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Original Article

An easy and practical method for isolation, culture and identification of mouse skeletal muscle satellite cells

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Abstract: Satellite cells are skeletal muscle stem cells that have the ability of proliferation and differentiation, they are important for the homeostasis, hypertrophy and repair of skeletal muscle fibres. Finding a suitable method to isolate satellite cells is essential for understanding the biology of satellite cells, and skeletal muscle function. This chapter aims to provide convenient protocols used in our laboratory for the isolation, culture and identification of satellite cells from skeletal muscle. Our protocol isolate and characterize of satellite cells from mouse hind limb muscles, and the protocols are suitable for neonatal or adult mice, we can get enough dynamic satellite cells. And above all our protocol offer further advantages: cheap materials, simple operation and no need of special equipments, it can be applicable to the general basic laboratory.

Keywords: Skeletal muscle, satellite cells, desmin, myosin

Introduction

Satellite cells were first described in 1961 by their anatomical location between the basal lamina and sarcolemma of muscle fibers [1]. It was proved to be the myogenic precursor cell in postnatal muscle [2]. As we all know, myofibers are the basic contractile units of skeletal muscle, the development of myofibers and their regenerative potential depends on the availability of myogenic precursor cells [3]. So as the myogenic precursor cells, Satellite cells function are self-evident. For many years following its study, electron microscopy provided the only definitive method of identification. For the past few years, several molecular markers have been described that can be used to detect satellite cells, making them more accessible for study at the light microscope level, they are restricted to either the quiescent, activated, or proliferative state, such as pax7, Desmin [4]. And when satellite cell fusion into myotubes, it expression of muscle-specific structural proteins such as Myogenin, Sarcomeric Myosin [5]. In the juvenile growth phase, when muscles enlarge, satellite cells are proliferative and add nuclei to growing myofibers; however in most adult muscles they are typically quiescent, it can be activated when needed to generate myoblasts, which eventually differentiate to provide new myonuclei for the homeostasis, hypertrophy and repair of muscle fibres, or fuse together to form new myofibres for regeneration [3]. All in all, satellite cells are vital to skeletal muscle homeostasis and regeneration throughout life, and understanding the function of myogenic stem cells will likely provide valuable insights into muscle wasting in aging and disease. Primary satellite cells isolated directly from muscle tissue are more closely approximate myogenesis than established cell lines. Therefore, purification of a pure population of satellite cells from skeletal muscle is essential for understanding the biology of satellite cells, myoblasts and muscle regeneration, and for the development of clinical applications. Our study provides the method for isolation of mouse skeletal muscle satellite cells is simple reliable and fruitful, more important is that the satellite cells obtained by this method are more pure, besides this method is suit for neonatal or adult mouse.

Materials and methods

Animals, reagent and general equipment

Mouse (B129) was provide by Wannan Medical College. Cultures were prepared from muscles of neonatal (3-7-day-old) or adult (8-11-week-old) mouse.

Dulbecco's Modified Eagle Medium (DMEM) (Hyclone), 100× penicillin-streptomycin solution (Biosharp), Fetal bovine serum (FBS) (Gibco), Horse serum (HS) (hyclone), Phosphate buffer (PBS) (Hyclone), 70% ethanol, Trypsin (BOSTER), 4% paraformaldehyde (BOSTER), Saponin (SIGMA), primary antibodies included Rabbit-anti-desmin (Proteintech), Rabbit-anti-Myosin heavy chain 1 (Hope Biotechnology), secondary antibody included Alexa Fluor 594 chicken-anti-Rabbit (Invitrogen).

Standard humidified tissue culture incubator (37°C, 5% CO_2 in air), Tissue-culture laminar flow hood, dissecting microscope, Fluorescent inverted microscope (OLYMPUS), surgical scissors (include at least one Operating-scissors, one Mtezenbaum-fino, one Mayo-scissors), forceps (include at least one Dissecting-forceps and two Allis tissue forceps), 100 mm culture plate (Corning), six-well plate (Corning).

Note: The regent and tools need to be sterilized. The 20% FBS proliferation medium was prepared with DMEM and FBS supplemented with 1× penicillin-streptomycin solution in advance. The 2% HS differentiation medium was prepared with DMEM and HS. The 20% FBS and 2% HS were store at 4°C until using and pre-warmed to 37°C before using. SS-PBS was prepared with saponin, FBS and PBS (1 ml SS-PBS contain 0.02 ml 10% saponin dilute with PBS and 0.98 ml 10% FBS dilute with PBS).

Dissection of muscles

This procedure was slightly different in the neonatal and adult mouse, because the neonatal mouse was very small, it's difficult to obtain the pure muscle, so we harvest the pure muscle with the help of a dissecting microscope, While in adult mouse we can easily get the pure muscle fibres by eye. We show the detailed operation approach step by step. Note that you must keeping sterile operation.

1. Sacrifice one mouse according to institutional regulations.

2. For neonatal mouse: Spray the mouse with 70% ethanol, then pin the mouse (face up) to a dissecting board. Cut the abdominal skin with sterile surgical scissors. Tear off the skin by hand, and exposing the entire hind limb muscles. Cut the hind limb away from the skin, store the hind limb in a 100 mm culture plate in 9 ml PBS. Next, working under the dissecting microscope, carefully remove the bone, fascia, blood vessels and fat from the muscle. Finally, we get the pure muscles without connective tissue.

For adult mouse: Rinse the hind limbs with 70% ethanol, and fixed the mouse to a dissecting board. Cut through the entire hind limb and expose the muscle underneath with the help of scissors, remove the skin as well as any hair or fur. Then cut the fascia without damaging the muscles, expose the distal tendons by forceps, cut the distal tendons (the Tibialis Anterior (TA) muscle or the Extensor Digitorum Longus (EDL) muscle) with scissors. Hold the TA and EDL muscles by their tendons and carefully pull the muscles up towards the proximal end, last cut the proximal tendon and gently remove the muscles.

3. Transfer the pure muscles (obtain from the previous step) to a new culture plate, rinse with 6 ml PBS. Repeat 3 times.

4. Cut the muscles into small fragments (about 1 mm^3) but do not mince.

5. Transfer the small fragments to a new culture plate, rinse with 6 ml PBS. Repeat 3 times.

6. The small muscle fragments were evenly distributed in a six-well plate by forceps.

7. Moist tissue with 20% FBS, and put the 6-well plate upside down, then placed the plate in an incubator at 37°C with 5% CO_2.

8. After 2 hours, place the six-well plate up, add 1 ml 20% FBS to every well. Culture the cells undisturbed in the incubator.

Culture and pure of satellite cells

1. Replace the culture medium until the satellite cells were transformed from tissue pieces about 3 days later.

2. And then change the culture medium every 2 days. Note, cultures should be rinsed very gently avoid tissue pieces flaking off.

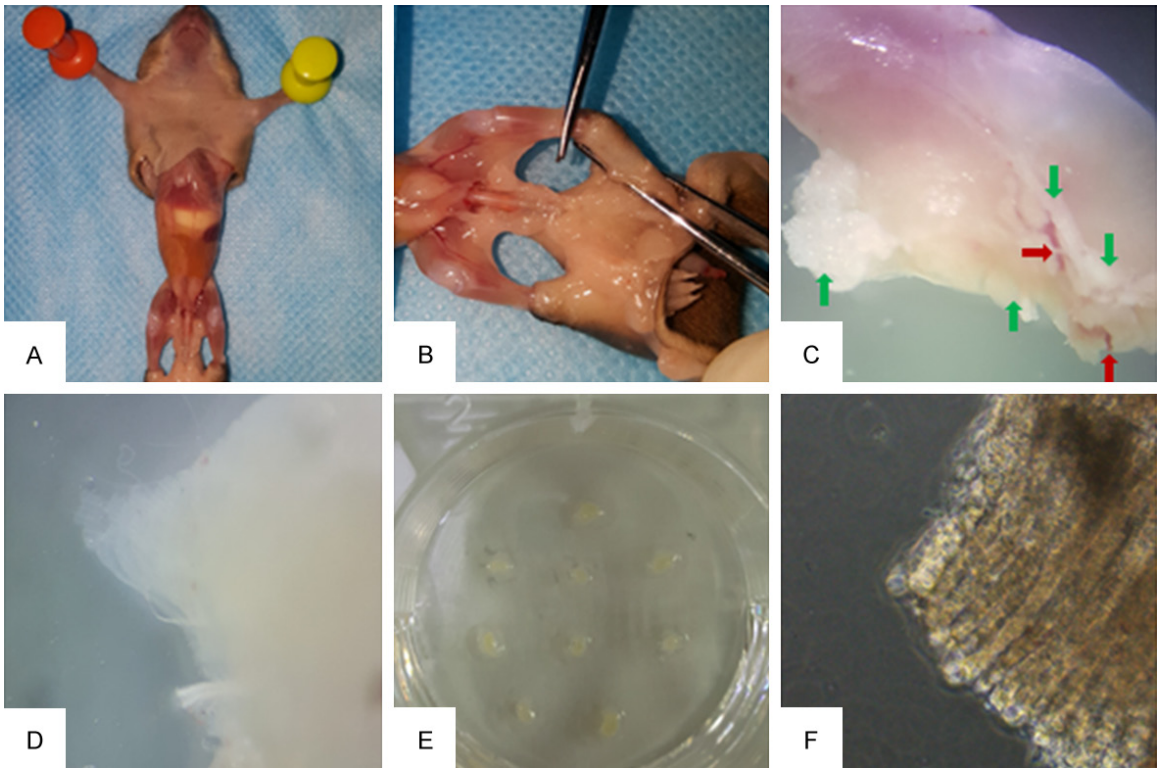

Figure 1. Muscle isolation from neonatal mouse hind limb. A. Fix the mouse to the dissecting board and expose the hind limb with skin removed. B. Cut the entire hind limb. C. Under the dissecting microscope to observe the hind limb, the red blood vessels (indicated by the red arrows) and white fat (indicated by the green arrows) connect with the muscle can be seen (×4). D. The pure muscle was harvested (×4). E. Small muscle fragments were distributed in a six-well plate. F. The obvious skeletal muscle fiber structure can be seen (×100).

3. About seven days later, when cells cultured to 70% confluent, taking the muscle fragments out. Then the satellite cells were digested by trypsin, plate the cells by transferring to a fresh 6-well plate and incubate 30 min, transfer the supernatant cells to a new plate and continue culturing (differential attachment), and the first "fresh 6-well plate" can be discarded. Repeat this step during the initial week of culture expansion. Note, the time of tryspin digestion must be mastered strictly, observed the cells under a microscope and it's the time to stop digest when the cells begin to go round.

Identification of satellite cells

To identify the mouse skeletal muscle satellite cells, the pured cells were seeded on the 12 mm glass coverslips in 6-well plates at a density of 10^5 cells/mL per well and cultured until to 60% confluent. Then, immunofluorescence stain the cells with specific antibodies desmin which is the commonly used molecular marker to characterize the proliferating muscel satellite cells [6, 7].

1. Fixation: Washed cells twice with PBS; fixed with 300 ul 4% paraformaldehyde for 30 min at room temperature, and then rinse three times with PBS.

2. Staining: Permeabilize/block cells by incubating at room temperature with 250 ul SS-PBS for 30 min. Remove the SS-PBS, then wipe the water around the coverslips,and be careful keep the cells wet. Add primary antibody desmin diluted in SS-PBS (1:100) 12 ul/coverslips, leave at room temperature for 60 min.

3. Wash coverslips three times with PBS, 10 min each wash.

4. Remove PBS, moist coverslips with SS-PBS, then wipe the SS-PBS around the coverslips, Add second antibody Alexa Fluor 594 chicken-anti-Rabbit diluted in SS-PBS (1:100) 12 ul/coverslips, leave at room temperature for 30 min in the dark.

5. Wash coverslips three times with PBS (in the dark), 10 min each wash.

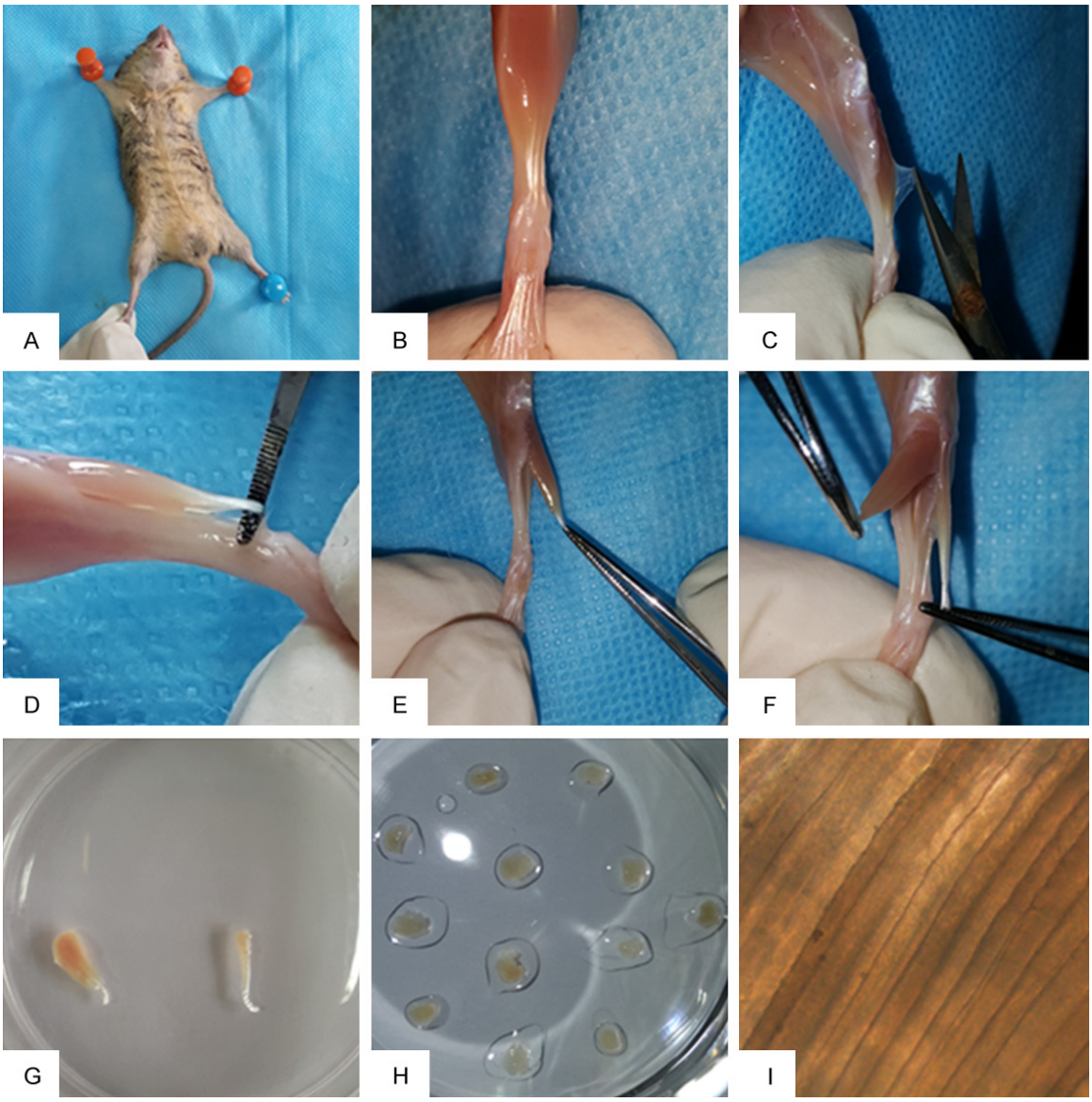

Figure 2. Muscle isolation from adult mouse hind limb. A. Fix the mouse to the dissecting board. B. Removed the skin, expose the muscle and tendons which cover with fascia. C. Fascia covering the hind limb muscles is removed to allow access to tendons. D. The tendon of the TA is carefully exposed and isolated at the ankle. E. Once isolated and foot insertions are cut, the TA tendons are pulled from the foot toward the knee. F. In a similar way, the EDL was dissected; anatomical position of the TA muscle and the EDL muscle. G. The proximal and distal tendon is cut and the TA and EDL is released. H. Small muscle fragments were distributed in a six-well plate. I. The obvious skeletal muscle fiber structure can be seen (×100).

6. Examined by fluorescent inverted microscope.

Differentiation of skeletal muscle satellite cells

To verify the differentiation characteristics of satellite cells, the cells were plated into the 6-well plates at a density of 10^4 cells/ml per well and were cultured with 20% FBS until 70% confluent. Then, the cells were cultured with 2% HS to induce differentiation and observed the cells morphology and replace the differential medium daily within 4 days to 1 week. The differentiation characteristics of satellite cells were detected with differentiation biomarker protein anti-myosin heavy chain (anti-MHC) (1:100) using immunostaining. The protocol was performed according to the method mentioned above.

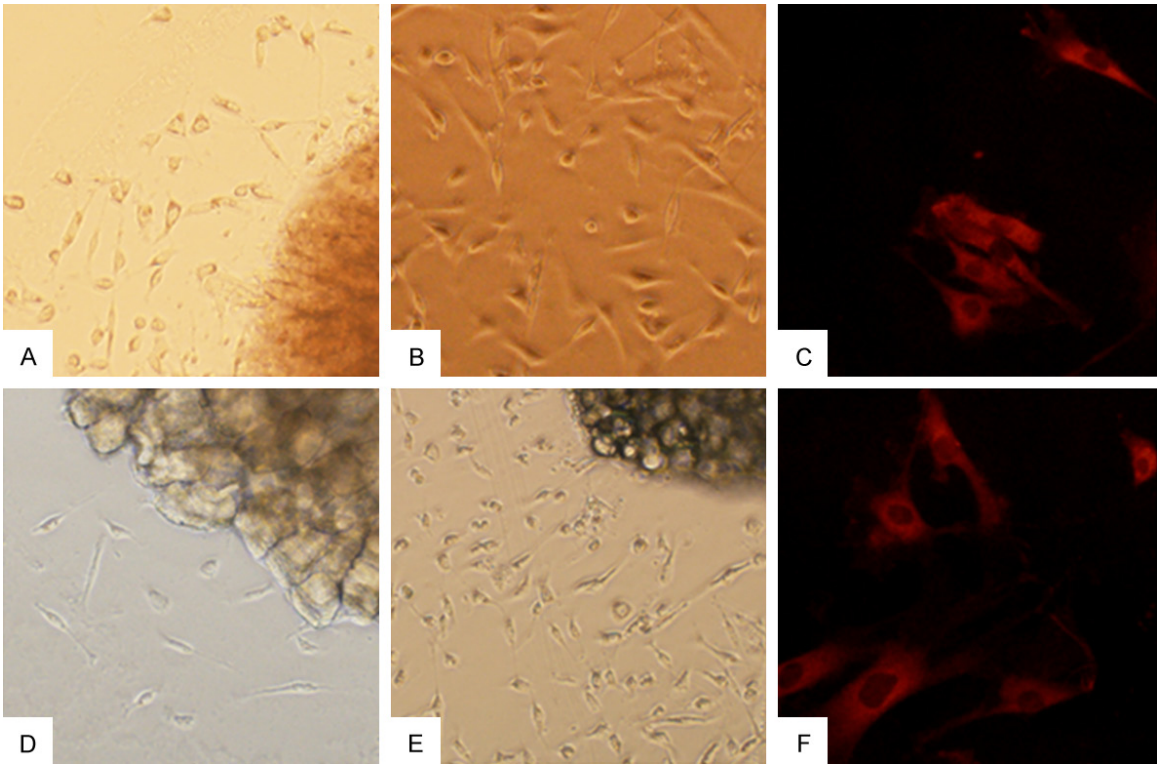

Figure 3. Morphological characteristics of isolated mouse skeletal muscle satellite cells under microscope. A. About three days later, several cells were seen around the neonatal muscle fragments (×100). B. After seven days, cells from the neonatal muscle cultured to 70% confluent (×100). C. Imunofluorescence analysis of Desmin expression on proliferating skeletal muscle satellite cells from neonatal mouse (×200). D. About three days later, several cells were seen around the adult muscle fragments (×100). E. After seven days, cells from the adult muscle cultured to 70% confluent (×100). F. Imunofluorescence analysis of Desmin expression on proliferating skeletal muscle satellite cells from adult mouse (×200).

Results

Harvest pure muscle from neonatal mouse

First we described the isolation of hind limb skeletal muscle from neonatal mouse (7 days old). We shows a step by step graphical representation of "dissected hind limb skeletal muscle". **Figure 1A**, **1B** show the way we got the entire hind limb without skin and hair. Under the dissecting microscope show in **Figure 1C** we can clearly see connective tissue such as the blood vessels and fat, it will take some time and energy to remove the connective tissue. With the help of surgical scissors and forceps, we obtain the pure muscle, and with a 100× magnification to show details of the muscle fiber structure (**Figure 1D**, **1F**). The small muscle fragments were distributed in a six-well plate was show in **Figure 1E**.

Harvest pure muscle from adult mouse

Then we show the isolation of hind limb skeletal muscle from an 8 weeks adult mouse, it would be much easier. Here we show a step by step graphical representation of the "tendon to tendon" isolation. In **Figure 2A-C**, we remove hair and fascia, exposed the muscle fiber bundle. In **Figure 2D-G**, we dissect the TA muscle and EDL muscle from the hind limb. Then we cut the pure muscle in to small fragments and culturing (**Figure 2H**, **2I**).

Culture and identify of satellite cells

Under this method, several cells were found scattered around the tissue after three days, and there were no significant difference between neonatal and adult mouse. It was seen under the microscope that the cells grew well with round or fusiform appearance, and the cellular plasma plenty and bright (**Figure 3A**, **3D**). About seven days later, a marked increase in cell number, the cells stretching full, and present a spindle shaped (**Figure 3B**, **3E**). At high cell density, the cells were in the concentric growth and arranged in order. And after subcul-

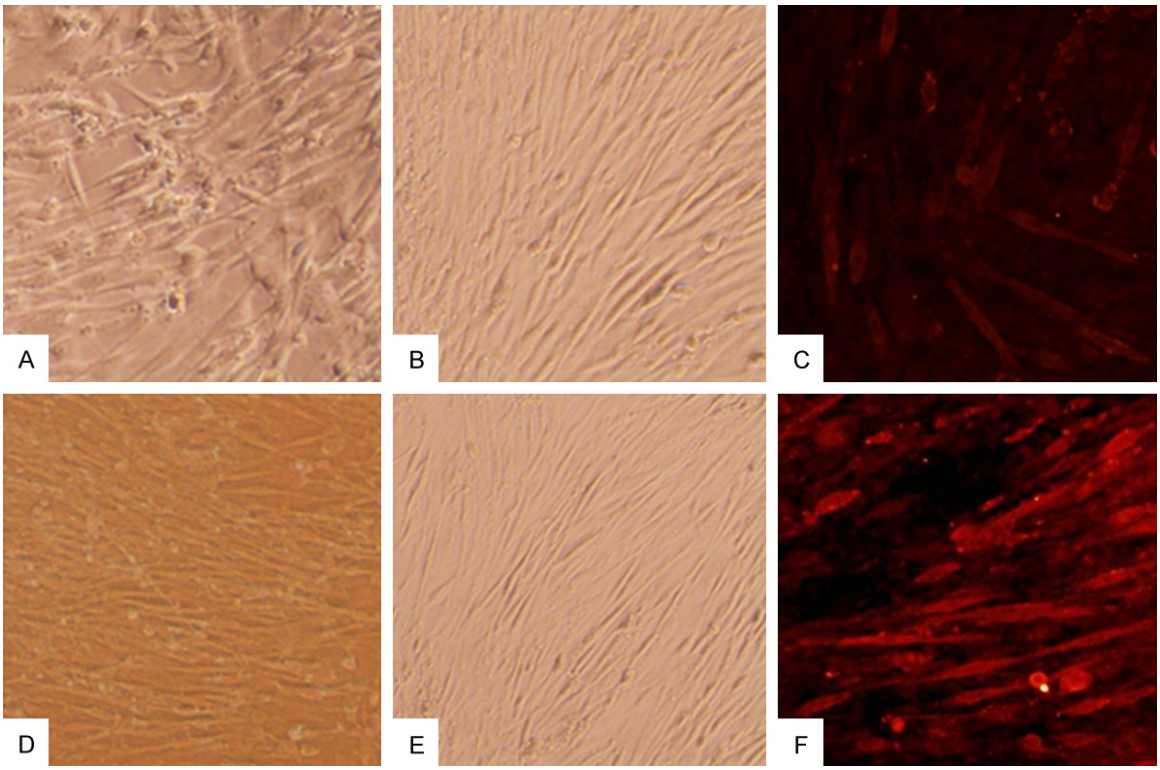

Figure 4. Differention of satellite cells and immunostaining of myotubes. A. 3 days differentiation of satellite cells from neonatal mouse (×100). B. Differentiated myotubules from neonatal muscle satellite cells after seven days differentiation (×100). C. Immunofluorescence analysis of MHC expression on differentiated mouse skeletal muscle satellite cells from neonatal mouse (×100). D. 4 days differentiation of satellite cells from adult mouse (×100). E. Differentiated myotubules from adult muscle satellite cells after seven days differentiation (×100). F. Immunofluorescence analysis of MHC expression on differentiated mouse skeletal muscle satellite cells from adult mouse (×100).

turing, the cells spread much more and most cells became spindle shaped. These cells can be passaged by trypsinization every 4 days, the cell proliferation rate is reduced after 5 times of passages. Desmin antibodies were used to characterize the proliferating positive muscle satellite cells, Desmin was strongly positive in the skeletal muscle satellite cells. While fibroblasts and other cells can't be stained with fluorescence. The representative results from immunofluorescence were shown in **Figure 3C**, **3F**.

Differentiation of skeletal muscle satellite cells

The cell proliferation rate were significantly decreased after change into the differentiation medium. After two days, a small amount of satellite cells began fuse, and most of the cell fusion into multinucleated myotubes as time went on (**Figure 4A**, **4D**). By seven days in differentiation medium (**Figure 4B**, **4E**), satellite cells undergo differentiation into myosin heavy chain (MHC)-positive myotubes, the representative immunofluorescence results were shown in **Figure 4C**, **4F**.

Discussion

At present, there are two main methods for isolate skeletal muscle satellite cells, one is to release the muscle satellite cells by mincing and enzymatic digesting connective tissue and myofibers [8]. This is the most common and efficient method to obtain enough muscle satellite cells, however, this method has an obvious defect: it may obtain a heterogeneous population of precursor cells. The other one is to isolate the muscle satellite cells from single intact muscle fiber, which can obtain relatively pure muscle satellite cells [9, 10]. Yet, there are also disadvantages in this method, this method take a longer time to obtain enough cells. While, both methods need enzyme digestion, the di-

gestion time and concentration were optimized to achieve the satisfactory dissociation and viability cells according to the characteristics of muscle tissues and the product manual. So these methods are more trouble, more expense, more hurdles. Our protocol was improved based on the method of releasing muscle satellite cells from single muscle fiber, and operate on the principle of skeletal muscle satellite cells have the ability to release, and migrate from the muscle tissue blocks. At the same time this article does not use enzymatic digestion, only pure muscle was provided, maximum extent simplify the experiment and reduce cost. Skeletal muscle satellite cells are a kind of skeletal muscle stem cells, therefore postnatal stages were appropriate for the success of skeletal muscle satellite cells. The previous studies indicated that the absolute numbers of muscle satellite cells were increased gradually in the few months after birth. However, the relative proportion of skeletal muscle satellite cells decreased with the increasing age after birth [11, 12]. Therefore it is better to select newborn animal to obtain a high proportion of muscle satellite cells. And our experiments also achieved good results both in neonatal and adult mouse, we can get a considerable number of cells. While in different parts of body the number of muscle satellite cells may considerable different. For example the number of satellite cells in soleus muscle (SOL) is greater than in extensor digitorum longus (EDL) from 2 to 3 months old mouse [13]. Usually, the hind limb muscles are used to isolate the skeletal muscle satellite. In our study the muscle satellite cells can be isolated from the whole hind limb muscles in neonatal mouse, and in adult mouse, TA muscle and EDL muscle was separate for research. Although we did not provide the evidence indicate which one is better. In this experience, the most important step for successful satellite cells isolation is the pure muscle isolation (steps 2 in the "*Dissection of muscles*" protocol text). This guarantees that pure muscle can be got with minimal or no damage muscle fibers thus increasing their performance in the following steps. And steps 7 to 8 in the "*Dissection of muscles*" protocol text also require attention, cause the time of "6-well plate upside down" shall be strictly control, neither short nor long. Too short the muscle tissue can't stick wall and float in the culture media, too long it would be damage the cells, affect the migration of satellite cells. Under these conditions, satellite cells initially remain between the basal lamina and sarcolemma of muscle fibers, later migrate away, and cause proliferating and differentiating progeny. From what has been discussed above, these results show that the optimized method can obtain enough pure skeletal muscle satellite cells which can be used as a cell model to study the molecular mechanisms of skeletal muscle growth and development.

Conclusions

In our study, we provide a new protocol for the isolation, culture and identification of satellite cells from skeletal muscle. this method was simple reliable and fruitful, more important is that the satellite cells obtained by this method are more pure, besides this method is suit for neonatal or adult mouse, it can be applicable to the general basic laboratory.

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Disclosure of conflict of interest

None.

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