged the solution at $1000\times g$ at 4°C for 5 min and discarded the supernatant. The cell pellet was washed with D-PBS and re-suspend with 7–9 ml IM solution.

Replating

The cell suspension was passed through 100- μm , 70- μm , and 40- μm cell strainers successively. The cells were plated on a 100-mm culture dish and incubated at 37°C in a humidified incubator under 5% CO_2 for 12 h.

After incubating for 12 h, the adhering cell population was labeled as PP0 (preplate 0). The supernatant containing non-adherent cells was transferred into a new culture dish and labeled PP1 (preplate 1). Six milliliters of IM were added to PP0 and incubated for 24 h. If the supernatant contained a high percentage of fibroblast-like cells and muscle fibers, the procedure was repeated one more time.

After incubating for 24 h, the supernatant of the PP1 dish was transferred into a 15-ml tube and centrifuged at $1000\times g$ at 4°C for 5 min. The supernatant was discarded, and the pellet was re-

suspended with 10 ml PM. The cell suspension was centrifuged at $500\times g$ at 4 °C for 1 min to remove the remaining fiber debris.

The supernatant was transferred into a 96-well culture cluster, 100 µl per well containing 200–300 cells. The cluster was labeled as PP2, and the cells were incubated at 37 °C in a humidified 5% CO₂ incubator. Fifty percent of the culture medium was replaced with fresh PM every 2 days while avoiding moving the cluster as much as possible.

After incubating PP2 for 1 week, the cell population reached $1.5\text{--}1.7 \times 10^4$ cells/well, which was sufficient for further analysis and expansion.

Cell growth assay

In the modified method, we randomly selected cells in triplicate and completely digested the cells with 100 µl 0.1% trypsin–EDTA every 2 days after the adherence of PP3. Meanwhile, in the traditional preplate group, we transferred PP6 cells into a 24-well plate and cultured the cells for 24 h after adherence. We performed cell counts in triplicate and completed random selection and digestion as described previously. We counted cell number by a hemocytometer continually for at least 2 weeks, repeated the whole counting process three times and recorded the average value.

Flow cytometry

The percentages of CD34, Sca-1 and PAX7 positive cells in our method were analyzed by flow cytometry. Cultured cells were detached using 0.1% trypsin–EDTA, spun down, washed in cold 0.1% bovine serum albumin buffer (BSA, from BD Bioscience, San Jose, CA, USA) in PBS and counted by hemocytometer. The cells were suspended with 1 ml 0.1% BSA buffer and equally divided into four groups. 1 µl of fluorochrome-conjugated anti-mouse antibodies CD34 (from BD Bioscience), Sca-1 and PAX-7 (from Santa Cruz Biotechnology) was added and the cells were incubated for 20–30 min in a covered ice bucket. Excess antibody was washed away with 1 ml 0.1% BSA buffer following staining, cells were re-suspended with 500 µl 0.1% BSA buffer and passed through 40-µm cell strainers just before analysis. A minimum of 10,000 live cells were collected with a FACS Aria II flow cytometer (from Becton–

Dickinson, San Jose, CA, USA) and analyzed with FlowJo software (from Becton–Dickinson). The flow cytometry analysis was repeated three times for each group.

Neural induction

Cell clusters were divided into two groups. In one group, PM was removed, cells were washed with D-PBS gently, and then 100 µl of neural induction medium was added per well. PM was kept to act as a control group. Cells were incubated at 37 °C in a humidified 5% CO₂ incubator, observed and medium was changed every 2 day. We compared the induced PP1 and PP2 cells harvested from our method with the same methods given previously.

Immunofluorescence analysis

We performed immunofluorescence analysis on both non-differentiated and induced PP2 cells. All cells were washed with D-PBS 3 times, fixed by 3.5% formaldehyde for 15 min, permeabilized by 0.2% Triton X-100 in PBS for 5 min, and then pre-incubated for 40 min with 10% HS in PBS. Non-differentiated cells were washed in PBS and incubated in a dilution of primary antibody (Sca-1, CD34, Desmin, 1:100 in primary antibody dilute, all from Abcam, Cambridge, MA, USA) at 4 °C for 8 h. For the induced group and control group, cells were washed in PBS and incubated in a dilution of primary antibody (MBP, S100, β-III tubulin, 1:100 in primary antibody dilute, all from Abcam) at 4 °C for 8 h. Then cells were washed with D-PBS and incubated with corresponding secondary antibodies at 37 °C for 40 min. Nuclei were stained with DAPI (Abcam) for 30 s and observed under a microscope. Analysis of fluorescence was processed by ImageJ software (National Institutes of Health, <http://rsbweb.nih.gov/ij/index.html>).

Real-time polymerase chain reaction

Total RNAs were isolated using Trizol reagent (Invitrogen). We performed reverse transcription reactions with 500 ng/14 µl RNA in DEPC water and 1 µl Oligo(dT) (Promega, Madison, WI, USA). Reactions were heated in a 70 °C heat block for 5 min and then chilled in ice water for at least 5 min. 1 µl MLV, 1 µl dNTP, 1 µl Oligo(dT), 2 µl DEPC water,

and 5 µl buffer were added to each reaction followed by mixing. Annealing was proceeded in a heat block at 25 °C for 5 min, and extension continued at 42 °C for up to 1 h to obtain cDNA. The real-time polymerase chain reaction (PCR) included 2 µl cDNA, 0.6 µl each of forward and reverse primers (SBS Genetech, Beijing, China; sequences are shown in Table 2), and 10 µl SYBR Green Master Mix. We performed the program for 40 cycles (95 °C for 20 s, 60 °C for 20 s, 72 °C for 40 s) in triplicate and used mouse GAPDH as an internal control. We analyzed relative expression of mRNA using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen 2001).

Statistical analysis

We performed statistical analyses with SPSS Statistics Software 19.0 (Chicago, IL, USA). All data are shown as mean $\pm$ standard deviation (SD) of at least three independent experiments. We determined differences using independent t tests followed by Scheffe's post hoc tests; $p < 0.05$ was considered statically significant. We performed all detections and analyses synchronously on cell populations obtained using our modified protocol and the traditional preplate method, respectively.

Results

1. Comparison of the morphology, growth activity, and expression of specific markers in both methods.

We observed and compared the morphology of cells at every plating stage obtained from the two methods. The earliest adhering cell populations from the two methods were composed of fibroblasts and endothelial cells, and this continued into PP3–PP4 in

the traditional method. In our method, however, most of the fibroblasts were eliminated during the first plating (Fig. 1A and E). This was due to the isolation medium we used, which promotes the adherence of fibroblasts and simplifies the preplating process. The typical spindle morphology of muscle satellite cells appeared in the second plating stage (PP1), which had limited differentiation capacity (Figs. 1F, 4A). Meanwhile, in the traditional process, PP1 still presented fibroblast-like morphology (Fig. 1B). The comparison of PP1 cell morphology indicated the difference of adhering speed. We also observed small, rounded, floating cells in the medium of PP1. We then changed the isolation media to proliferation medium and transferred 100 µl of cell suspension per well to a 96-well culture cluster. In the PP6 stage of the traditional method and in PP2 of our modified method, cells were spherical and refractive and, in some cases, clustered as spheroids (Fig. 1C and G). Comparing the morphology and density of the last adhering cells from the two techniques, our technique obtained more cells in higher density and larger cell spheroids within the same expansion time (Fig. 1C, D, G and H). In the growth curve analysis, there were statistically significant differences between the two methods, and the adherent time for cells was 4 days prior to that in the traditional method. Comparisons between growth curves indicated that the proliferative activity of MDSCs obtained from the modified method was higher than that from the traditional method. Two weeks after biopsy, the total cell number in our procedure reached 17.5×10^5 , which is sufficient for further analysis, while we harvested less than 10×10^5 cells with the traditional method over the same timeframe (Fig. 1J). Additionally, we used the modified method to obtain MDSCs from GFP mice. The modified protocol generated sufficient MDSCs for observation and further grafting after a short period (Fig. 1K–N).

Immunocytochemistry performed on PP2 cells from our method and PP6 cells from the traditional method showed that both were Sca-1-, CD34-, and desmin-positive (Fig. 2A–L), indicating that late-adhering cells are muscle-derived and have stem cell properties that meet the standards of MDSCs. We performed semi-quantitative analysis of immunocytochemistry using ImageJ software and found differential expression of specific markers between the two cell groups (Fig. 2M).

Table 2 Real-time PCR primer sequences

Nestin	F: GCTCTGGGCCAGCACTCTAG R: TGTAGACAGGCAGGGCTAGCA
P75	F: GGCACCTGCTGCTCTGTAAT R: GTCTGAAGCTGGTGTCTGCT
Nrg1	F: AAGCAGACACCAGC1TCAGA R: ACAGTGCATTACCACGCAGA

F Forward primer, R Reverse primer

Fig. 1 The cellular morphology variation in different preplating stages and the growth curve of both methods. **A–D** Use of the traditional preplate culture methods; **A** Appearance of first adhering cells (PP0) on petri dish. PP0 are almost fibroblast-like cells. **B** Rapidly adhering cells (PP1) after one replating were mainly made up by fibroblast-like cells. **C** Slowly adhering cells (PP6) after repeating the preplate process six times were rounded and refractive. **D** After 2 weeks of culture, clustered cells formed some colonies, but the density was still low. **E–I** Use of the modified method; **E** PP0 mainly contained fibroblast-like cells. **F** In PP1 myoblast-like spindle

cells appeared. **G** PP2 consisted of rounded cells of refractive appearance which formed small cells sphere. **H** Colonies cultured for 14 days, the colonies grew and the cell number of each colony increased. **I** Higher magnification after 14 days of culture showed the specific morphology and details of the colonies. **J** Growth curve of two methods, X axis starts from the biopsy and the cell counting started from the adherence (**, $p < 0.01$, $n = 3$); **K, L** Use of the modified method to obtain GFP-MDSC, after 7-days of culture, we could obtain sufficient cell amount. **M, N** GFP-MDSC under higher magnification. Scale Bar = 50 μ m

To clarify the cell subpopulations in every preplating stage, we performed a flow cytometry analysis to demonstrate the Sca-1, CD34 and PAX-7 expression levels in PP0, PP1 and PP2 obtained from the modified method (Fig. 3). The portion of Sca-1-positive (Fig. 3B, C, D) and CD34-positive (Fig. 3F, G, H) cells increased following the preplating process. In early preplate PP0, only $4.5 \pm 1.8\%$ and $6.0 \pm 2.1\%$

($n = 3$) of the cells expressed Sca-1 and CD34, respectively (Fig. 3B and F). After being cultured with isolation medium, about $23.7 \pm 5.7\%$ ($n = 3$) of the cells expressed Sca-1, and $15.5 \pm 6.3\%$ ($n = 3$) of the cells expressed CD34 (Fig. 3C and G). When we transferred suspension cells into 96-well flasks and cultured them for 7 days, over 90% (Sca-1-positive = $91.9 \pm 6.6\%$, CD34-positive = $90.4 \pm 7.3\%$,

Fig. 2 Immunofluorescence staining of specific markers CD34, Sca-1 and Desmin. **A–F** Immunofluorescence staining showed the late adhering cells obtained by modified method were CD34, Sca-1 and Desmin positive which are specific markers of MDSCs. **G–L** Immunofluorescence staining showed the late

adhering cells obtained by the traditional method were positive as well. Omission of primary antibody served as negative control. **M** The semi-quantitative analysis of immunofluorescence staining by using ImageJ software, (* $p < 0.05$, $n = 5$). Scale Bar = 50 μm

$n = 3$) of the cells expressed Sca-1 and CD34 in PP2 (Fig. 3D and H, A and E were negative controls). The percentage of PAX7-positive cells showed the proportion of satellite cells in different preplating stages (Fig. 3J–L, I as negative control), which reached the peak in PP1 with $48.5 \pm 9.2\%$, ($n = 3$) cells (Fig. 3K). In PP2, there were only about $10.5 \pm 3.7\%$ ($n = 3$) cells expressing PAX7 (Fig. 3L). Taken together, these results suggest that our method can generate purified MDSCs in a more efficient and useful manner.

2. Comparison of neural differentiation potential between populations of muscle-derived cells.

To investigate and compare the neural differentiation potential of early adhering cells (PP1) and late-adhering cells (PP2), we induced the two cell groups and performed analyses of morphology, immunocytochemistry, and immunoblotting. We applied neural induction medium as described in previous methods (Lucas et al. 1995; Kelc et al. 2013). We recorded morphological changes daily. One week after induction, we observed evident morphological changes in

Fig. 3 Flow cytometry showed the positive rate of specific markers in PP0, PP1 and PP2 after cultured for 7 days. **B–D** Expression of Sca-1 in PP0, PP1 and PP2, **F–H** Expression of CD34 in PP0, PP1 and PP2. **A, E** show negative controls. There were only 3.36% cells expressing Sca-1 and 3.29% cells expressing CD34 in the first preplate PP0. Afterwards, the expression of Sca-1 and CD34 increased along with preplating

and over 90% cells expressed Sca-1 and CD34 in PP2. **J–L** Show the expression of PAX7 in every stage, **I** negative control. The expression of PAX7 reached a peak in PP1 with 56.5% cells, and in PP2 there were only 10.1% cells expressing PAX7. **J** Bar graph shows the average of positive cells (percentages $\pm$ SD, $n = 3$) in PP0, PP1 and PP2, respectively

PP1 cells (Fig. 4A–C). Morphological changes, however, in PP2 cells started to occur within 3 days after induction and were remarkable after 5 days, even with few undifferentiated colonies remaining (Fig. 4D–F). Immunocytochemistry showed that both PP1 cells induced for 2 weeks and PP1 cells induced for 5 days expressed P75, MBP, and Beta-III (Fig. 5A–L). Semi-quantitative analysis displayed higher average optical density of PP2 cells for all markers, even with a shorter induction time (Fig. 4M). We performed real-time PCR to measure mRNA levels of neurotrophin

receptor (P75), neural progenitor cell marker (Nestin), and glial cell marker (Neuregulin 1, NRG1) in differentiated PP1 and PP2 cells. Expression of p75 and Nestin was higher in induced PP2 cells relative to GAPDH, whereas the expression of neuregulin was not significantly different ($p > 0.05$) between the two cell groups (Fig. 5N). These results indicate that both PP1 and PP2 cells are capable of differentiating into neural lineages, although PP2 has stronger neural properties after induction.

Fig. 4 Neurogenic differentiation of diverse muscle-derived cell lineages. **A–C** Morphology of PP1 cells from the modified method treated with neural induction medium for 24 h, 48 h, 72 h. **D–F** Morphology of PP2 cells from the modified method

were treated with neural induction medium for 24 h, 48 h, 72 h. The typical morphology of neural lineages could be observed. Scale Bar = 50 μ m

Discussion

MDSCs, long categorized as satellite cells, are characterized by self-renewal and can regenerate muscle tissue (Tamaki et al. 2008). Recent research has identified a cell population with distinct expression of CD34 and Sca-1 and the ability to differentiate into other lineages. These are now categorized separately as muscle stem cells (Gussoni et al. 1999; Asakura et al. 2001; Lau et al. 2014). Based on these markers, several methods to isolate MDSCs have been reported, including fluorescence-activated cell sorting (FACS) and magnetic cell sorting (Kallestad and McLoon 2010; Agley et al. 2015). These techniques can isolate MDSCs in simple and fast processes with some degree of success. However, marker-oriented methods could be biased and limited because different cell populations may have common molecular markers (Jankowski et al. 2002). Furthermore, the expression of cell surface markers used to define and obtain MDSCs are variable and change under cell culture conditions (Machida et al. 2004; Zheng et al. 2007). Furthermore, the expression of cell surface markers used to define and obtain MDSCs are variable and change under cell culture conditions (Machida et al. 2004; Zheng et al.

2007; Lorant et al. 2018; Seta et al. 2018; Tamaki et al. 2015, 2016). These surface marker differences limited the techniques for MDSC purification that are based on marker expression such as flow cytometry sorting and magnetic-activated cell sorting. Human MDSCs share similar morphology, multi-potential and therapeutic ability in vitro and vivo (Lorant et al. 2018; Seta et al. 2018), but the surface markers are different from mice MDSCs. Unlike mouse muscle derived cells, which could be divided by Sca-1, CD34 and Pax7, Tamaki et al. (2015) indicated that the human muscle derived cells can be distinguished by CD34, CD45 and CD29. The mouse satellite cells that have strong myogenic potential are CD34(–)/Sca-1(–)/Pax7(+), whereas multi-potential MDSCs express CD34, Sca-1 but not Pax7. In cultures of human muscle-derived cells, satellite cells are CD34(–)/CD45(–)/CD29(+) and hMDSCs are CD34(+)/CD29(–) (Tamaki et al. 2016). These surface marker differences limited the techniques for MDSC purification that are based on marker expression such as flow cytometry sorting and magnetic-activated cell sorting. The classic method to obtain MDSCs is the preplate technique based on the specific location and slow adherence of the cells. Even though several other methods have been reported, the

Fig. 5 The immunofluorescence analysis of PP1 and PP2 cell groups obtained through the modified method, respectively, after 7-day induction. Cells were stained for immature Schwann cells marker P75, the myelin formation marker MBP and early neuronal marker neuronal class III β -tubulin, and DAPI for the nucleus. **A–F** Expression of PP2 acquired through the modified

method after Lee et al. (2012) induction. **G–L** Expression of PP1 after induction. **M** Semi-quantitative analysis of immunofluorescence performed with ImageJ software ($*p < 0.05$, $n = 5$). **N** Real-time PCR detected the relative mRNA level of P75, neuron marker Nestin and nueregulin-1 (NRG), ($*p < 0.05$). Scale Bar = 50 μ m

preplate technique is still the most common one because of its higher efficiency (Asakura et al. 2001; Gharaibeh et al. 2008; Park et al. 2014).

Our laboratory started with the traditional preplate method to harvest purified MDSCs, yet we were not able to acquire the desired quantity and quality of MDSCs within a reasonable time. We speculated that chronic preplating might reduce the activity of MDSCs (Khosravi-Farsani et al. 2015; Tamaki et al. 2015); hence, we have taken several measures to shorten the preplate duration (Table 1). Our first observation was that the culture medium for MDSCs

was not favorable for fibroblast and satellite cells to adhere, which may delay the purification step. After attempts with different media, we determined that isolation medium, which favors the adhesion of impure cells without affecting the activity of MDSCs (Montarras et al. 2005; Vukusic et al. 2013), was optimal. The most rapidly adhering cells were eliminated after repeating the preplate procedure two or three times, significantly shortening the isolation process compared with the traditional method, which requires at least six repetitions (Li et al. 2010; Lavasani et al. 2013).

Moreover, we compared culture dishes with or without collagen coating and found no significant advantage with coated dishes. Hence, we removed the collagen-coating step to simplify the process. Low-density purified MDSCs in a culture dish or flask may not be sufficient to form colonies, which limits MDSC proliferation (Lee et al. 2000; Gharaibeh et al. 2008). High-density culturing has been reported to improve colony formation and improve differentiation (Spradling et al. 2001; Xue et al. 2013). To improve the density and maintain a homeostatic environment for MDSCs, we moved the last adhering MDSCs into 96-well plates to promote cell clusters. We found that the 96-well clusters provide a more effective system to significantly improve MDSC growth. We speculate that the 96-well cluster provides a more stable physical environment, minimalizing the impact from movement during experiments, such as when changing medium, which might shorten the adherence time of MDSCs. MDSCs are located beneath the basement membrane, in the intermuscular space, where the stem cell niche is comparatively specific and constant (Lee et al. 2000; Qu-Petersen et al. 2002; Chen et al. 2013; Beane et al. 2014). In our lab, the modified protocol could be repeated by different investigators and MDSCs were able to maintain characters and stemness over 20 passages. The differentiation assessment indicated that slow-adhering cells have a stronger neural differentiation potential compared to that of fast-adhering cells, which mainly contain satellite cells and fibroblast cell. The life span and cell viability would affect the stemness and self-renewal ability (Lu et al. 2012; Kalvelyte et al. 2013); in this case, the modified method is able to shorten the life span and maintain the differentiating capacity of MDSCs, which would benefit applications in regenerative medicine. Further investigations into the extracellular matrix and key growth factors such as VEGF, IGF, and PDGF in different containers are required (Li et al. 2013; Ozasa et al. 2014; Wang et al. 2017). In some former studies, considering the difficulty of acquiring MDSCs, fast-adhering muscle-derived cells were applied as cell resources in regenerative medicine (Jackson et al. 2013; Saito et al. 2015). We compared the neural differentiation capacity of fast-adhering (PP1) and slow-adhering (PP2) cell populations. The morphological and genetic expression assessments indicated that slow-adhering cells, which mostly consist of MDSCs, exhibited a stronger neural

differentiation potential, meaning that once the obstacle of the culture process is settled, MDSCs could be more ideal than other muscle-derived cell groups as seed cells.

In conclusion, many special characteristics of MDSCs are still unknown, and future studies will reveal more properties and improve culture techniques. Our modified method is an effective and simplified approach to obtain MDSCs in a short time, the procedures and material are easy to reach as well, although further researches and analysis are needed.

Author's contribution ZX and LY performed the biopsy and cells culture, HL and WF took charge of the immunofluorescence and flow cytometry, LC and JZ accomplished Statistical analysis. ZX drafted the manuscript. Co-corresponding authors XY and ZQ designed and guided the study. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare they have no competing interests.

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