Separation and Purification of Bovine Milk Fat Globule Membrane Protein and Its Effect on Improvement of C_2C_{12} Mouse Skeletal Muscle Cell Proliferation

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Abstract

A novel method to improve the proliferation activity of C_2C_{12} cell by bovine milk fat globule membrane (MFGM) protein was established in this study. The MFGM protein was extracted and isolated into 4 fractions by an electric cream separator, and purified by cellulose DEAE-52 column. Fraction 2, accounted for 57.8% of total MFGM protein, and was used in the following study. MTT assay showed that it induced cell proliferation activity, increased cell survival rate and cell number using flow cytometer and fluorescence microscope analysis. There were only subtle changes in morphology as observed by confocal scanning laser microscopy, but the number of mitochondria were significantly increased by transmission electron microscopy analysis respectively. Furthermore, the mRNA expression of MyoD, cyclinD1, p70S6K and mTOR were up-regulated by utilizing quantitative real-time PCR assay, and the activation of Akt and mTOR phosphorylation was up regulated by using *Western blot* assay. The main protein in fraction 2, assayed by 1-D gel electrophoresis and MALDI TOF-TOF, was identified as milk fat globule-EGF factor 8, the content was 65.6% of total protein in fraction 2. The results elucidate a new molecular mechanism of MFGM protein fraction 2: the

activation of the Akt signal pathway in promoting cell proliferation.

Keywords: MFGM protein; proliferation; C_2C_{12} cell; milk fat globule-EGF factor 8; *Western blot*; Akt signal pathway

1. Introduction

Sarcopenia is defined as the deficiency of both muscle mass and its function.[1, 2] It is caused by an imbalance of protein degradation and synthesis.[3] It is accompanied by a loss of strength which can compromise the functional abilities. Eventually, skeletal muscle strength declines, equilibrium ability and metabolic rate reduce.[4] C_2C_{12} mouse skeletal muscle cell has been wildly used as a sarcopenia model to study myoblast differentiation, neuromuscular junction formation.[5] Increasing C_2C_{12} muscle cell apoptosis declines in regenerative potential, also contributes to aging-associated sarcopenia.[6] Thus a combined approach of increasing regenerative potential and proliferation activity, may present a framework for therapeutic intervention of sarcopenia. Histological, biochemical and molecular biological research have shown that sarcopenia is linked with several risk factors, e.g. hormone level changes, oxidative injury, cell apoptosis, as well as poor dietary protein intake etc.[3, 4, 7] These factors affect the synthesis and decomposition of muscle protein.

Dairy protein-derived branch chain amino acids, have shown the enhancement of muscle protein synthesis, increasing lean body mass and improvement of skeletal muscle function.[8] Studies also showed that the phosphorylation of mTOR, and its downstream targets implicated in translation/initiation of P7S6K.[9] Milk fat globule membrane (MFGM), the major nutrient component (about 0.2%) of raw milk, is a mixture consisting primarily of lipids and membrane-specific proteins.[10] Except for its effect of anticancer, cholesterol lowering, anti-bacterial toxins and anti-infection etc, MFGM attracts much attention in protection against sarcopenia.[11] In senescence-accelerated P1 mice, which is a naturally occurring animal model for accelerated aging after normal development and maturation, exercise plus dietary MFGM can improve

muscle function through neuromuscular development.[12] Clinical trials in frail women[13] or in healthy adults [14] have both shown that the supplementation with MFGM alone, or combined with exercise did reverse the deficits in muscle mass, function, and improved muscle mass and strength. However, there were several hundreds of proteins identified in MFGM, e.g. 268 in human milk,[15] 269 [15] and 966 [16] in bovine milk. Their functions are related to lipid metabolism (e.g. AGT, A0MBP), inflammation (e.g. APOA4, BTN1A1) and immunity (e.g. CD14, CD36), etc.[16]

In this study, bovine MFGM protein was extracted and isolated by an electric cream separator, and purified by cellulose DEAE-52 column. MTT assay was then used to screen the best effect of MFGM protein fraction on cell proliferation. The effect of MFGM protein on cell proliferation, apoptosis, morphology, as well as cell mRNA and protein expression were further analysed, in order to explore its mechanism of preventing sarcopenia in the C_2C_{12} cell model.

2 Materials and methods

2.1 Materials and chemicals

C_2C_{12} cell was supplied by Chinese Academy of Agricultural Sciences (Beijing, China). Cow's milk was purchased from the local dairy farms (Harbin, Heilongjiang, China). Cellulose DEAE-52 column (2.6 × 60 cm) was purchased from Whatman (UK). Dulbecco's Modified Eagle's medium (DMEM, GIBCO, USA), fetal bovine serum (FBS) and trypsin-EDTA solution (approximately 0.25% trypsin and 0.02% EDTA) were purchased from GIBCO (USA). All other chemicals and reagents were analytical grade.

2.2 Extraction of MFGM from cow's milk

The method was based on He[17] with a slight modification. Sucrose (5 g) was dissolved in fresh milk (100 ml), then centrifuged with an automatic cream separator, 3500 g at 38 °C for 30 minutes. The buttermilk, was initially adjusted to pH 4.8 using 0.01 mol/L HCl in order to allow MFGM to precipitate, and then neutralized with 0.01

mol/L NaOH. Finally, the MFGM pellet was resuspended, its supernatant was frozen dried and stored at -20 °C before analysis.

2.3. Separation and purification of MFGM protein

The method was described by Wang[18] with a slight modification. MFGM crude extract (0.2 g) from Section 2.2 was dissolved in PBS buffer (10 ml, *pH* 8.0) completely, the supernatant was loaded onto a DEAE cellulose DE-52 column followed by a linear gradient elution with NaCl (0.2 M, 0.5 M and 0.8 M) in the same buffer, the protein fraction was collected into a vial every 5 minutes (the elution flow rate was 1 ml/min) then absorbance of 4 fractions (P1, P2, P3, and P4, Figure 2A) were measured at 280 nm by HD-93-1 spectrophotometer (Purkinje General Instrument Co. Ltd., Beijing, China). The MFGM protein fractions were dialysed, and then they were further concentrated and freeze-dried; The protein content was determined by bicinchoninic acid assay kit (BCA, Solarbio, China); The yield rate of MFGM protein was calculated as in the following equation: (mass of dry MFGM protein/(mass of MFGM loaded on a DEAE cellulose DE-52 column) × 100%.

2.4 Cell culture and MTT assay

C_2C_{12} cells were grown in DMEM with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/mL of streptomycin (Invitrogen) in a humidified incubator containing 5% CO_2 at 37°C. Effect on cell proliferation by proteins from 4 different fractions was firstly measured using the MTT assay.[19] The C_2C_{12} cells were inoculated with a density of 5×10^4/ml in 96-well plates and cultured overnight at 37°C. C_2C_{12} cells were then cultured in the same medium with 200 μg/mL of each of four fractions (Five samples per trial) for 24 h. Then 20 μl of MTT (5 mg/ml, Sigma) working solution was added to each well and incubated for 4 h at 37°C. After the MTT solution was removed, the formazan formed inside the cells was dissolved in DMSO. The absorbance was measured at 490 nm using a microplate reader (Model 550, Bio-Rad USA). Among the four fractions, fraction 2 (MFGM P2) showed the highest cell proliferation (Fig. 2C). To further test the dosage-efficacy, MFGM P2 (100, 200 or 300 μg/mL) and MFGM (200 ug/mL as a control) were further assessed, based on the above method, and cells

were cultured at 24, 48, and 72 h. The cell proliferation rate was calculated as in the following equation: (OD_{490} experiment group - OD_{490} control group)/OD_{490} control group (Five samples per trial).

2.5 ***Analysis of MFGM protein composition***

MFGM P2 showed the biggest effect on increasing cell viability compared to other 3 fractions in the MTT assay (Fig. 2C). Thus, the composition of MFGM P2 was further investigated.

MFGM P2 as well as MFGM were separated with a 1-D SDS gel (12% polyacrylamide) using a Bio-Rad electrophoresis system (Bio-Rad, USA).[17] The sample (2 mg) was suspended in 200 μl of reducing buffer [250 mM pH 6.8 Tirs-HCl, 10%（W/V) SDS, 2.5% (W/V) bromophenol blue, 50% (V/V) glycerol, 5% (W/V) β-mercaptoethanol], and denatured for 15 min in a boiling water bath and then centrifuged at 3 000 rpm for 15 min, 10 μg protein sample was loaded onto the gel. The gel was stained with 0.1 g/L Coomassie brilliant blue R-250 and subjected to a gel imaging system using a LKB 2400 GelSCan XL software (Bio-Rad, USA). The density of a protein band with molecular weight of 45 KDa from the SDS gel was 80% of the total bands in same lane. It was excised and subjected to further analysis with MALDI-TOF/TOF, the method was based on Reiz[20] with a slight modification. The protein gel band was digested with trypsin (Promega), then mixed with the matrix solution of α-Cyano-4-hydroxycinnamic acid. The peptides were analyzed using a 4800 Plus MALDI-TOF/TOF^{TM} Analyzer (AB Sciex, Concord, Ontario). Ionization was performed with a diode-pumped Nd:YAG laser operated at 355 nm. The peptide ion peak selection and mass assignment were done automatically using Mascot 2.2 software in the 4800 Plus system. The components of the protein were retrieved from NCBI database.

The protein components in the band were further analysed with capillary LC-MS/MS based on the method of Churchwelll.[21] The peptides were separated on an Agilent 1200 chromatographic system (Agilent Technologies, Wilmington, DE) after concentrating

and desalting the products, which used a Zorbax 300SB-C18 trapping column (5 mm × 0.3 mm, Agilent Technologies, Wilmington, DE) at a 4 mL min^{-1} flow rate using 2% (*v*/*v*) acetonitrile and 0.1% formic acid in water. The samples were separated on a Zorbax 300SB-C18 analytical column (150 mm × 75 mm, Column Technology Inc.). Solvents A and B for chromatography were 0.1% formic acid/water and 0.1% formic acid/acetonitrile water (84% of acetonitrile), respectively. The gradient elution was: 0 min, 4% B; 30 min, 50% B; 34 min, 100% B; 35 min, 100% B. The flow rate was 300 nL min^{-1}. MS detection was performed using a Q Exactive mass spectrometer (Thermo Fisher). The LC-effluent was directly introduced to a Triversa NanoMate ESI (electrospray ionisation) source (Advion, Ithaca, NY, USA) with a positive mode, working in a nano-LC mode and equipped with Dchips where on a 1.55 kV voltage was supplied. The Q Exactive mass spectrometer was calibrated with caffeine, MRFA and UltraMark before measurement. Mass spectra were recorded in full scan and MS^2 was triggered by a data-dependent threshold. LC-ESI-FT-MS/MS raw data were analyzed using Mascot Daemon v.2.2 (Matrixscience, London, UK).

2.6 Effect of MFGM protein on cell cycle, apoptosis, cell proliferation and morphology

The C_2C_{12} cells were inoculated in a density of 1×10^5 cells per well in a six-well plate and cultured overnight at 37□. C_2C_{12} cells were then treated with 200 μg/mL of MFGM, MFGM P2, or control (PBS) for 48 h at 37□. In each group, cells were harvested for the following experiments.

***Cell cycle and apoptosis*:** the cells were harvested and fixed with 70% ice-cold ethanol overnight at -20□. After centrifugation, cells were washed with ice-cold PBS, and stained with propidium iodide (PI, BD) solution for 30 min at room temperature in a dark environment. Cell cycle and apoptosis were analyzed using a BD FACS scan flow cytometer (Bio-Rad) and Cell-Quest software (BD Biosciences).

***Cell apoptosis*:** the method was based on Chen[22] with a slight modification. A fluorescein-conjugated Annexin V (Annexin V-FITC) staining assay was performed

according to the manufacturer's protocol (BD Biosciences Pharmingen, San Jose, CA, USA). Briefly, cells were stained with 5 μl of Annexin V-FITC and 5 μl of PI in each sample. After incubation for 15 min at room temperature in a dark environment, the degree of apoptosis was quantified as a percentage of the Annexin V-positive and PI-negative cells by flow cytometer.

Cell proliferation (survival and apoptosis cells) assay: the Hoechst 33342/PI detection kit (Solarbio). Cells were washed with PBS (pH 7.2), then incubated with 1 ml of staining buffer, 5 μl of Hoechst 33342 (excitation 360 nm/emission 465 nm) buffer and 5 μl of PI staining (excitation 488 nm/emission 620 nm) buffer for 30 min at 4 °C in the dark. The survival and apoptosis cells were observed using a fluorescence microscope (ZEISS, Germany) and a confocal scanning laser microscope (Leica, Germany) respectively.

Morphology examination: C_2C_{12} cells were fixed with 4% glutaraldehydeat at 4□ for 12 hours. The fixed cells were collected using cell scrappers followed by centrifugation at 1 000 rpm for 5 min. The cell pellets were fixed for an additional 2 h in 2.5% glutaraldehyde, and for 2 h with 1% osmium tetroxide. The cell pellets were dehydrated in ascending ethanol serial washes and embedded in Epon 812. Serial ultrathin sections were examined using Zeiss 900 electron microscope (ZEISS, Germany) with magnifications of 7 000 to 30 000.

mRNA expression of cyclin D1, MyoD, mTOR and S6K: Total RNA was isolated, then the RNAprep Pure Cell/Bacteria Kit (TIANGEN) was used for qRT-PCR analysis. cDNA was synthesized by using 3 μg of RNA with PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa) following the manufacturer's protocol. The assay was carried out on an ABI 7300 Real-Time PCR system (Applied Biosystems) with SYBR® Premix Ex TqaTM II (TaKaRa, Japan). Relative expression was first quantified using a standard curve, and data was normalized to GAPDH mRNA. Primers used in the study were shown as followings.[22-24] GAPDH: 5'-ACCA-CAGTCCATGCCATCAC-3', 5'-TC-CACCACCCTGTTGCTGTA-3'; CyClinD: 5'-T

-AGGCCCTCAGCCTCACTC-3', 5'-CCACCCCTGGGATAAAGCAC-3'; mT-OR: 5'-CTGGGACTCAAATGTGTGCAGTTC-3', 5'-GAACAATAGGGTGAATGATCCG-GG-3'; p70S6K: 5'-GGGAAGGCTTTGCAGTTTAC-3', 5'-TCCAGTCCCTCACGA-ACAAA-3'. Myod: 5'-TCAGGTGCTTTGAGAGATCGAC-3', 5'-CGAAAGGACAG-TTGGGAAGAGT-3'.

2.7 Western blot assay

The C_2C_{12} cells were treated with 200 μg/ml MFGM or MFGM P2 for 48 h at 37□, then collected and washed twice with PBS, and homogenized in lysis buffer. Total protein was extracted and harvested by scraping with a modified Radio Immunoprecipitation Assay (RIPA) buffer and phenylmethylsulfonyl fluoride (PMSF) for 30 min. Following centrifugation 10 000 rpm at 4□ for 15 min, the supernatant was then sonicated. Protein concentration was quantified using BCA kit. 100 μg protein was loaded onto a 1-D SDS gel (10% polyacrylamide). Then proteins were transferred to nitrocellulose filter membrane (PPLYGEN, China) using a wet electrotransfer system (Bio-Rad, USA） for 4 h at 200 mA. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST) buffer for 1 h at room temperature followed by incubation with primary antibodies (Santa CruzZ，USA) for each protein, for 1 h at 37□ or overnight at 4□ according to the requirements. The concentrations of antibodies were GAPDH (1:500), Akt (1:500), p-Akt Ser 473 (1:800), p-mTOR Ser 2248 (1:500) respectively. The membrane was washed 3×5 min with TBST followed by incubation with anti-mouse or anti-rabbit secondary antibody (1:2 000; Santa Cruz Biotechnology, Inc) at 37□ for 1 h. The membrane was washed with TBST twice and with TBS once, 5 min each, then incubated with alkaline phosphatase until an appropriate signal level was obtained. Protein bands were detected by FluorChem Imaging Systems (Alpha Innotech, Corp., San Leandro, CA, USA).

2.8 Statistical analysis

All experiments unless stated somewhere else, were tested and analyzed in triplicate. An analysis of variance (ANOVA) was identified to determine the significant differences ($P < 0.05$) between means. The statistical analysis was done by using a

General Linear Model procedure of SAS 9.1.3 software from SAS Institute Inc., Cary, NC, USA.

3. Results

MFGM protein was extracted by an electric cream separator, and four fractions of MFGM protein were collected by a linear gradient elution; the effect of each fraction on cell proliferation was initially assessed. Amongst four fractions, the second fraction showed the best effect based on the yield of MFGM protein and the cell proliferation rate, thus, its composition was analyzed; and its effects on cell proliferation, apoptosis, morphology, as well as cell mRNA and protein expression were further investigated (Working Flow Chart 1).

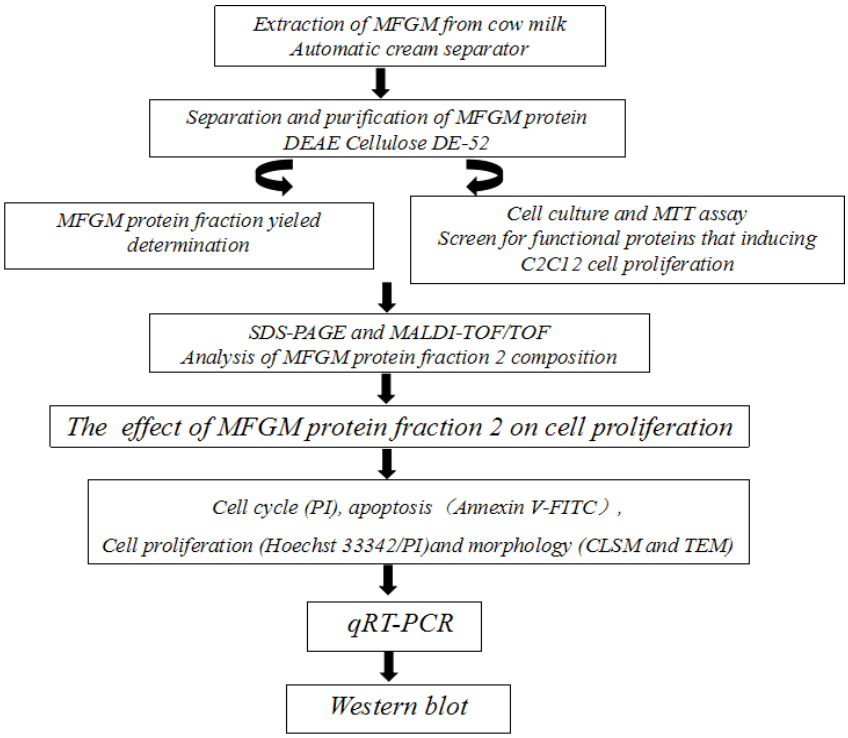


Fig. 1 The flow chart of this study

3.1 Protein amount in four fractions of MFGM protein and their effects on cell proliferation

MFGM protein was separated by DEAE-52 (Fig. 2A). Four fractions (P1 to P4) were collected based on elution time, and protein amount in each fraction is shown in Fig.

2B. After dialysis, the order of protein recovery rate (%) was: MFGM P2 (23.3%) > MFGM P3 (12.6%) > MFGM P4 (3.6%) > MFGM P1 (1.2%). Among the four fractions, MFGM P2 accounted for about 57.8% of total protein, even more, the proliferation of C_2C_{12} cell in MFGM P2 was the highest among all fractions (Fig. 2C). Thus, MFGM P2 was used to carry out the following experiments.

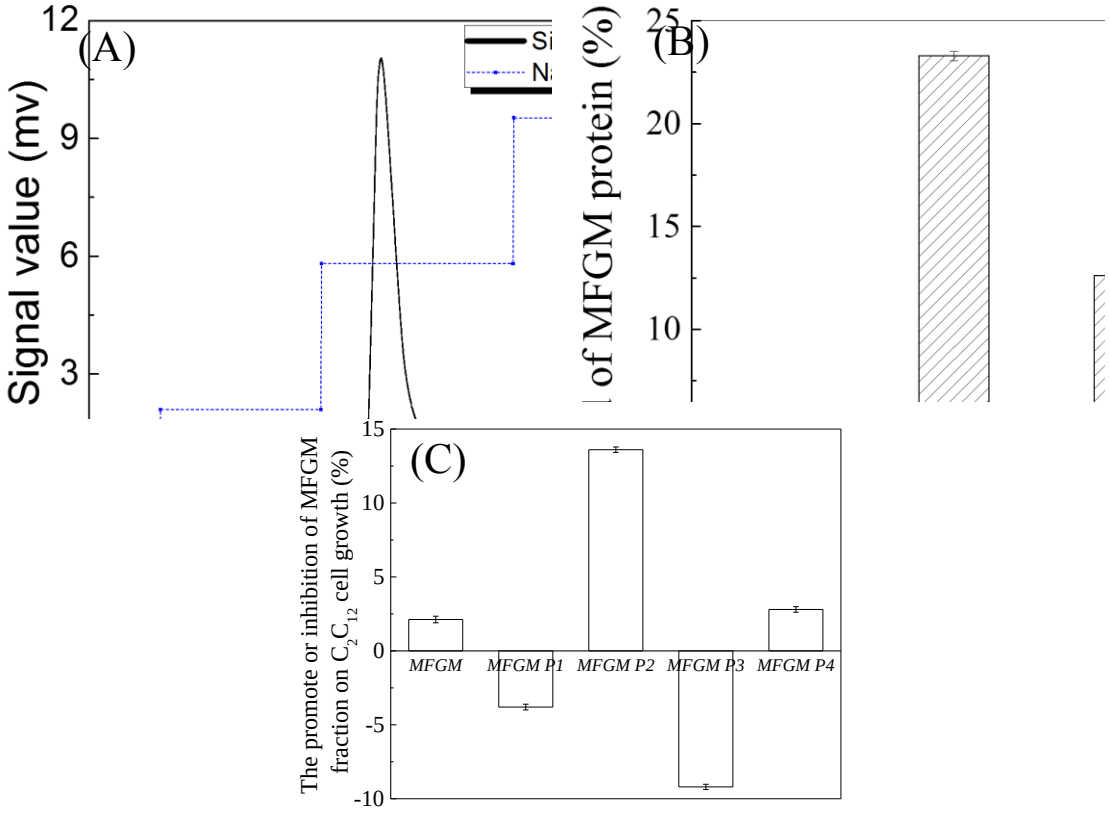


Fig. 2 The analysis of separation and screening of functional MFGM protein. A) MFGM protein separated by a cellulose DEAE-52 column; B) the yield of MFGM protein fraction; C) the effect of MFGM protein on C_2C_{12} growth.

3.2 The composition of MFGM P2

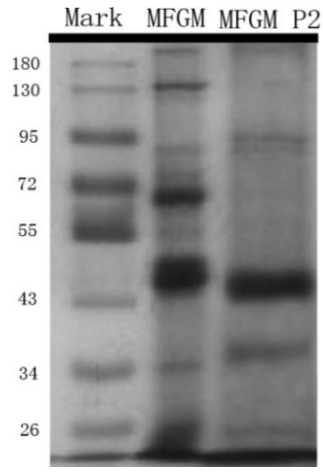


Fig. 3 SDS-PAGE analysis of the MFGM P2 extracted by the cellulose DEAE-52 column. Lane 1: molecular weight marker; lane 2: crude extraction of MFGM; lane 3: MFGM P2 purified by the cellulose DEAE-52 column.

The MFGM P2 was first analysed by 1-D SDS-PAGE (Fig. 3) followed by MALDI TOF/TOF (Fig. 4). There were eight major bands in the lane of MFGM protein, and four bands in the lane of MFGM P2, ranging from 26 to 225 kDa in molecular weight. The major protein band of 45 kDa in the MFGM P2 lane had a density of 80%, was further analysed by MALDI TOF/TOF. There were 42 proteins identified (data not shown) in the band, and among them, one protein, milk fat globule-EGF factor 8 protein (MFG-E8), was quantified as 82 % of total protein (raw data not shown) in the band. Thus, MFG-E8 accounted for 65.6% of total protein in fraction 2. This was based on the calculation of [82% (the amount of MFG-E8 in the main protein band at 45 kDa in MFGM P2) x 80% (density of MFG-E8 band to density of total protein bands)].

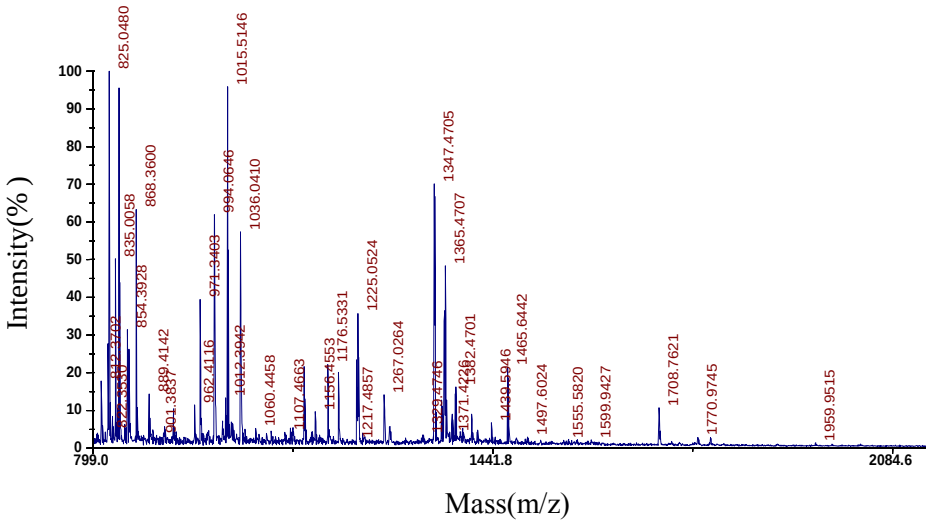


Fig. 4 The analysis of MFGM protein by MALDI TOF/TOF.

3.3 MTT Assay for appropriate concentration of MFGM P2

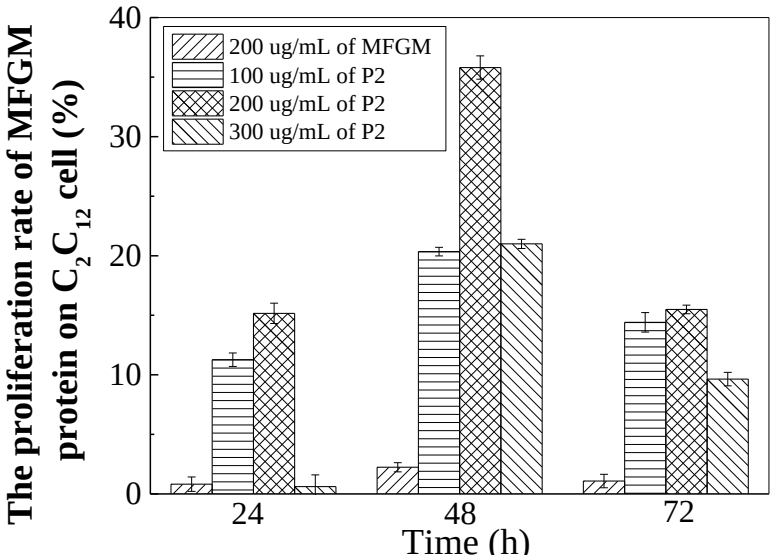


Fig. 5 MTT assay analysis of the effect of MFGM and MFGM P2 on cell proliferation.

C_2C_{12} cells were treated with 100, 200 and 300 μg/mL of MFGM P2 in 96-well plates for 24, 48 and 72 h at 37□, and cell proliferation was measured subsequently by MTT assay (Fig. 5). Results showed that the cell proliferation activity increased with time as well as with concentration, but decreased at 72 h. The proliferation rate reached at

maximum (35.8%) at 200 μg/mL at 48 h. The order of proliferation rate was shown as followings: 200 μg/mL of MFGM P2 >100 μg/mL of MFGM P2 > 200 μg/mL of MFGM > control > 300 μg/mL of MFGM P2. Thus, the concentration of 200 μg/mL MFGM P2 and incubation time at 48 h were applied to the following experiments.

3.4 The effect of MFGM P2 on cell proliferation and apoptosis

To determine the effect of MFGM P2 on the cell cycle, FACS analysis was performed in C_2C_{12} cells treated with each of MFGM P2 (200 μg/mL) or MFGM (200 μg/mL) for 48 h. Compared with the control group, the G0/G1 and S population decreased by 8.63% and 2.94%, respectively, while the G2/M population increased by 11.72% in cells treated with MFGM P2 (Fig. 6A), suggesting that MFGM P2 induces the cell cycle. However, there was a 5.12% increase in S population, and 2.93% and 2.36% decrease in G0/G1 and G2/M population in cells treated with MFGM (200 μg/mL) (Fig. 6A), suggesting that MFGM induces S-phase arrest. The results demonstrated that MFGM P2 induced the cell cycle, whereas MFGM induced S-phase arrest.

The cell apoptotic status, affected by MFGM and MFGM P2, was further observed by a fluorescence microscope, the results are shown in Fig. 6. There were less apoptosis cells stained with Annexin V-FITC-PI, but the cell density was higher in the MFGM P2 group than MFGM group (Fig. 6B); the result of flow cytometer showed that scarcely any apoptosis of C_2C_{12} cells were found in control group, MFGM group and MFGM P2 group (Fig. 6C). The result indicated that MFGM and MFGM P2 had no toxicity effect on C_2C_{12} cells, the result was collated with the cell cycle experiment.

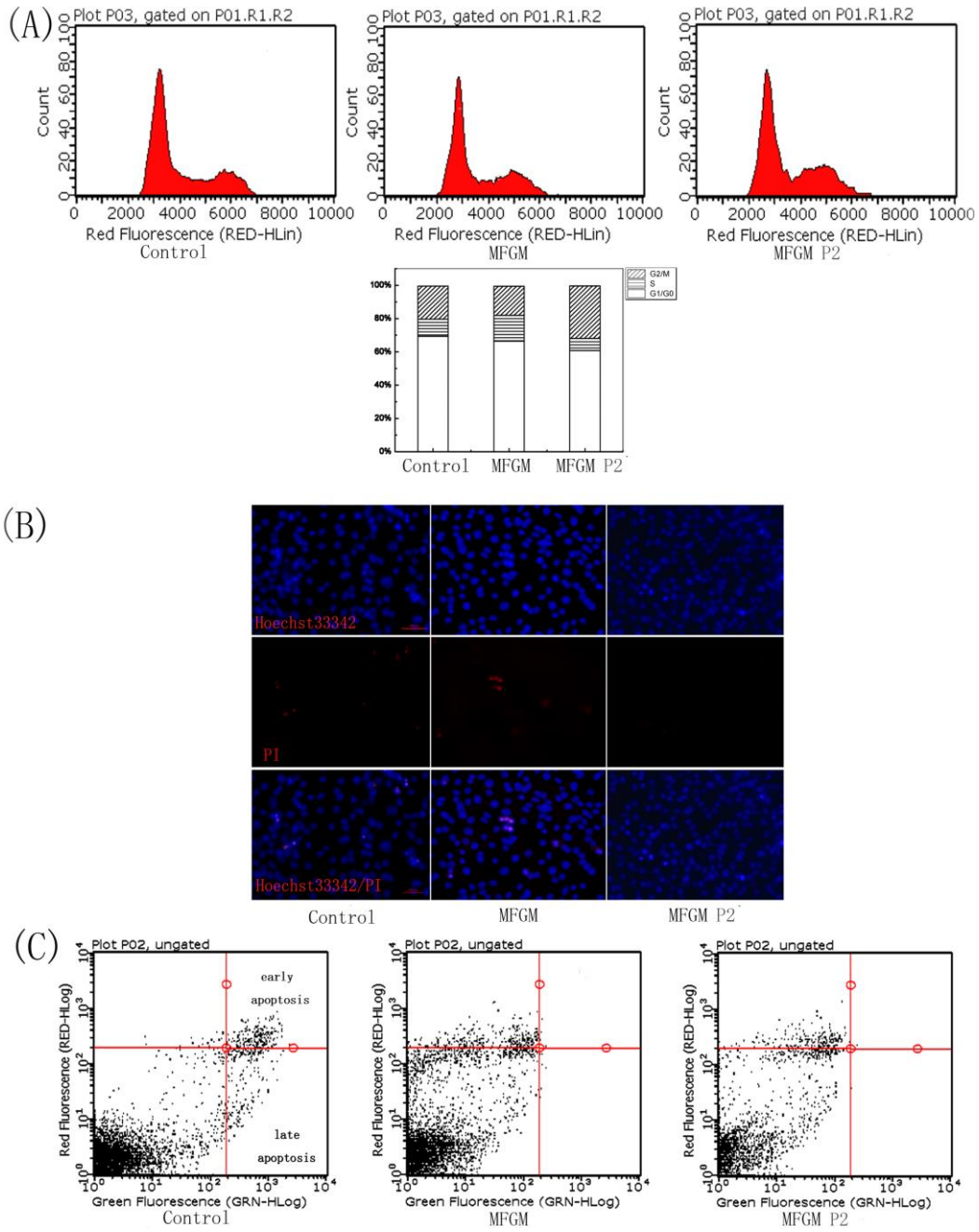
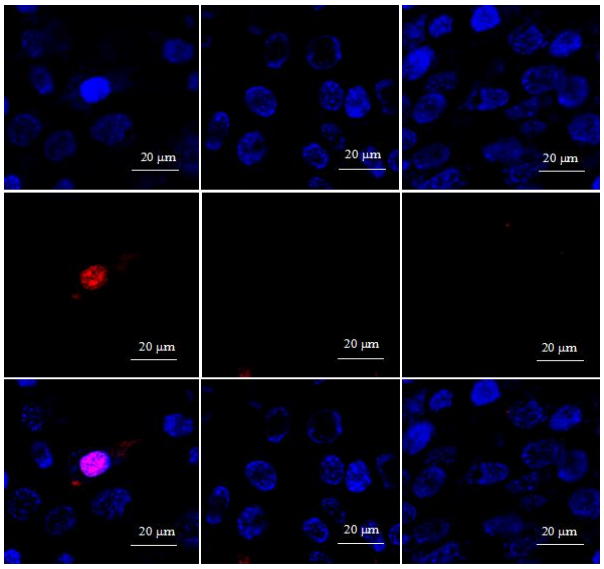


Fig. 6 The effect of MFGM protein on cell proliferation. (A) the effect of MFGM protein on cell cycle; (B) the analysis of Hoechst 33342/PI staining; (C) the effect of MFGM protein on cell apoptosis.

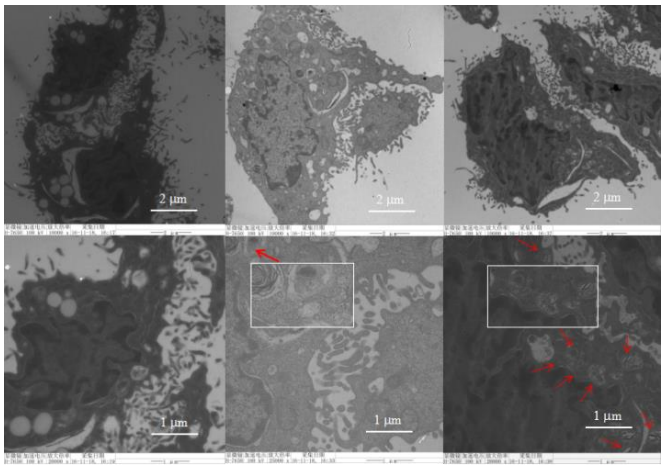
3.5 Effect of MFGM P2 on cell morphology

The effects of MFGM P2 on cell morphology and internal structure were shown in Fig. 7. The results showed that there was no difference among control group, MFGM

group and MFGM P2 group, which indicated the MFGM and MFGM P2 did not affect the surface morphology of the C_2C_{12} cell (Fig. 7A). However, the results from transmission electron microscopy (TEM) did show that the inner structures were changed (Fig. 7B), although there was a similar morphology of the C_2C_{12} cell in all groups (Fig. 7A).



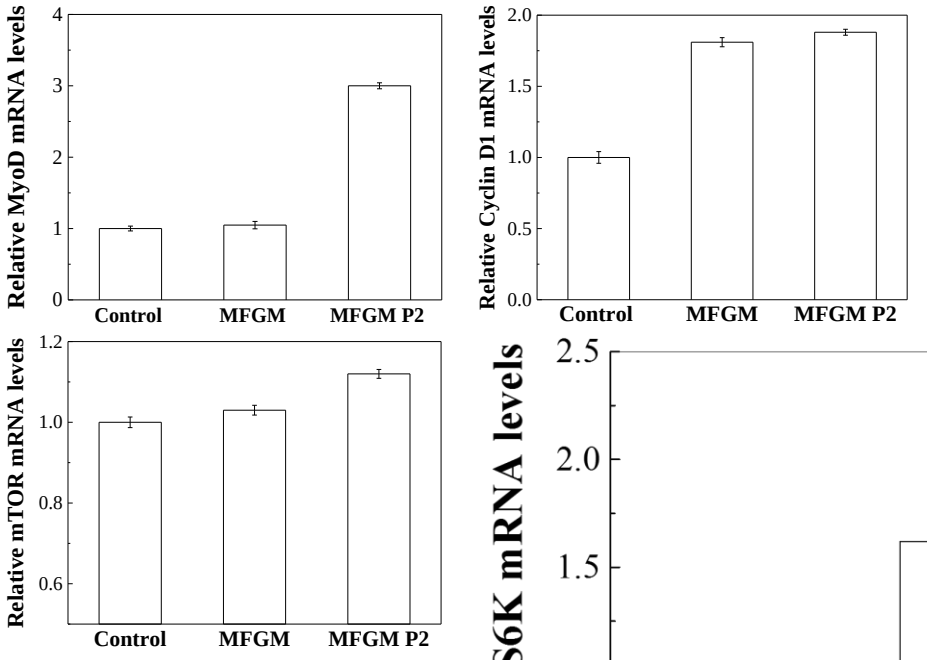
(A)



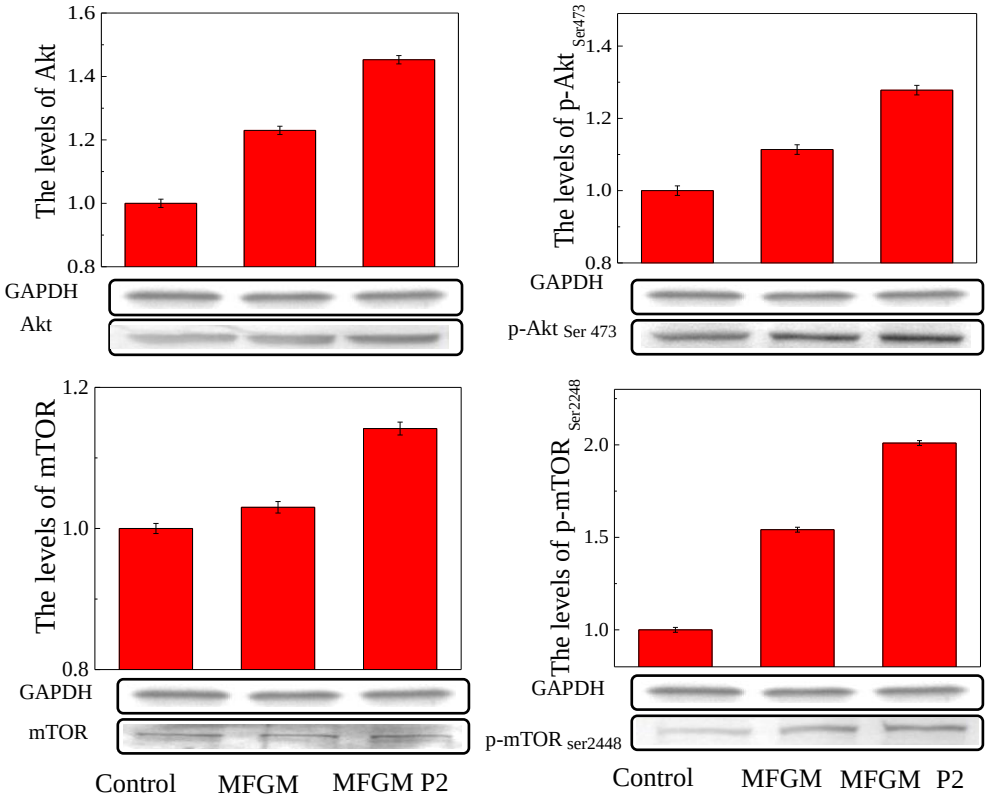
(B)

Fig. 7 Transmission electron microscopy (TEM) and CLSM imaging of C_2C_{12} cells incubated for 48 h with MFGM or MFGM P2 modified medium. (A)The analyisis of CLSM; (B) The TEM micrograph shows C_2C_{12} with the presence of rounder shaped mitochondria (arrows).

3.6 Effect of MFGM P2 on the mRNA expression of cyclin D1, MyoD，mTOR and p70S6K; and the protein expression of Akt, p-Akt, mTOR and p-mTOR



(A)



(B)

Fig. 8 The effect of MFGM P2 on mRNA and protein expressions of some regulators during C_2C_{12} cell proliferation at 48 h. (A) The response of cyclin D1, MyoD, mTOR

and p70S6K mRNA levels; (B) The effects of MFGM and MFGM P2 on Akt, p-Akt Ser 473, mTOR and p-mTOR Ser 2448 expression.

The mRNA levels of MyoD，cyclin D1, mTOR and p70S6K were determined by quantitative real-time PCR experiments (Fig. 8A). Compared with control group, MyoD, cyclin D1, mTOR and p70S6K mRNA levels in the MFGM group were increased by 3%, 82% ($P<0.01$), 10% ($P<0.01$) and 63% ($P<0.01$), respectively; Compared with the MFGM group, myoD and p70S6K levels in MFGM P2 group were significantly increased by 1.37 and 1.86 fold (both $P<0.01$) respectively, the cyclin D1 and mTOR mRNA levels were increased by 3.5% and 5.8%, respectively.

Corresponding to the results of qRT-PCR, some proteins were analysed further with *Western blot* (Fig. 8B). The total amount of Akt in MFGM P2 group increased 1.46 fold over control and MFGM, whereas the level of its phosphorylated form was increased 1.33 fold.

4 Discussion

Previous studies showed that crude MFGM could improve skeletal muscle strength or capability in animal and humans.[13, 14] However, the specific protein wasn't known yet, and the amount of MFGM protein in dietary sources is very low, therefore, this study not only found a way to enrich the protein but also investigated the effect and mechanism of antisarcopenia of the best effective protein, MFGM P2.

Results from Section 3.1 showed that the method successfully extracted and separated proteins into four fractions, and importantly, about 57.8% of total protein was in the second fraction, was MFGM P2. In a 1-D gel electrophoresis of MFGM P2, 42 proteins of 45 kDa were identified in the main protein band, of which MFG-E8 accounted for about 82%. Thus, MFG-E8 accounted for about 65.6% of total protein in MFGM P2. MFG-E8 is known as lactadherin, a protein encoded by the MFG-E8 gene in humans. It is identified as a major glycoprotein of the milk fat globule, a protein and triglyceride rich membrane-bound vesicle secreted from the mammary epithelium

346 during milk production.[25] In this study, rather than further extraction and purification
347 this MFG-E8 protein, whole protein of MFGM P2 was used to analyse the
348 antisarcopenia effect. Because MFG-E8 is composed of 65.6% of total protein, the
349 effect of MFGM P2 was considered as a potential effect of MFG-E8, even though more
350 research is required for verification.

351 MTT assay suggested that MFGM P2 had both a proliferative and antiproliferative
352 effect on C_2C_{12} cells, the results were similar to the effect of insulin, shown by the
353 research of Cheng[23] who found that insulin has both proliferative and antiproliferative
354 effect on C_2C_{12} cells, depending on insulin concentration. Our results found an optimal
355 MFGM P2 dosage for cell growth at 200 μg/mL. This dosage would be a good reference
356 for further clinical trials. FACS analysis demonstrated that MFGM P2 induced the cell
357 cycle, whereas MFGM induced S-phase arrest; V-FITC-PI analysis showed that
358 although scarcely any apoptosis of C_2C_{12} cell was found in the control group, MFGM
359 group and MFGM P2 group, but cell density was higher in the MFGM P2 group than
360 the MFGM group. The results indicated that MFGM P2 showed more enhancement of
361 cell proliferation by promoting cell cycle progression and no toxicity. Therefore,
362 MFGM P2 was more beneficial to muscle protein synthesis.

363 Results from confocal scanning laser microscopy and TEM showed MFGM and
364 MFGM P2 did not change the surface morphology of the C_2C_{12} cell, but the
365 mitochondrial number was increased in MFGM P2 group by TEM assay (Fig. 7B).
366 Inherent or acquired mitochorndrial disorders can cause major disruption of cell
367 survival and whole body metabolic homeostasis.[26, 27] Mitochondria play crucial roles in
368 energy metabolism.[28, 29] Mitochondrial dysfunction in skeletal muscle has been
369 considered as a crucial step in the development of metabolic diseases. Protein kinase B,
370 also known as Akt, a serine/threonine kinase, is a critical signaling component for the
371 regulation of cellular metabolism, growth, and survival in multiple systems.[30] The
372 activity of Akt is in response to numerous stimuli, e.g. growth factors and hormones.
373 Akt can also be activated by increasing intracellular Ca^{2+} or cAMP, which occur with

increasing muscle contractile activity.[31]

Our findings showed that promoting cell proliferation by MFGM P2 might be related to Akt signal pathways. Results showed that MFGM P2 regulated the transcription and the expression of cyclin D1, MyoD, p70S6k, mTOR and Akt to affect cell growth. As 65.6% of protein in MFGM P2 was MFG-E8, we deduced that the action caused by MFGM P2 was mainly from MFG-E8. Previous research reported the role of MFG-E8 in cell growth, invasion, and metastasis.[32] MFG-E8 promoted resistance to apoptosis, an epithelial mesenchymal transition, and angiogenesis through the activation of the PI3K/Akt/mTOR signal pathways. However, there has been no report about the effect of MFG-E8 on C_2C_{12} cell growth.

MyoD is considered to be a marker for terminal specification to the muscle lineage.[33] Its activity has been shown to correlate with the induction of the CDK inhibitor p21.[34] PI3K/Akt mediated signal pathway can increase the MyoD by enhancing its transcriptional activity.[35] Here, we demonstrated that MFGM P2 could induce MyoD expression and cell growth. Results also showed that MFGM P2 regulated the transcription and expression of cyclin D1 to affect cell growth. Cyclin D1 plays a key role in controlling the cell cycle progression.[36] It regulates progression through the G1 phase of the cell cycle by simulating the activity of the cyclin D-dependent kinases (CDK) 4 or 6.[37] The activities of CDK serve to integrate extracellular signaling during the G1 phase with the cell-cycle engine that regulates DNA replication and mitosis.[38] Akt can regulate Cyclin D1 and CDK 4 activity and induce cell proliferation.[39] The relevance of the MFG-E8-cyclin D1 pathway in the proliferation of vascular smooth muscle cells has also been demonstrated by Haruka[40], who showed that MFG-E8 expression is associated with a tendency for increasing expression of vascular smooth muscle cell proliferation marker, cyclin D1.

Akt activation prevents muscle atrophy including sarcopenia.[41] Moreover, the activation of Akt in myoblasts increases their cell proliferation rate and rescues them from cell death. mTOR is one of the key kinases in cell signal transduction, playing an

important role in cell growth, metabolism and the cycle process. Previous research found that PI3K mediates G1 progression and cyclin expression through activation of the AKT/mTOR/p70S6K signaling pathway,[42] mTOR regulation protein synthesis, via phosphorylation and activation of p70S6K.[43] Phosphorylation of p70S6K leads to the activation of pathways promoting protein synthesis and translation initiation.[44-46] So essentially, the mTOR signaling pathway is critical for cellular growth and survival in skeletal muscle, and is activated in response to growth factors such as insulin-like growth factor-I.[47, 48] In the current study, phosphorylation of Akt increased significantly with incubation in MFGM P2 (Fig. 8). A significantly higher Akt-dependent phosphorylation of mTOR was observed after 48 h with MFGM P2 treatment. The result indicated that MFGM P2 treatment has a proliferation effect on translation via the proliferation of Akt and mTOR phosphorylation, which is necessary for the blockade of MFG-E8 induced translational change.

The results demonstrated that MFGM P2 could up-regulate mRNA levels of Cyclin D1, MyoD, mTOR and p70S6K expression, and protein levels of Akt and mTOR expression. The mechanism of MFGM P2 on cell proliferation might be through PI3K/Akt/mTOR/p70S6K signal pathway. This may be caused by the effect of MFG-E8 via regulation on the expression of the Akt/mTOR signal pathway. To the best of our knowledge, there were no report about MFGM protein promote C_2C_{12} cells proliferation previously, in this study, we describe this growth function of MFGM P2, and discuss its downstream target, Cyclin, MyoD, p70S6k, mTOR and Akt, which play important roles in cell proliferation.

Conclusion

Our data suggest that MFGM protein extracted and isolated by an electric cream separator, and purified by cellulose DEAE-52 column is an effective way, which can potently promote cell growth and inhibit apoptosis of C_2C_{12} cells by upregulation of the expression of Akt and mTOR protein kinase. These findings represent a novel mechanism of MFGM protein in cell growth and have new clinical implications in

designing MFGM protein therapies.

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Conflict of interest statement

The authors declare no conflicts of interest.

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