

Original Research

Mechanical ventilation regulated the differentiation and proliferation of diaphragm satellite cells

Junying Ding^{1,2,*}, Qian Li², Feng Liang², Salyan Bhattarai², Qingquan Liu^{1,*}

¹Beijing Key Laboratory of Basic Research with Traditional Chinese Medicine on Infectious Diseases, Beijing Hospital of Traditional Chinese Medicine, Capital Medical University, Beijing Institute of Traditional Chinese Medicine, 100010 Beijing, China, ²Meakins-Christie Laboratories and Translational Research in Respiratory Diseases Program, McGill University Health Centre and Research Institute, Montreal, QC H4A 3J1, Canada

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1 MV intervention
 - 3.2 Isolation of diaphragm satellite cells
 - 3.3 BrdU staining
 - 3.4 Immunofluorescence
 - 3.5 Real-time PCR
 - 3.6 Statistical analysis
4. Results
 - 4.1 MV inhibited the proliferation of diaphragm satellite cells
 - 4.2 MV promoted the differentiation of diaphragm satellite cells
 - 4.3 MV upregulated MyoD and myogenin expression in diaphragm satellite cells
5. Discussion
6. Conclusions
7. Author contributions
8. Ethics approval and consent to participate
9. Acknowledgment
10. Funding
11. Conflict of interest
12. Data availability
13. References

1. Abstract

Aim: This study aimed to determine the effect of mechanical ventilation (MV) on the differentiation and proliferation of diaphragm satellite cells. **Methods:** Diaphragm satellite cells were isolated from C57 mice receiving 6 h of MV with optimized magnetic-activated cell sorting (MACS) approach. The cells were stained with BrdU or antibody for differentiation marker MYH3. The expression of MyoD and myogenin was detected by real-time PCR. **Results:** Diaphragm satellite cells were successfully isolated from mice by using MACS with a set of optimized parameters. About 1.5×10^5 cells could be harvested from a diaphragm. Upon MV, the proliferation rate of diaphragm satellite cells was decreased from 88.74% to

81.92%, while the differentiation rate was increased from 17.94% to 27.58%, compared to controls. Moreover, the expression of MyoD and myogenin were significantly up-regulated upon MV. **Conclusions:** We established a practical method to purify diaphragm satellite cells, and demonstrated that MV regulated the differentiation and proliferation of diaphragm satellite cells.

2. Introduction

Mechanical ventilation (MV) is known to cause damage to diaphragm muscle [1]. Especially, weaning from MV is challenging for the recovery after serious illness. Moreover, weaning failure is associated with life-

threatening complications, leading to increased morbidity and mortality [2]. Accumulating evidence has indicated that prolonged MV longer than one week leads to respiratory muscle weakness because of contractile dysfunction and atrophy, especially in the diaphragm [3–6]. Diaphragm dysfunction plays a key role in weaning failure from MV, and such a condition is named as ventilator-induced diaphragmatic dysfunction (VIDD) [7].

Diaphragm satellite cell proliferation is a promising driving force to reverse VIDD. Satellite cells are usually mitotically quiescent but can be activated upon injury, leading to the production of myogenic precursor cells or myoblasts to promote muscle regeneration [8]. Stem cells are known to have great potential of differentiation and regeneration [9, 10]. Satellite cells are the primary myogenic cells that contribute to skeletal muscle regeneration after muscle injury [11, 12]. Myogenic cells in dystrophic muscle show accelerated differentiation [13]. However, it remains unknown whether such a phenomenon occurs in diaphragm muscle.

Previous study reported that pro-myogenic factors or the transplantation of muscle satellite cells (MuSCs) can enhance skeletal muscle regeneration [14]. Several protocols have been published to isolate MuSCs from skeletal muscle [15, 16]. However, the diaphragm is distinct from other skeletal muscles because it is activated constantly to drive ventilation and diaphragm satellite cells are myogenic cells with potential for proliferation and differentiation [17]. Therefore, it is difficult to isolate diaphragm satellite cells because of the challenge to preserve the quiescence state of diaphragm satellite cells during the isolation. To overcome the problem, in this study we established an optimized method to purify diaphragm satellite cells, and determined the effects of MV on the differentiation and proliferation of satellite cells.

3. Materials and methods

3.1 MV intervention

Male C57 mice (10–12 weeks of age) were housed under standard conditions, and divided randomly in two groups: control group and MV group ($n = 6$). Mice in control group were fasted for 6 h with free access to water. Mice in MV group underwent 6 h of controlled MV as described previously [18].

3.2 Isolation of diaphragm satellite cells

All the animals were sacrificed using CO₂ asphyxia method. Diaphragm muscles were dissected, and blood, fat, and tendons were removed. The pure diaphragm muscles were maintained in F12 medium, and chopped into a fine slurry until it could pass through a 10-mL pipette freely. The fine slurry was transferred into a 15-mL Falcon tube and digested with trypsin at 37 °C for 35 min with gentle agitation. Finally, the digested muscle suspension was

filtered through an 80-micron nylon cloth and centrifuged at 1800 rpm (688 g) for 10 min at 4 °C. The cells in the supernatants were resuspended and labeled with the specific antibody at 4 °C for 15 min. MS column was placed on magnetic rack, followed by pre-equilibration using PBS containing 1% FBS. The cell suspension was mixed with the column, and after centrifugation the cell pellet was resuspended at a density of 10⁶ cells per 80 μL buffer, and mixed with 20 μL anti-integrin α7 microbeads, followed by incubation at 4 °C for 15 min. Another MS column was placed on magnetic rack, followed by pre-equilibration. Labeled cells were loaded onto the column. The column was then taken from magnetic field and washed by 1 mL PBS. The diaphragm satellite cells were collected and seeded into 12-well plates precoated with Matrigel. The cells were cultured for 4 days at 37 °C.

3.3 BrdU staining

The cells were stained with 0.03 mg/mL BrdU (Cat # B5002, Sigma, St. Louis, MO, USA) for 24 h, and sequentially incubated with acetone and 1.5 M HCl. Next, the cells were fixed with 1:5000 Hoechst and covered using coverslips, the cells were observed under Zeiss M2 fluorescence microscope. The proliferation of satellite cells was determined according to the intensity of BrdU staining.

3.4 Immunofluorescence

The cells were fixed in 4% PFA, washed and then incubated with primary antibody for MYH3 (Cat # sc-53091, Santa Cruz Biotech, Santa Cruz, CA, USA) and rabbit anti-mouse secondary antibody Alexa Fluor 488 (Cat #A11059, Invitrogen, Carlsbad, CA, USA). Next, cells were fixed with 1:5000 Hoechst and covered using coverslips. The cells were observed under Zeiss M2 fluorescence microscope.

3.5 Real-time PCR

Total RNA was isolated from satellite cells using TRIzol reagent (Cat #15596018, Invitrogen, Carlsbad, CA, USA) and RT-PCR was performed using iScript reverse transcription supermix for RT-qPCR (Cat # 1708840, Bio-Rad, Hercules, CA, USA). HPRT1 was selected as the housekeeping gene. The primer sequences were as follows: MyoD 5'-AGAATGGCTACGACACCGCC-3', 5'-GCTGTCTGTGGAGATGCGCT-3'; myogenin 5'-GAGGAGCGCGATCTCCGCTA-3'; 5'-GTCAGCCGCGAGCAAATGAT-3'; HPRT1 5'-CATGCCATGGCATAACAGTGAAAAGGTGATC-3'; 5'-TGACAAGCTTGTGTGCCGCTGGGCACT-3'. The parameters were denaturation at 94 °C for 10 min, and then melting at 94 °C for 30 s and annealing at 60 °C for 1 min (40 cycles). The relative expression of target genes was calculated using 2^{-ΔΔC_t} method.

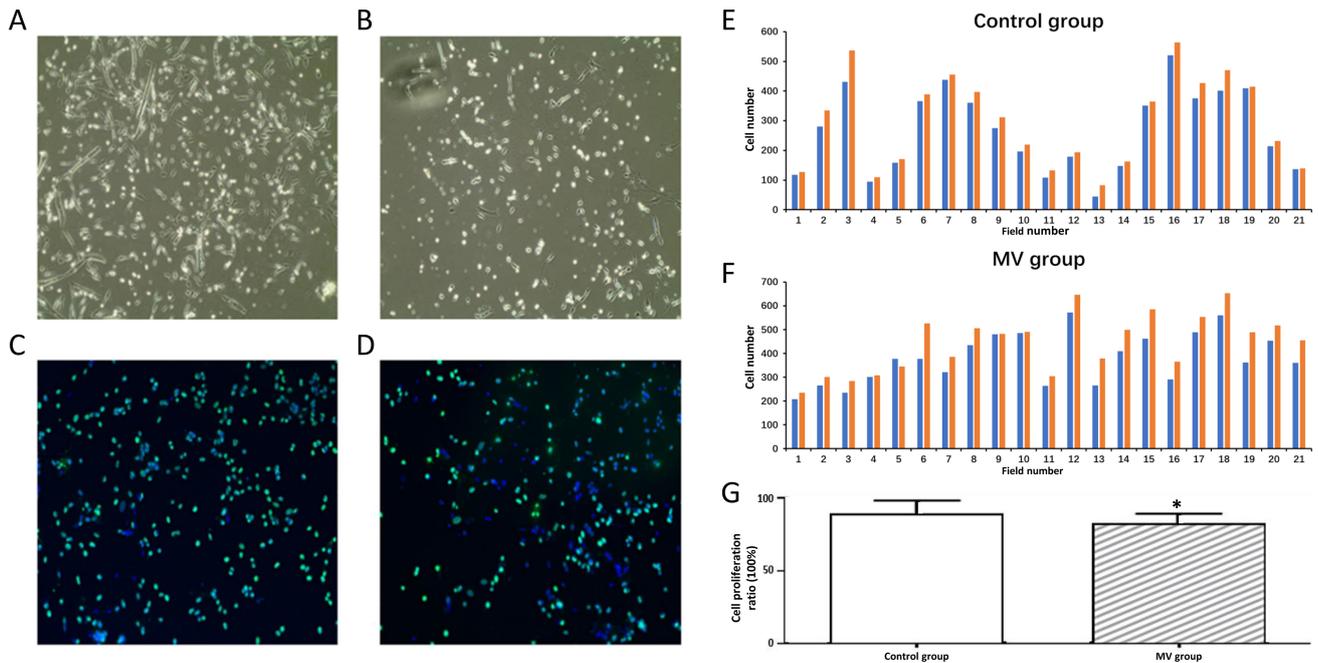


Fig. 1. Proliferation of diaphragm satellite cells. (A) Diaphragm satellite cells in control group on day 4. (B) Diaphragm satellite cells in MV group on day 4. (C) Diaphragm satellite cells in control group stained by BrdU (green) after 5 days. (D) Diaphragm satellite cells in MV group stained by BrdU (green) after 5 days. The nuclei were stained as blue by DAPI. Magnification 400. (E) Number of cells in proliferation in control group from 21 randomly selected areas in the slides. (F) Number of cells in proliferation in MV group from 21 randomly selected areas in the slides. Green indicated positive BrdU staining and blue indicated positive DAPI staining. (G) Proliferation ratios of diaphragm satellite cells. Proliferation ratio: number of BrdU positive cells/number of DAPI positive cells $\times 100\%$. * $p < 0.05$ compared to Control group.

3.6 Statistical analysis

The data were shown as mean \pm SD, and an independent sample *t*-test was used to analyze the variables. $p < 0.05$ indicated significant difference.

4. Results

4.1 MV inhibited the proliferation of diaphragm satellite cells

Using MACS, 1.5×10^5 diaphragm satellite cells could be isolated from a diaphragm using the optimized parameters. We found that the morphology of satellite cells in control group was normal with active proliferation. However, the proliferation of satellite cells in MV group was significantly reduced, exhibiting apparent disintegration (Fig. 1A,B). BrdU staining showed that the number of positively stained cells was higher in control group than in MV group (Fig. 1C,D). Furthermore, we counted the numbers of cells stained positive for proliferation in randomly selected areas in control group and MV group (Fig. 1E,F). We found that the proliferation ratio of diaphragm satellite cells was decreased from 88.74% to 81.92% by MV ($p < 0.05$) (Fig. 1G). Collectively, these data confirmed that MV reduced the proliferation of diaphragm satellite cells.

4.2 MV promoted the differentiation of diaphragm satellite cells

All satellite cells were detected with the antibody for differentiation marker MYH3. We found that the diaphragm satellite cells in the control group exhibited spindle-shaped morphology, and the nucleus was distributed orderly in fiber bundles. However, the satellite cells of the MV group were not spindle-shaped, and the nucleus was irregular and scattered, indicating that the cells of the MV group tended to differentiate (Fig. 2A–D). Furthermore, we counted the numbers of cells stained positive for differentiation in randomly selected areas in control group and MV group (Fig. 2E,F). We found that the differentiation ratio was increased from 17.94% to 27.58% by MV ($p < 0.05$) (Fig. 2G). Collectively, these data confirmed that MV promoted the differentiation of diaphragm satellite cells.

4.3 MV upregulated MyoD and myogenin expression in diaphragm satellite cells

MyoD and myogenin are markers of muscle cell differentiation. Real-time PCR analysis showed that MyoD and myogenin mRNA levels were significantly higher in diaphragm satellite cells from MV group compared to control group (Fig. 3). These results supported that MV promoted the differentiation of diaphragm satellite cells.

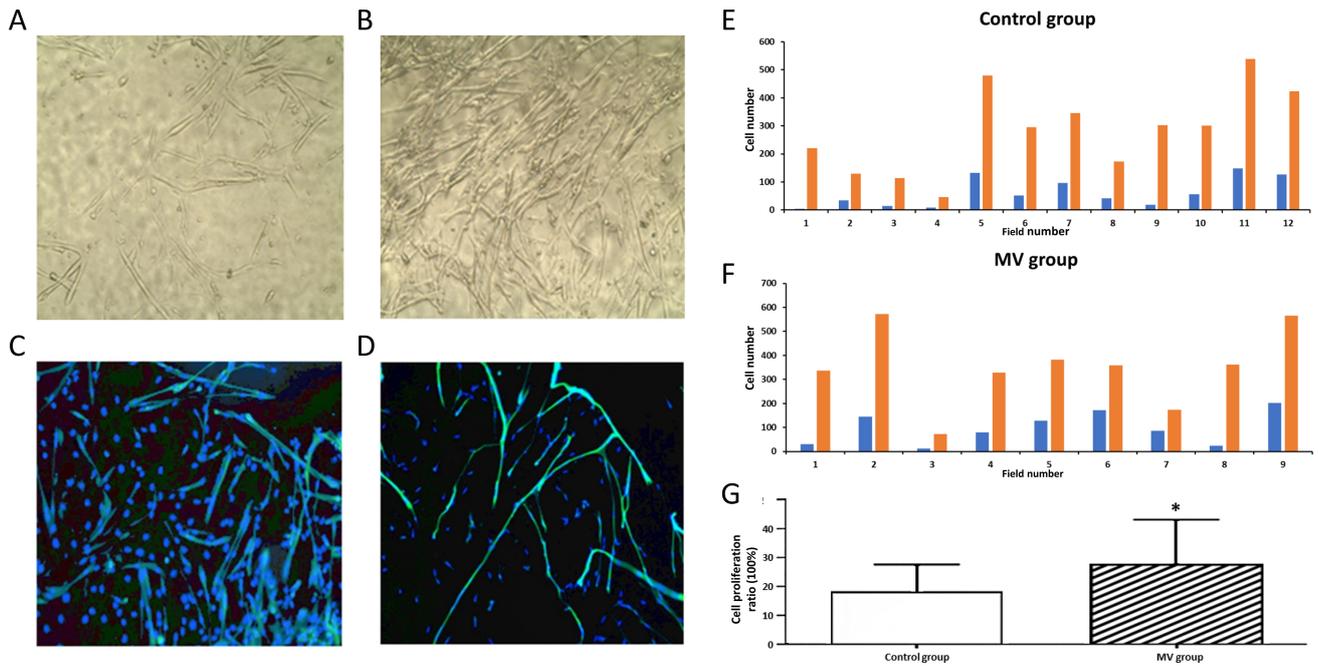


Fig. 2. Differentiation of diaphragm satellite cells. (A) Diaphragm satellite cells in control group on day 4. (B) Diaphragm satellite cells in MV group on day 4. (C) Diaphragm satellite cells in control group stained by antibody for differentiation marker MYH3 (green) after 5 days. (D) Diaphragm satellite cells in MV group stained by antibody for differentiation marker MYH3 (green) after 5 days. The nuclei were stained as blue by DAPI. Magnification 400. (E) Number of cells in differentiation in control group from 12 randomly selected areas in the slides. (F) Number of cells in differentiation in MV group from 9 randomly selected areas in the slides. Green indicated positive MYH3 staining and blue indicated positive DAPI staining. (G) Differentiation ratios of diaphragm satellite cells. Differentiation ratio: number of MYH3 positive cells/number of DAPI positive cells \times 100%. * $p < 0.05$ compared to Control group.

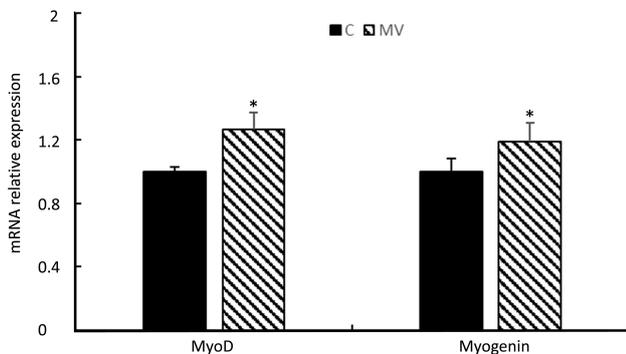


Fig. 3. MyoD and myogenin mRNA expression in diaphragm satellite cells. Data were presented as mean \pm SD ($n = 3$) after MyoD and myogenin mRNA levels were normalized to housekeeping gene *HPRT1*. C, Control group; MV, MV group. * $p < 0.05$ compared to Control group.

5. Discussion

Currently, no reliable approach is available to isolate diaphragm satellite cells, which has been a challenge for further investigation on diaphragm satellite cells. In present study, we developed a method to isolate diaphragm satellite cells with MACS. Based on parameter optimization, we successfully established a stable method to obtain diaphragm satellite cells. The muscle weight of a single diaphragm from C57 mouse was about 150 mg, and 1.5×10^5 satellite cells could be harvested from a diaphragm.

MV is a life-saving supportive therapy for patients with respiratory failure, and has been associated with some complications such as infection, barotrauma, tracheal injury, and ventilator-induced lung injury [19]. A previous study indicated that diaphragmatic strength and endurance played critical role in successfully weaning patients from MV [20]. MV can cause sudden interruption of diaphragmatic contractile activity [21]. Taken together, the mechanisms underlying MV-induced diaphragm damage may involve oxidative stress, muscle atrophy and muscle fiber remodeling, but need further investigations.

In present study, we speculated that the differentiation and proliferation of diaphragm satellite cells played a critical role in weaning difficulties from MV. It is known that in adult muscle, satellite cells are myogenic progenitors [13]. It has been reported that the atrophy of the diaphragm was faster compared with other skeletal muscles under adverse stimulation [18]. Interestingly, myogenic cells from the dystrophic mouse showed accelerated differentiation [13]. In present study, we found that diaphragm satellite cells tended to differentiate after 6 h of MV. This result was consistent with the reduced diaphragmatic flexibility, disuse atrophy, and dysfunction after MV.

Myogenic regulatory factors (MRFs) are important to regulate myocyte proliferation and differentiation, and mainly include Myf5, MyoD, myogenin, and MRF4 [13].

Among them, MyoD can regulate skeletal muscle differentiation via the interaction with chromatin modifying complexes [22]. Myogenin is a target of MyoD and directs satellite cell regeneration to skeletal muscle [23]. In present study, we confirmed that MyoD and myogenin expression in diaphragm satellite cells significantly increased after 6 h of MV. These findings might help explain diaphragm cell differentiation tendency and diaphragm dysfunction after MV.

6. Conclusions

In conclusion, we developed a feasible and efficient approach to isolate diaphragm stem cells through optimized MACS and demonstrated that diaphragm stem cells tended to differentiate after MV, accompanied by increased expression of MyoD and myogenin.

7. Author contributions

JD performed experiments, conducted data analysis, and drew the manuscript. QL (Qian Li), FL and SB took part in the animal experiments and contributed to the tests. QL (Qingquan Liu) provided financial support.

8. Ethics approval and consent to participate

All animal-related protocols were approved by the institutional animal care and use committee (IACUC) of McGill University Health Centre (Approval No. 20180911, date 2018-09-11), and efforts were made to minimize the suffering of animals according to the recommendations by European Commission (1997).

9. Acknowledgment

This work was performed at McGill University Health Centre. The authors are grateful to Basil Petrof for scientific advice.

10. Funding

This work was supported by the Beijing Natural Science Foundation [No. 7182071] and National Natural Science Foundation of China [No. 81503399].

11. Conflict of interest

The authors declare no conflict of interest.

12. Data availability

All data are available from the corresponding author upon request.

13. References

- [1] Major VJ, Chiew YS, Shaw GM, Chase JG. Biomedical engineer's guide to the clinical aspects of intensive care mechanical ventilation. *Biomedical Engineering Online*. 2018; 17: 169.
- [2] Sanfilippo F, Di Falco D, Noto A, Santonocito C, Morelli A, Bignami E, *et al.* Association of weaning failure from mechanical ventilation with transthoracic echocardiography parameters: a systematic review and meta-analysis. *British Journal of Anaesthesia*. 2020; 126: 319–330.
- [3] Formenti P, Umbrello M, Dres M, Chiumello D. Ultrasonographic assessment of parasternal intercostal muscles during mechanical ventilation. *Annals of Intensive Care*. 2020; 10: 120.
- [4] Dres M, Demoule A. Diaphragm dysfunction during weaning from mechanical ventilation: an underestimated phenomenon with clinical implications. *Critical Care*. 2018; 22: 73.
- [5] Liang F, Emeriaud G, Rassier DE, Shang D, Gusev E, Hussain SNA, *et al.* Mechanical ventilation causes diaphragm dysfunction in newborn lambs. *Critical Care*. 2019; 23: 123.
- [6] Larsson L, Friedrich O. Critical Illness Myopathy (CIM) and Ventilator-Induced Diaphragm Muscle Dysfunction (VIDD): Acquired Myopathies Affecting Contractile Proteins. *Comprehensive Physiology*. 2016; 7: 105–112.
- [7] Petrof BJ, Jaber S, Matecki S. Ventilator-induced diaphragmatic dysfunction. *Current Opinion in Critical Care*. 2010; 16: 19–25.
- [8] Verma M, Asakura A. Efficient single muscle fiber isolation from alcohol-fixed adult muscle following β -galactosidase staining for satellite cell detection. *The Journal of Histochemistry and Cytochemistry*. 2011; 59: 60–67.
- [9] He Y, Guo L, Ding J, Lv H, Ma Q, Li C, *et al.* Down-regulation of N-methyl-D-aspartate receptor subunits 1 affects neurogenesis of hippocampal neural stem cells. *BIOCELL*. 2021; 45: 417–426.
- [10] Tang X, Yu M, Huang R, Lan S, Fan Y. Comparative characterization of human fetal neural stem cells and induced neural stem cells from peripheral blood mononuclear cells. *BIOCELL*. 2020; 44: 13–18.
- [11] Randolph ME, Pavlath GK. A muscle stem cell for every muscle: variability of satellite cell biology among different muscle groups. *Frontiers in Aging Neuroscience*. 2015; 7: 190.
- [12] Maeda Y, Yonemochi Y, Nakajyo Y, Hidaka H, Ikeda T, Ando Y. CXCL12 and osteopontin from bone marrow-derived mesenchymal stromal cells improve muscle regeneration. *Scientific Reports*. 2017; 7: 3305.
- [13] Yablonka-Reuveni Z, Anderson JE. Satellite cells from dystrophic (mdx) mice display accelerated differentiation in primary cultures and in isolated myofibers. *Developmental Dynamics*. 2006; 235: 203–212.
- [14] Han WM, Mohiuddin M, Anderson SE, García AJ, Jang YC. Co-delivery of Wnt7a and muscle stem cells using synthetic bioadhesive hydrogel enhances murine muscle regeneration and cell migration during engraftment. *Acta Biomaterialia*. 2019; 94: 243–252.
- [15] Pasut A, Jones AE, Rudnicki MA. Isolation and culture of individual myofibers and their satellite cells from adult skeletal muscle. *Journal of Visualized Experiments*. 2013; e50074.
- [16] Keire P, Shearer A, Shefer G, Yablonka-Reuveni Z. Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. *Methods in Molecular Biology*. 2013; 946: 431–468.
- [17] Stuelsatz P, Keire P, Almuly R, Yablonka-Reuveni Z. A contemporary atlas of the mouse diaphragm: myogenicity, vascularity, and the Pax3 connection. *The Journal of Histochemistry and Cytochemistry*. 2012; 60: 638–657.
- [18] Mrozek S, Jung B, Petrof BJ, Pauly M, Roberge S, Lacampagne A, *et al.* Rapid onset of specific diaphragm weakness in a healthy murine model of ventilator-induced diaphragmatic dysfunction. *Anesthesiology*. 2012; 117: 560–567.

- [19] Blough JT, Purnell CA, Chow I, Gosain AK. Wound Complications, Additional Ventilation Requirement, Prolonged Stay, and Readmission in Primary Palatoplasty. *Plastic and Reconstructive Surgery*. 2019; 144: 1150–1157.
- [20] Liu Y, Li L. Ventilator-induced diaphragm dysfunction in critical illness. *Experimental Biology and Medicine*. 2018; 243: 1331–1339.
- [21] Marini JJ, Rocco PRM, Gattinoni L. Static and Dynamic Contributors to Ventilator-induced Lung Injury in Clinical Practice. Pressure, Energy, and Power. *American Journal of Respiratory and Critical Care Medicine*. 2020; 201: 767–774.
- [22] Matteini F, Andresini O, Petrai S, Battistelli C, Rossi MN, Maione R. Poly(ADP-ribose) Polymerase 1 (PARP1) restrains MyoD-dependent gene expression during muscle differentiation. *Scientific Reports*. 2020; 10: 15086.
- [23] Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Seminars in Cell & Developmental Biology*. 2017; 72: 19–32.

Keywords: Diaphragm; Proliferation; Differentiation; Mechanical ventilation; Satellite cell

Send correspondence to:

Junying Ding, Beijing Key Laboratory of Basic Research with Traditional Chinese Medicine on Infectious Diseases, Beijing Hospital of Traditional Chinese Medicine, Capital Medical University, Beijing Institute of Traditional Chinese Medicine, 100010 Beijing, China, Meakins-Christie Laboratories and Translational Research in Respiratory Diseases Program, McGill University Health Centre and Research Institute, QC H4A 3J1, Canada, E-mail: Dingjunying@bjzhongyi.com

Qingquan Liu, Beijing Key Laboratory of Basic Research with Traditional Chinese Medicine on Infectious Diseases, Beijing Hospital of Traditional Chinese Medicine, Capital Medical University, Beijing Institute of Traditional Chinese Medicine, 100010 Beijing, China, E-mail: Liuqingquan@bjzhongyi.com