Bovine Milk Fat Globule Epidermal Growth Factor VIII Activates PI3K/Akt Signaling Pathway and Attenuates Sarcopenia in Rat Model Induced by D-galactose

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Abstract

To develop a more effective and safer treatment for sarcopenia, this research investigated the anti-sarcopenia mechanism of Milk Fat Globule Epidermal Growth Factor VIII (MFG-E8) from the liver function and metabolism in sarcopenic model rat. After 4 weeks nutritional intervention experiment, MFG-E8 can significantly increase the gastrocnemius mass in rat. The mechanism of MFG-E8 in improving sarcopenia was related to its promotional capacity to the activities of superoxide dismutase (SOD) activity in serum, Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in liver. Meanwhile, MFG-E8 could also down-regulate obesity-related indicators, such as triglyceride (TG) and Non-esterified fatty acid (NEFA). The analysis of liver and gastrocnemius histopathology found that MFG-E8 could reduce the accumulation of fatty vesicles, improve liver function, thereby
alleviating gastrocnemius tissue inflammation. *In vitro* experiments, myoblasts obtained from gastrocnemius tissue showed that MFG-E8 could reduce mitochondrial autophagy and inhibit cell apoptosis. In addition, MFG-E8 could up-regulate the phosphorylation level of PI3K via activating PI3K/Akt signaling pathway in gastrocnemius tissue, and promote the formation of muscle fibers, thereby increasing muscle mass. Moreover, MFG-E8 could also promote the formation of neuromuscular junctions by up-regulating the mRNA and protein expression of MusK in gastrocnemius.

**Keywords:** MFG-E8; Sarcopenic model rat; Myoblasts; PI3K/Akt signalling pathway.

1 Introduction

Sarcopenia is a kind of aging-related degenerative diseases that occurs mainly in the elderly populations. It is caused by imbalance of skeletal muscle protein anabolism and catabolism, and also associated with metabolic chronic diseases, such as diabetes and obesity, which pose threats to physical performance and life expectancy (Viana et al. 2013). Traditional hormonal drugs for treating sarcopenia have significant effects. However, many steroid hormones drugs, for example, glucocorticoids, testosterone and nandrolone, are poorly nutrient absorption and digestion, as well as a vicious circle, which poses great risks to human health (Sakuma et al. 2017; Sakuma and Yamaguchi 2018). Resistance exercise is an effective way to increase muscle mass, loss weight, and cardiometabolic health. It can enhance the activity of mitochondria and myoblasts activity, but resistance exercise is dangerous for some elderly people (Elena et al. 2015; Joe 2019). Therefore, new safer and more effective anti-sarcopenia nutrients are needed. Currently, natural products, such as bioactive food components, have emerged as new therapies that can overcome incidental risk of drugs (Sakuma and Yamaguchi 2018; Yuan et al. 2018). Traditional food-borne nutrients, such as polyphenols, proteins, bioactive peptides, are regarded as alternative methods in preventing and treating sarcopenia.

Milk is the main source of nutrition in newborn mammals, milk fat globule membrane (MFGM) is a mixture of primarily membrane proteins, phospholipid and sphingomyelin (Nguyen et al. 2016). MFGM has attracted widespread attention in
anti-sarcopenia due to its potential nutritional value, e.g. clinical trials in frail women and healthy adults demonstrated that resistance exercise combined with dietary supplementation and MFGM can reverse the deficits in skeletal muscle protein metabolism imbalance and improve muscle mass and function.

Milk fat globule epidermal growth factor VIII (MFG-E8), a cysteine rich secretory glycoprotein distributed in various tissues in mice and other mammalian species, has been broadly used as Food functional factors and drugs for a long time (Aziz et al. 2017; Cheyuo Cletus et al. 2019). MFG-E8 contains two N-terminal epidermal growth factor-like domains (EGF domains), and C-terminal discoid in domains similar to the blood coagulation factor-V/VIII (C₁ and C₂ domains)(Aziz et al. 2017; Cheyuo Cletus et al. 2019). EGF₁ contains an Arg-Gly-Asp sequence and binds to integrins αvβ3 and αvβ5, besides, C₂ domain has high affinity for membranes that contains phosphatidylserine. The functions of MFG-E8 mainly focused on angiogenesis, phagocytosis of apoptotic cells and cell matrix adhesion. The important health benefits of MFG-E8 include suppressing autoimmune diseases, anti-tumor, suppressing Alzheimer's disease, promoting angiogenesis and repairing intestinal function (Cheyuo Cletus et al. 2019; Aziz et al. 2017). We have previously demonstrated in vitro C₂C₁₂ cells that MFG-E8 could promote cell proliferation and differentiation (He et al. 2017; Li et al. 2017). Based on the results of cell differential proteomics, we clarified that MFG-E8 promoting cell proliferation is related to 60S ribosomal protein L29, Hsp 40, integrin α-V, serine/threonine protein phosphatase 2A and insulin degrading enzyme expression, mediating PI3K, MAPK, AMPK and Oxidative phosphorylation signaling pathways cascade, and PI3K plays a major role (Li et al. 2019). In vitro experiment preliminarily demonstrated the possible benefits of MFG-E8 on sarcopenia (Li et al. 2018). However, to the best of our knowledge, the research of MFG-E8 on sarcopenia has focused on the in vitro myoblasts model, so far, there are no related reports about MFG-E8 in vivo (Li et al. 2017; Li et al. 2018).

Organs involved in the development of sarcopenia mainly include the liver, kidney, skeletal muscle, brain and adipose tissue (Ponziani and Gasbarrini 2018; Sun et al.
The liver, which is the primary organ for lipid and carbohydrate metabolism (Qi-chen et al. 2019). Skeletal muscle and liver tissue are the primary site of insulin-mediated glucose uptake (Manning and Toker 2017). Insulin-like growth factor (IGF-I) regulates the proliferation and differentiation of cells with insulin-like metabolism and nutrition, and is closely related to animal muscle growth and development. PI3K/AKT pathway play a central role in the molecular mechanisms, which regulate cellular energy metabolism and glucose metabolism (Zecic and Braeckman 2020). Mitochondrial oxidative capacity was significant reduced in ageing liver, which may act as an important contributor to the ageing process (Rygiel et al. 2017; Joe 2019). Akt, known as protein kinase B, is a serine/threonine kinase, operates downstream of PI3K, and has a key responsibility in regulation of energy metabolism at both organismal and cellular levels (Qi-chen et al. 2019; Li et al. 2018). In addition, IGF-I can regulate the differentiation of myoblasts by inducing the expression of key factors such as MyoD, MEF2 and p21. The biological activity of IGF-I is regulated by IGF-I receptors and IGF binding proteins, which play roles in promoting mitosis, growth differentiation and insulin-like metabolic effects (Yu et al. 2015). Therefore, it seems that insulin and IGF-I are in a tight relationship in terms of intracellular activities. However, there are very limited data about the effect of MFG-E8 on IGF-I in age-related sarcopenia subjects.

Aging is manifested as the decline of self-functions, decreased physical strength, stress ability and energy metabolism accompanied by the occurrence of various aging-related diseases (Zhou et al. 2016; Haifeng et al. 2005). Excessive injection of D-gal can induce toxic and side effects on the body, mainly involve mitochondrial dysfunction, generation of a large number of free radicals and lipid peroxidation, accelerate the aging process of rats, and cause abnormal metabolic function of important organs such as heart, liver and kidney. This impair syndrome exhibited senile degenerative changes, and ultimately lead to varying degrees of damage and atrophy of skeletal muscle fibers, highly similar to the clinical symptoms of sarcopenia. The rat model of sarcopenia constructed by D-gal is reversible, and can be relieved and treated
through drug and nutritional intervention (Zhang et al. 2018; Sakuma and Yamaguchi 2018). Because of its small damage to the body and high survival rate, it can simulate the sarcopenia caused by natural aging more accurately, so it is widely used in the screening of senile sarcopenia drugs (Fan et al. 2017; Liao et al. 2014).

In this study, a sarcopenic model rat induced by injection of D-gal was established to evaluate the repair and regeneration effects of MGF-E8. The effects of MFG-E8 on liver and gastrocnemius dysfunction and histological lesion caused by sarcopenia were carried out. Furthermore, considering the important role of the PI3K/Akt pathways in metabolism, the effects of MFG-E8 on the activation of the PI3K/Akt pathways were also investigated.

2. Materials and methods

The MFGM protein was separated from cow’s milk (Harbin, Heilongjiang, China) by an electric cream separator, MFG-E8 from the MFGM was prepared and separated based on the cellulose DE-52 ion exchange chromatography treatment (He et al. 2017). Sprague Dawley (SD) rats were purchased from the animal facility of the Second Affiliated Hospital of Harbin Medical University (Harbin, Heilongjiang, China), permission number: SCXK 2013-001.

2.1 Experimental design

2.1.1 Animal model

Sarcopenic model rats were carried out by following the literature procedure (Zhang et al. 2018; Sun et al. 2018). 200 mg/kg D-galactose (D-gal) was administered daily for 6 weeks through subcutaneous injection, control group was subcutaneous injection same volume of 0.01 mol/L Phosphate buffered saline (PBS). Each rat was kept in an isolated cage with free access to food and water (Table 1).

2.1.2 Nutrition intervened

Rats were randomly assigned to 3 groups (Table 1), MFGM or MFG-E8 was administrated orally daily for 4 weeks days after 6 weeks of D-gal injection, asodium pentobarbital anesthesia, while the rests were sacrificed by cervical dislocation, and the blood, organs and tissues were taken out for biochemical assays. All surgeries were
performed under sodium pentobarbital anesthesia, and all the efforts were made to minimize suffering. The body weight of the rats was measured weekly. All animal protocols were approved by the animal ethics committee of Harbin Institute of Technology, School of Life Science and Technology.

### 2.2 Physiological and biochemical analysis

#### 2.2.1 Tissue collection

In the end of the experiments, the muscle weight (gastrocnemius and soleus), fat tissue weight (epididymal and perienal) and tissue weight (liver, spleen and kidney) were measured and harvested. Liver and gastrocnemius tissues were rapidly placed in 10% formaldehyde solution fixative as a biopsy for microscopy assessment.

#### 2.2.2 Biochemical analysis

Blood was collected from anesthetized rat heart under non-fasted conditions via chlora hydrate vein. Plasma glucose, triglyceride (TG) and Non-esterified fatty acid (NEFA) contents were measured with glucose, TG and NEFA testing kit (Lengton Biosciencei, Shagnhai, China). The enzymatic activities of superoxide dismutase (SOD) and the levels of Malondialdehyde (MDA) were measured with SOD and MDA testing kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s instructions. Insulin like growth factor 1 (IGF-1) was measured with a rat IGF-1 Immunoassay kit (Lengton Biosciencei, Shagnhai, China). Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) activity in liver were measured with GOT and GTP assay kits (Nanjing Jiancheng Bioengineering Institute, China).

#### 2.3 Histological analysis

Histopathological determine was performed by a pathologist at the hospital of Harbin Institute of Technology. The gastrocnemius and liver samples were pre-fixed in 10% formaldehyde solution and washed with PBS buffer. The muscle samples were vertically embedded in paraplast. Block prepared, frozen and cut muscle fragments into 5 µm thick sections. These sections were well-mounted on pre-silanated slides and dried at 60 °C for 1 h, followed by dewaxing, hydration and staining with hematoxylin–eosin
These stained slides were dehydrated and mounted in Permount. For the quantification of nuclei and muscle fibers and area measurement of these fibers were magnified from 10 to 100x.

2.4 The In vitro Assessment of MFG-E8 on myoblasts of gastrocnemius

2.4.1 Isolation and identification of myoblast

The myoblasts were isolated according to Alessandra et al.’s(Alessandra Sacco 2008) with a slight modification. The large hind limb gastrocnemius of rats was isolated, then non-muscle tissues were removed under a section microscope. The gastrocnemius was subjected to enzymatic dissociation with 0.2% collagenase Type II (Solarbio, China) for 60 min, then with 0.04 U/mL dispase II (Solarbio, China) for 45 min. The cell suspension was filtered through a cell strainer.

Evaluation of myoblast proliferation and differentiation ability was according to our previous research methods(Li et al. 2017). Myoblasts were grown in DMEM with 10% (v/v) FBS, 100 U mL\(^{-1}\) penicillin, and 100 mg mL\(^{-1}\) of streptomycin (Invitrogen) in a humidified incubator containing 5% CO\(_2\) at 37°C. The myoblasts were fused into myotubes at 50% confluence by shifting the growth medium to a differentiation medium consisting of DMEM supplemented with 2% horse serum. The morphological changes of myoblasts were observed by light microscope.

2.4.2 Effect of MFG-E8 on Cell cycle

Cell cycle experiments were carried out on a BD FACS scan flow cytometer (Bio-Rad) and Cell-Quest software (BD Biosciences), which describe in our previous research(He et al. 2017).

2.4.3 Effect of MFG-E8 on mitochondria

Myoblasts were fixed with 4% glutaraldehyde at 4 °C for 12 hours, which were collected by centrifugation. The cell pellets were fixed in 2.5% glutaraldehyde for 2 h and with 1% osmium tetroxide for 2 h, then dehydrated in ascending ethanol serial washes and embedded in Epon 812. Serial ultrathin sections were characterized by transmission electron microscope (TEM, Zeiss 900, Germany).
2.5.4 Effects of the MFG-E8 on the myoblasts proliferation and morphology

Myoblasts were incubated with 1 mL of staining buffer, 5 mL of Hoechst 33342 (excitation 360 nm/emission 465 nm) buffer and 5 ml of PI staining (excitation 488 nm/emission 620 nm) buffer for 20~30 min, then myoblasts were observed by confocal laser scanning microscope (CLSM, Leica, Germany).

2.5 qRT-PCR

Total RNA in gastrocnemius was extracted using a RNAPrep pure animal tissue kit (Tiangen Biotech, Beijing). cDNA was synthesized using 3 mg of RNA with a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Ishiyama, Japan) according to the manufacturer’s instructions. The assay was carried out on an CFX96 Real-Time PCR system (Bio-Rad Laboratories, USA) with iTaq™ Universal SYBR Green Supermix (BIO-RAD, CA, USA). The qRT-PCR conditions were as follows: 95 °C for 5 s, followed by 40 cycles of 95°C for 10 s, and 60 °C for 30 s. Relative expression was first quantified using a standard curve and 2-\Delta\Delta CT formula, and normalised to GAPDH. The primers used in this study were shown in Table 2.

2.6 Western blot

The method was based on our previous research (Li et al. 2018), total protein in gastrocnemius was extracted by RIPA buffer with 10 mM PMSF, then the concentration of protein was determined by BCA protein assay kit (Solarbio, China), and 10 mg protein was electrophoresed in 10% SDS-PAGE gel. The proteins in gel were transferred to nitrocellulose (NC) membrane carried out by Bio-rad electrottransfer system. The NC membranes were incubated at 37 °C temperature for 1 h in blocking buffer. Primary antibodies for each protein was incubated at 4 °C for 2 h. The concentrations of the primary antibodies were anti-MusK (Bioss, China, 1:600), anti-PI3K (Santa Cruz, USA, 1:500), anti-Akt (Santa Cruz, USA, 1:500), anti-p-Akt (Santa Cruz, USA, 1:800), anti-mTOR (Santa Cruz, USA, 1:300) anti-p-mTOR (Santa Cruz, USA, 1:500), anti-P70S6K (Santa Cruz, USA, 1:800), anti-p-P70S6K (Santa Cruz, USA, 1:800), anti-4E-BP (Santa Cruz, USA, 1:300), anti-p-4E-BP (Santa Cruz, USA, 1:400), and GAPDH (Santa Cruz, USA, 1:500). The NC membrane was washed by
TBST, following by incubation with secondary antibody (1:2000; Santa Cruz, USA) for 1 h at 37 °C. The NC membrane was washed by TBST and TBS, then incubated with alkaline phosphatase. The protein band was detected by FluorChem Imaging Systems (Alpha Innotech, Corp., San Leandro, CA, USA).

2.7 Statistical analysis

All experiments, except described 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 analyses were done by using SPSS 19.0 (SPSS Science, USA).

3 Results

The MFG-E8 was extracted using an electric cream separator, and separated according to cellulose DEAE-52 ion exchange chromatography method. Our previous work has demonstrated that MFG-E8 can promote cell proliferation and differentiation via the PI3K/Akt and ERK signal pathway in the in vitro C$_2$C$_{12}$ cells (Li et al. 2019). In this study, we focused on the in vivo rat experiment to investigate the effect of MFG-E8 on age-related sarcopenia. The work flow was shown in Fig. 1.

3.1 Construction and evaluation of sarcopenic model rats

D-gal is a nutrient mainly obtained from lactose in milk, however, excessive intake of D-gal can accelerate the aging process, accompanied by tissue and organ dysfunction, resulting in metabolic abnormalities, which are the pathological characteristics of aging-related sarcopenia (Zhang et al. 2018; Sun et al. 2018). Therefore, D-gal has been widely used in exploring the targets of aging-related sarcopenia model and drug testing. D-gal was injected into the subcutaneous of the rats for 6 weeks, based on the changes of the rat's physical characteristics, physiology, biochemistry and organ coefficients to evaluate whether the sarcopenic rats model was successfully constructed.

3.1.1 Effects of D-gal on physiological and biochemical indices in rat

To investigate the effect of D-gal on the basic physiological indexes of rats, we recorded the data of initial weight, final weight gastrocnemius and soleus coefficient of rats in 6 weeks (Table 3). Compared with normal group, the weight of rat increased
more slowly in D-gal group (Fig. 2A), after 6 weeks, the final weight of rats in D-gal was also significantly lower than normal group (Table 3, \( p<0.05 \)). The gastrocnemius and soleus coefficients were calculated based on the final weight of the rats, results showed that gastrocnemius and soleus coefficients of D-gal group were reduced by 11.3% and 12.5%, respectively (\( p<0.05 \)), suggesting that D-gal induces impairment of skeletal muscle mass in rat.

The tissue coefficients of kidney and liver were significantly lower in the D-gal (Fig. 2B, \( p<0.05 \), versus normal group), and the kidney and liver were increased by 12.8 and 22.8%, respectively. However, the coefficient of spleen, epididymis adipose and perirenal fat was no significantly different in D-gal (\( p>0.05 \), versus normal group).

SOD and MDA were used as indicators to evaluate the degree of aging, which can regulated the dynamic imbalance among antioxidant capacity, free radical generation and the degree of lipid peroxidation. SOD is the first enzymatic line of antioxidant and an important enzyme in the antioxidant system, as shown in Fig. 2C, SOD activity in the D-gal group was reduced by 22.6% (\( p<0.05 \), versus normal group). However, the content of MDA in the D-gal group was increased by 38.6% (\( p<0.05 \), versus normal group).

These results were similar to zhang et al.\'s (Zhang et al. 2018) who have reported that 200 mg/kg D-gal in the model group, which caused metabolic disorders and free radical imbalance in the rat, accelerated the aging process of the rat, and resulted in a decrease in the rate of increase in the gastrocnemius and soleus mass of the rats.

3.1.2 gastrocnemius histopathology analysis

The status of muscle fibers of gastrocnemius, affected by D-gal was further observed using an inverted fluorescence microscope, and the results were shown in Fig. 2D. The muscle fibers of the gastrocnemius were seen to be arranged in fascicles surrounded by connective tissue of normal appearance, with each fiber also surrounded by connective tissue. Compared with normal group, the number of fibers and nuclei decreased in the gastrocnemius of the D-gal treated rats (\( p<0.05 \)), however, the diameter of cells was increased and oedema of the cells was more serious in D-gal group. Besides,
there was significant difference in the total cross-sectional area, cells arrangement irregularity, various forms of muscle fibers, and nuclei of the gastrocnemius between the control and D-gal group (Fig. 2D). Combined with the results of physiological and biochemical indicators, these results revealed that D-gal of 200 mg/kg could lead to soleus and gastrocnemius injury, and the aging-related sarcopenic model rat was successfully constructed by D-gal.

3.2 MFG-E8 improved gastrocnemius and soleus in sarcopenia rats

After MFGM or MFG-E8 treatment for 4 weeks, the average food intake of sarcopenic model rat fed the normal diet (as control group) was not significantly different from that of rats fed with MFGM and MFG-E8 (Table 4). Accordingly, the rat body weight increased significantly after 4 weeks of MFGM or MFG-E8 treatment ($p<0.05$, versus control group). The order of weight was as follows: MFG-E8 (474.33±18.43g) > MFGM (464.00±12.46g) > Control (455.67±10.34g). The gastrocnemius and soleus coefficient was significantly lower than that in the MFGM (increased by 6.2% and 10.3%, respectively) or MFG-E8 (increased by 22.1% and 44.6%, respectively) group ($p<0.05$, versus control group); the gastrocnemius and soleus coefficient was increased 28.8% and 10.7%, respectively ($p<0.05$, versus MFGM group). The order of gastrocnemius coefficient was as follows: MFG-E8 (0.083±0.002) > MFGM (0.075±0.005) > Control (0.068±0.008).

3.3 Effect of MFG-E8 on tissue weights.

The results of physiological indices demonstrated that the tissue coefficients of kidney and liver were significantly higher in the MFGM and MFG-E8 group (Fig. 3A, $p<0.05$, versus control group), and the kidney and liver were increased by 11.9% and 6.0%, respectively ($p<0.05$, versus MFGM group). In contrast, the epididymis adipose and perirenal fat tissues mass were lower in MFGM and MFG-E8 group, but the coefficient of epididymis adipose, perirenal fat, spleen and testis was no significantly difference ($p>0.05$, versus control group).

3.4 MFG-E8 improved antioxidant activity, liver function and lipid profile

IGFs stimulate responses in skeletal muscle that include effects on carbohydrate
and lipid metabolism, protein turnover, growth, and differentiation. To assess more
c characteristics directly related to aging, IGF-I cytokines in rat were measured by ELISA
(Fig. 3B). Compared with controls, IGF-I in MFGM or MFG-E8 group was increased
by 5.7% and 48.6%, respectively (Fig. 3B, \( p<0.05 \)).

The effects of MFG-E8 on the antioxidant activity were summarized in Fig. 3C,
the SOD activity in the MFGM or MFG-E8 group was increased by 13.6% and 33.6%,
while MDA content was reduced by 12.4% and 41.9%, respectively (\( p<0.05 \), versus
control group). It shows that MFGM, especially MFG-E8, could improve the
scavenging ability of superoxide anion free radicals in rats, reduce the accumulation of
harmful substances in the cells of rats, and delay the aging process.

Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase
(GPT) exist in the mitochondria and cytoplasm of all cells. The increase in the activity
of GOT and GPT correlates in general well with the extent and severity of cellular
damage, and is a basic procedure for diagnosing and monitoring hepatocellular diseases
or muscle damage. Liver function, as represented by GOT and GPT activities, improved
in model rat and this effect was significantly increased by MFG-E8 consumption (Fig.
3D).

In addition, MFG-E8 feeding decreased obesity-related metabolic parameters in rat,
including the epididymis adipose, perirenal fat, serum TG and serum NEFA (Fig. 3E,
epididymis adipose and perirenal fat coefficient: \( p>0.05 \), TG and NEFA:\( p<0.05 \), versus
control and MFGM group). We noticed that MFGM and MFG-E8 showed no effect on
the rat’s food intake, but it can effectively increase body weight, gastrocnemius and
soleus coefficient and improve obesity-related metabolic parameters (Table 4, \( p<0.05 \),
versus control group).

3.5 MFG-E8 ameliorated pathological changes of liver and gastrocnemius

Histological examination of liver sections by staining with haematoxylin–eosin
revealed a loss of cellular integrity and an excessive fat accumulation in the liver of
control group, which exhibited more and large hepatic lipid droplets and more severe
steatosis compared with the MFGM and MFG-E8 treated groups. The hepatic lipid
droplets were disappeared almost completely, and hepatocytes were increased after MFGM, especially MFG-E8 treatment (Fig. 4A). Hepatic morphology exhibited that MFG-E8 treatment suppressed hepatic fatty acid accumulation and deposition, and thereby preventing the formation of lipid droplets in hepatocytes. This trend is similar with the effect of MFG-E8 administration on liver and plasma lipid profiles, which suggest that MFG-E8 can down-regulate lipid accumulation in liver.

In comparison with control group, the spacing of muscle fiber became smaller and dense, and cell nucleus was significantly increased in model rats treated by MFGM and MFG-E8 (Fig. 4B); In addition, the rat also showed the direct evidence of muscle atrophy in control group, eg: MFGM and MFG-E8 could effectively alleviate oedema of the cells and muscle fiber atrophy. Compared with control, MFGM, especially for MFG-E8 showed an effective way to alleviate muscle atrophy as a nutritional element. Muscle atrophy or degenerative changes was significantly improved in the MFG-E8 group. These results indicated MFG-E8 could improve aging-related muscle atrophy.

3.6 The In vitro Assessment of MFG-E8 on myoblast of sarcopenic rats

3.6.1 Isolation and Characterization of Myoblast

The cell morphology and differentiation ability were observed through an inverted microscope (Fig. 5). The cells isolated from the gastrocnemius of the three groups of rats exhibited an elliptical/spindle shape (Fig. 5A) and had the ability to differentiate into muscle fibers (Fig. 5B), and the MFG-E8 group had the strongest cell differentiation ability ($p<0.05$, versus control group). The results proved that the cells isolated from the gastrocnemius tissue were myoblasts.

3.6.2 Effects of MFG-E8 on the myoblast

To determine the effect of MFG-E8 on myoblast, the changes of MFG-E8 treated in cell cycle, cell activity, cell morphology and structure were analyzed. There was a 2.87% decrease in the G0/G1 population, and 0.54% and 12.77% decreases in S and G2/M populations in myoblast treated with MFGM (Fig. 6A). Compared with the control group, the G0/G1 and S population decreased by 11.90%, while the S and G2/M population increased by 17.94% and 8.00% in cells treated with MFG-E8.
Mitochondrial dysfunction in skeletal muscle has been considered as a crucial step in the development of metabolic diseases. Inherent or acquired mitochondrial disorders can cause major disruption of cell survival and metabolic homeostasis (Joe 2019). Further to investigate the effect of MFG-E8 on mitochondrial of myoblast was shown in Fig. 6B. The mitochondria appeared to autophagy, vacuoles and formed unclear edges in controls. Compared with control group, the mitochondrial number increased and mitophagy was alleviated in the MFGM and MFG-E8 group.

The effect of MFG-E8 on myoblast morphology was further detected by CLSM (Fig. 6C). The microscopic observation results showed that cell density significant increased in MFG-E8 group, and the surface morphology of myoblasts showed no significant change in MFGM and MFG-E8 group. However, the proportion of apoptotic cells decreased (Fig. 6C). Combined with cell cycle, CLSM and TEM results, the cell repairment and anti-apoptotic effect conducted by MFG-E8 may cause by the increase of mitochondria.

Taken together, the measurement of gastrocnemius weight showed that gastrocnemius coefficient leads to 15%-20% muscle loss in the sarcopenic model rats (Table 3, \( p < 0.05 \), versus control group); while MFGM (~6.5%), especially for MFG-E8-treated group, significantly reduced the loss of muscle mass (~44.63%) in sarcopenic model rats (Table 4, \( p < 0.05 \), versus control group). Consistent with the in vitro morphological data, the MFG-E8 could exert its protective effect against muscle atrophy in rat muscle fibers as well.

3.7 Effect of MFG-E8 on mRNA and protein expression levels

The mRNA levels of MyoD, MyoG, Cyclin D1, IGF-I, Dok-7 and MusK were determined by qRT-PCR (Fig. 7). the levels of Cyclin D1, MyoD and MyoG mRNA in the MFGM group were increased by 15%, 88% and 63%, respectively, while the levels of IGF-I and MusK mRNA in the MFGM group were significant increased by 25% and 21%, respectively (Fig. 8A, \( p < 0.05 \), versus control group). The levels of MyoG, Cyclin D1, IGF-I and MusK in the MFG-E8 group were significantly increased by 65%, 39.2%, 41.7%, 14.9% and 78.1%, respectively, while the MyoD mRNA level was reduced by
Muscle-specific tyrosine kinase (MusK) and Dok-7, both of which play essential roles in synapse formation at the neuromuscular junction (NMJ), lack of MusK and Dok-7 can lead to the failure of NMJ formation (Punga et al. 2015). Corresponding to the results of qRT-PCR, MusK proteins were analysed further with western blot assay (Fig. 7B). The expression of MusK in the MFG-E8 group up-regulated 39.4% over control and MFGM. This result was similar to Haramizu (Haramizu et al. 2014a) who has reported that MFGM, especially for resistance exercise combined with dietary nutritional supplementation with MFGM, could improve muscle function deficits via neuromuscular development in aging-related sarcopenia mouse.

3.8 Effect of MFG-E8 on the activation of PI3K/AKT signaling pathway

Mitochondria play crucial roles in energy metabolism, the PI3K/Akt signaling pathway plays a crucial role in a variety of basic cell processes, including proliferation, apoptosis, cell survival and metabolism (TF et al. 2003). To investigate the possible effect of MFG-E8 activates PI3K/Akt signaling pathway, the expressions of PI3K protein and phosphorylation levels were determined in gastrocnemius. Compared with control group, MFGM did not significantly affect protein expression of PI3K, Akt and mTOR (Fig. 8A-C, p > 0.05, versus control group) but could significantly up-regulated the expression of phosphorylated PI3K, Akt and mTOR, especially MFG-E8 group (p < 0.05). MFGM contains more than four hundred proteins (Ji et al. 2017), among them, MFG-E8 is a high abundant protein in MFGM protein (~30%), indicating that MFG-E8 is a key functional factor in MFGM that could activate the PI3K signaling pathway.

MFG-E8 not only increase the total PI3K, AKT mTOR and P70S6K protein levels but also increase the phosphorylated PI3K, AKT, mTOR and P70S6K protein levels (Fig. 8A-D, p < 0.05, versus control), whereas the transcriptional activities of p-4E-BP were decreased (Fig. 8E, p < 0.05, versus control). This result was similar to our previous research in vitro cell experiment (Li et al. 2018), it can be speculated that MFG-E8 may have a certain impact on the translation process of the protein, that is, there may be a mechanism that promote protein synthesis and inhibit protein degradation. the
p-70S6K/p-70S6K and p-4E-BP/4E-BP levels in the MFG-E8 group were up-regulated by 37.3% and down-regulated by 11.5%, respectively. The results indicated that MFG-E8 could promote the proliferation, differentiation and myofibroblast formation of myoblasts by activating the PI3K signaling pathway, thereby achieving the purpose of repairing the damaged gastrocnemius.

4. Discussion

Sarcopenia is closely related to many metabolic diseases, such as obesity, abnormal glucose metabolism or diabetes, hypertension and dyslipidemia (Angulo et al. 2016). Although there is a clear classification of drugs for sarcopenia, side effects are still serious during clinical use. So the choice of more effective and safer treatment for sarcopenia has been become attractive. In recent years, more and more researches focus on natural products that tried as novel therapeutic agents for sarcopenia, most of them involved herbal plants extracts or foodborne plants, such as epigallocatechin gallate, saponins flavonoids, curcumin, and ginsenosides, etc (Sakuma and Yamaguchi 2018). Only a few studies have focused on the role of milk protein in preventing sarcopenia, such as whey and MFGM (Minegishi et al. 2016). The branched-chain amino acids of the hydrolysate of whey protein could promote the proliferation of myoblasts by activating the IGF-I/PI3K/Akt signaling pathway (Jean et al. 2014). MFGM produced by milk fat has an anti-sarcopenia effect caused by upregulating the expression of MusK and Dok-7 in clinical trials (Haramizu et al. 2014b; Hari et al. 2015); MFG-E8 isolated from milk exhibited more potential efficacy than MFGM in mitochondrial protection, and proposed that it could regulate C2C12 cell proliferation by activating PI3K, MAPK, Oxidative phosphorylation and AMPK signaling cascades.

MFGM has been used worldwide for many years as commercially available health foods and has been considered beneficial to health. MFG-E8 is secreted from the mammary epithelium during milk production as a high abundant protein in MFGM. MFG-E8 also expressed in a wide range of organ tissues by a large variety of cells including macrophages, fibroblasts, dendritic and epithelial cells (Li et al. 2017; Ji et al. 2017). In addition, a large number of literatures have reported the function, nutrition and
physiological characteristics of MFG-E8 in recent years. Based on the previous work, in this paper, we for the first time investigated the anti-sarcopenia mechanism of MFG-E8 \textit{in vivo}. The basic principles for establishing aging-related sarcopenia model based on current research were as follows: As an inducer of aging \textit{in vivo}, D-gal have been widely used for exploring the targets of aging D-gal and drug testing (Zhang et al. 2018; Sun et al. 2018). The aging rat induced by D-gal exhibited a dramatically aging-associated decline in endurance capacity, oxygen consumption, and fat oxidation associated with decreased fatty acid β-oxidation. Therefore, it could be concluded that this was a suitable model that conforms to similar pathogenesis in humans, and can thus be used to study the causes and pathogenesis of metabolic disorders involving sarcopenia and to screen for potential use as treatment. In this study, the results of our data in Section 3.1 showed gastrocnemius coefficient, kidney and liver coefficient, and SOD/MDA in sarcopenic model rats decreased along with the daily treatment, and inter-striated muscle cell edema and wax-like degeneration appeared, which meant the sarcopenia model was successfully established (Liao et al. 2014; Zhang et al. 2018).

Aging process is precisely controlled by a giant signaling network that included energy homeostasis, cellular metabolism and stress resistance (M; Nair K Sreekumaran; Kahn C Ronald; 2019). Oxidative stress is a physiological and pathological reaction in concert with increasing age, manifested as elevated free radicals leading to metabolic disorders and inducing myoblast apoptosis, which can further cause the imbalance and dysfunction of muscle protein (Zecic and Braeckman 2020). SOD and MDA are important parameters to evaluate the levels of free radicals and oxidative stress (Wei et al. 2016). MDA is the secondary product of lipid peroxidation used as reactive aldehyde that could make cross-linking between intramolecular and intermolecular and interact with proteins to exert a toxic effect on the cells (Ruiz et al. 2012). SOD is also regarded as the primary defense system against ROS and may reflect the ability of scavenging free radicals (Wei et al. 2016; b et al. 2020). In present study, MFG-E8 could enhance SOD activity and reduce MDA content, accelerate the scavenging of free radicals, and improve the antioxidant capacity, SOD and MDA was negatively correlated. In addition,
Fatty acid metabolism is mainly characterized by elevated levels of NEFA, a predominant raised TG and low high density lipoprotein cholesterol levels, small dense low-density lipoprotein cholesterol particles and raised apolipoprotein B values postprandial hyperlipidaemia may also be present. Some emerging evidence also suggests that the accumulation of lipids within skeletal muscle fibers may lead to metabolic disease, such as insulin resistance (Janssen and Ross 2005). The mTOR is a conserved serine-threonine kinase that regulates cell growth and metabolism in response to nutrient signals. mTOR play a significant role both in “energy sensing” and in regulation of energy production through profound effects on hepatic fatty acid metabolism in rat (Joe 2019; Yamane et al. 2017). The levels of NEFA and the content of TG in serum were decreased (Fig. 3E), in contrast, the liver coefficients, GTP and GOT activity were increased, and the content of hepatic lipid droplets was reduced in histopathological sections of liver (Fig. 3D). It suggested that MFG-E8 improve aging-induced oxidative stress, fatty acid metabolism and promote the repair and regeneration of gastrocnemius, which is related to the activity of intracellular mitochondria. It is similar to Świątecka who demonstrated that dairy proteins, eg: MFGM, whey protein or casein have the potential to suppress postprandial lipaemia due to their insulinotropic effects (Świątecka et al. 2017; Qi-chen et al. 2019). Based on these results, the repair and regeneration effect of MFG-E8 on sarcopenia is, at least partially, attributed to the protection of the liver.

Apoptosis, oxidative stress, mitochondrial dysfunction and inflammation are important factors in the occurrence of sarcopenia. Apoptotic cells and potentially toxic substances are the result of aging-related neurodegenerative diseases and normal aging processes. Effective removal of apoptotic substances is essential to protect surrounding tissues from damage by proteins released by apoptotic cells (Cheyuo Cletus et al. 2019). In the aging-related sarcopenia population, due to insufficient secretion of their own growth factors and hormone levels, insufficient intracellular ATP energy supply result in increased cytoplasmic Ca\(^{2+}\) concentration, finally leading to the number and activity of muscle cells decrease, and even cause cell apoptosis (He et al. 2017; Wang et al. 2016).
Based on *in vitro* gastrocnemius myoblasts experimental results, MFG-E8 has no significantly effect on the surface morphology of myoblasts. The structure of mitophagy and vacuolar was reduced, and the number of cells in S phase and G2/M phase increased, which promoted the synthesis of cell division-related enzymes and spindle filament proteins, accelerated the process of cell mitosis to promote cell proliferation. In addition, as the number of mitochondria increases, their activity, intracellular Ca\(^{2+}\) and cyclic adenylate content increases, which further enhance Akt phosphorylation (Rygiel et al. 2017; Joe 2019). Activated Akt can negatively affect autophagy by regulating the phosphorylation of mTOR, further promoting muscle protein synthesis and enhancing muscle contraction (Jianbo et al. 2019). Therefore, MFGM, especially for MFG-E8 increased the number of myoblast of the aged rats *in vivo* and *in vitro*, promoted their differentiation compared with controls, which could be the mechanisms that the skeletal muscle weight increased, and accelerated the regeneration of injured skeletal muscles in aged rats compared with controls.

IGF-I maintains the functions of skeletal muscle development mainly by: i) promoting protein anabolism; ii) inhibiting protein catabolism. Besides, IGF-I can regulate the differentiation of myoblasts by inducing the expression of key factors such as MyoD, MEF2 and p21. IGF-I can also mediate the physiological role of growth hormone (GH) in peripheral tissues. GH is secreted by pituitary gland and binds to GH receptor to stimulate local tissue synthesis and secretion of IGF-I, however, excessive IGF-I inhibits growth factor secretion, which limits the efficiency of IGF-I translation initiation/translation extension and reduces the rate of gastrocnemius protein synthesis (Webster et al. 2001; Yu et al. 2015). In this regard, MFG-E8 attenuates aging-related sarcopenia by promoting the level of IGF-I in serum and IGF-I mRNA expression in gastrocnemius, suggesting that MFG-E8 might attenuate dyslipidemia by improving lipid metabolism in liver tissue.

The PI3K/Akt signaling pathway plays a crucial role in a variety of basic cell processes, including proliferation, apoptosis, cell survival and metabolism (TF et al. 2003). The PI3K-related signaling pathway is not only related to the proliferation of
C2C12 cells, but also regulate the expression of myogenic regulatory factors: MyoD, MyoG and MyHC, and participate in the regulation of the proliferation, differentiation and fusion of muscle C2C12 cells to form multinucleated myotubes, which eventually develop into mature muscle fibers (Li et al. 2017). Akt is a serine/threonine kinase, operates downstream of PI3K, and plays a key role in the regulation of energy metabolism at both organismal and cellular levels. Akt was also found to alter PI3K/Akt signaling in the skeletal muscle of young men with low birth weight (Jensen et al. 2008). mTOR is a downstream effector of Akt. Akt was phosphorylated on Thr308 in the catalytic domain and Ser473 in the C-terminal domain by PDK1 and PDK2, respectively, which were critical for Akt activation (He L et al. 2019; Li et al. 2018). Phosphorylated Akt can improve the function of mTOR by phosphorylation at Ser2448, which could up-regulate the expression of P70S6K phosphorylation and down-regulate the expression of 4E-BP phosphorlyation to regulate protein metabolism balance (Li et al. 2018). Based on these research, we investigated the mechanism of action of MFG-E8 on the expression pattern of gastrocnemius in an in vivo rat model with qRT-PCR and western blot. It was also observed that activated Akt phosphorylates the 2448 position of mTOR, mTOR was activated, which regulates gastrocnemius protein synthesis and degradation through two ways: (i) up-regulate the phosphorylated expression of P70S6K protein to promote protein synthesis; (ii) down-regulate the phosphorylated expression of 4E-BP1 to inhibit protein degradation.

The process of myogenic proliferation and differentiation is largely controlled by the transcription factors of myogenic regulatory factor (MRF) family, such as MyoD, MyoG and Myf5, which regulate the expression of several muscle-specific genes (Conerly et al. 2016; Zammit and S. 2017). MyoD is a skeletal muscle-specific protein that is able to induce myogenesis in a large variety of cell types, and considered as a marker for terminal specification to the muscle lineage (Conerly et al. 2016). MyoG not only can regulate its own expression, but also can interact with other transcription factors of MRF, such as MyoD and Myf5, which is necessary for myoblast differential and fusion in vivo (Zammit and S. 2017). MyoD activity has been shown
to correlate with the induction of the CDK inhibitor p21 (Li et al. 2017). Our previous results indicated that the PI3K/Akt-mediated signaling pathway can regulate the activity of MyoD and MyoG by enhancing its transcriptional activity. Cyclin D1 is a key target of proliferation signals, which could activate CDK4 by changing the G1 components. Akt could induce cell proliferation by regulating its downstream cyclin D1, cyclin D2, CDk 4 and p21 levels (Jianbo et al. 2019). The relevance of the AKT/GSK3β/CyclinD1 pathway in the proliferation of hepatocellular carcinoma cells has also been demonstrated by Xi, who showed that Akt expression is associated with a tendency for increasing the expression of the hepatocellular carcinoma cells proliferation marker, cyclin D1 (Jianbo et al. 2019). In this research, MFG-E8 can accelerate the differentiation and fusion process of myoblasts by up-regulating MyoG gene expression in myoblasts of gastrocnemius tissue, which is consistent with the results of HE staining, and these results were similar to our previous studies, therefore, MFG-E8 can effectively promote the proliferation, differentiation and fusion of myoblasts, and further promote the formation of gastrocnemius fibers.

In summary, in vivo, MFG-E8 treatment increased the gastrocnemius coefficient, regenerated capacity of injured muscles, improved oxidative stress and fatty acid metabolism compared with controls in sarcopenia rats via activating PI3K/Akt-signaling pathway. In vitro, myoblast proliferation, differentiation, apoptosis and mitochondrial autophagy were improved compared with controls in isolated myoblast from sarcopenia rats. These results indicated that MFG-E8 has a beneficial effect on preventing aging-related sarcopenia by activating the PI3K/Akt signaling pathway (Fig.9).

5 Conclusion

This study investigated the mechanism of MFG-E8 in improving sarcopenia in vivo for the first time. MFG-E8 regulated oxidative stress and fatty acid metabolism level through down-regulating the level of MDA, IGF-I, TG and NEFA in serum, up-regulating the SOD activity and SOD/MDA. Inter-striated muscle cell edema and wax-like degeneration were improved in gastrocnemius, and the fat content was reduced in liver. In vitro, MFG-E8 alleviated apoptosis and mitochondrial autophagy of
myoblasts isolated from rats. MFG-E8 also up-regulated mRNA and protein expression of IGF-I, MUSK, PI3K, Akt, mTOR and P70S6K, as well as down-regulated 4E-BP protein expression in gastrocnemius. This research puts in new knowledge about the potential mechanism of MFG-E8 in alleviating sarcopenia, which provides a new evidence for the scientific and rational use for MFG-E8 in the improvement of sarcopenia. Given these results, it can be affirmed that MFG-E8 could be a potential anti-sarcopenia function factor and improve liver condition.

**Conflict of interest**

The authors declare no conflicts of interest

**Acknowledgments**

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**Reference**


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Viana JU, Silva SL, Torres JL, Dias JM, Pereira LS & Dias RC (2013) Influence of sarcopenia and functionality indicators on the frailty profile of


Table

Table 1. Primer sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>TGGTGGACGCTCCTTCAGTTC</td>
<td>CTTCAGCGGAGCAGTACA</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>CTGACACCAATCTCCTCAACGAC</td>
<td>GCGGCCCCGTTCCACTTGAGC</td>
</tr>
<tr>
<td>MyoD</td>
<td>TCGGTGCTTTGAGAGATCGAC</td>
<td>CGAAAGGACAG-TTGGGAAGAGT</td>
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<td>MyoG</td>
<td>AGAGAAGCACCCTGCTCAAC</td>
<td>TGATCTCGGGTTGGGACC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCACAGTCCATGCCATCAC</td>
<td>TC-CACCACCTGTGTGCTGTA</td>
</tr>
<tr>
<td>MusK</td>
<td>AACAACATCCCGTCCATAACG</td>
<td>GTGAGGAGGACAAACCCG</td>
</tr>
<tr>
<td>Dok-7</td>
<td>TCTTTTCGCGCTCCTGCACCTC</td>
<td>TATCTGTGCTGAACGAGG</td>
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</table>

Table 2. Animal grouping and feeding conditions

Table 3. The effects of D-gal on soleus and gastrocnemius coefficient of rat
### Table 2. Animal grouping and feeding conditions

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Number of rats</th>
<th>Basic feed</th>
<th>Amount</th>
<th>Feeding cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construction of rat model (6 week)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>SPF animal feed</td>
<td>0.01 mol/L PBS</td>
<td>6 week</td>
</tr>
<tr>
<td>Model (D-gal)</td>
<td>36</td>
<td>SPF animal feed</td>
<td>200 mg/kg D-gal</td>
<td>6 week</td>
</tr>
<tr>
<td>Nutritional intervention (4 week)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>SPF animal feed</td>
<td>0.01 mol/L PBS</td>
<td>4 week</td>
</tr>
<tr>
<td>MFGM</td>
<td>12</td>
<td>SPF animal feed</td>
<td>16 mg MFGM</td>
<td>4 week</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>12</td>
<td>SPF animal feed</td>
<td>16 mg MFG-E8</td>
<td>4 week</td>
</tr>
</tbody>
</table>

### Table 3. Effect of D-gal on soleus and gastrocnemius coefficient of rats

<table>
<thead>
<tr>
<th>Construction of rat model (6 week)</th>
<th>Normal</th>
<th>Model (D-gal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>200±10.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200±12.56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>442.1±12.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>412.4±10.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soleus coefficient (%)</td>
<td>0.16±0.011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14±0.013&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gastrocnemius coefficient (%)</td>
<td>0.72±0.031&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60±0.024&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: values are means ± SEM of 10–12 rats. a-b means with different letters within a row are significantly ($p<0.05$).

### Table 4. Effect of MFG-E8 on gastrocnemius and soleus coefficient of sarcopenic model rats

<table>
<thead>
<tr>
<th>Nutritional intervention (4 week)</th>
<th>Control</th>
<th>MFGM</th>
<th>MFG-E8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>412.4±10.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>412.4±10.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>412.4±10.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>455.67±10.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>464.00±12.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>474.33±18.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soleus coefficient (%)</td>
<td>0.068±0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.075±0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.083±0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gastrocnemius coefficient (%)</td>
<td>0.522±0.048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.586±0.017&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.755±0.175&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: values are means ± SEM of 10–12 rats. a-b means with different letters within a row are significantly ($p<0.05$).
Figure Legend

**Fig. 1** The work flow in this study.

**Fig. 2** Construction of sarcopenic model rats. (A) Effect of D-gal on body weight of rats; (B) Effect of D-gal on tissue weights in rat; (C) Effect of D-gal on oxidation associated biomarkers in blood; (D) Effect of D-gal on muscle fiber morphology. The results are expressed as mean±SEM of 10~12 rats, the different letters (a-b) between control and D-gal gropus across all represent significant differences (p<0.05).

**Fig. 3** Effect of MFG-E8 on improvement of muscle atrophy. (A) Effect of MFG-E8 on tissues; (B) Effect of MFG-E8 on IGF-1; (C) Effect of MFG-E8 on SOD and MDA; (D) Effect of MFG-E8 on GPT and GOT; (E) Effect of MFG-E8 on TG and NEFA. The results are expressed as mean±SEM of 10~12 rats, the different letters between the three groups of them across all represent significant differences (p<0.05).

**Fig. 4** Pathological section of rat liver and gastrocnemius. (A) Histological examination of liver sections, black arrows indicate central veins, yellow arrows indicate lipid droplets. blue arrows indicate hepatocytes. (B) Histological examination of gastrocnemius sections.

**Fig. 5** Identification of myoblasts. (A) Morphology of myoblasts; (B) Myoblast differentiation.

**Fig. 6** The effect of MFG-E8 on myoblasts activity was evaluated by *in vitro*. (A) Effect of MFG-E8 on cell cycle; (B) Effect of MFG-E8 on mitochondria was observed by TEM; (C) CLSM observed the effect of MFG-E8 on cell apoptosis.

**Fig. 7** Effect of MFG-E8 on the formation of neuromuscular junction. (A) Effects of MFG-E8 on mRNA levels of MyoD, MyoG, cyclin D1, MusK, Dok-7 and IGF-1; (B) Effect of MFG-E8 on protein levels of MusK. The results are expressed as mean±SEM of 10~12 rats, the different letters (a-c) between the three groups of them across all represent significant differences (p<0.05).

**Fig. 8** Effect of MFG-E8 on PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, P70S6K, p-P70S6K, 4E-BP and p-4E-BP expression in rat. The results are expressed as mean±SEM of 10~12 rats, the different letters (a-c) between the three groups of them
across all represent significant differences ($p<0.05$).

**Fig. 9** The mechanism of MFG-E8 in gastrocnemius repair and regeneration. Based on *in vivo* and *in vitro* results, we proposed that MFG-E8 in gastrocnemius repair and regeneration might be described as follows: i) MFG-E8 promotes the regulatory and catalytic subunit of PI3K and promote myoblast proliferation via PI3K/Akt/mTOR/P70S6K and PI3K/Akt/mTOR/4E-BP signal pathway (Li et al. 2018); ii) MFG-E8 can activate the PI3K/Akt pathway during myoblast differentiation process, and regulate the expression of MyoD and MyoG (Li et al. 2017).
The diagram illustrates the process of constructing sarcopenia rats using D-galactose intervention. Nutrition intervention is shown in the upper left section, with MFG-E8 acting as a major glycoprotein of the milk fat globules, separated by DEAE-52. The control group uses PBS.

The process involves:
- Tissue Collection
- Biochemical analysis
- Histological analysis

Steps include:
- Fat liver kidney muscle heart
- Take blood from the heart
- Liver skeletal muscle
- Identification of myoblasts
- qRT-PCR
- Western blot

The analysis of cell activity includes Cell cycle, TEM, CLSM.

Fig. 1
Fig. 3

Fig. 4
Fig. 5

(A) Control  MFGM  MFG-E8

(B) Control  MFGM  MFG-E8

Fig. 6

(A) Control  MFGM  MFG-E8

(B) Control  MFGM  MFG-E8

(C) Apoptotic cell  Mitochondrial autophagy  Normal cell

MFGM  MFG-E8  MFG-E8

MFGM  MFG-E8  MFG-E8
Fig. 7
Fig. 8
Fig. 9