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MFG-E8 Induced Differences in Proteomic Profiles in Mouse C_2C_{12} Cells and Its Effect on PI3K/Akt and ERK Signal Pathways

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Abstract

Milk fat globule-EGF factor 8 (MFG-E8) is one of the major proteins in milk fat globule membrane. In this study, mouse-derived C_2C_{12} myoblast cells were served as an experimentally tractable model system for investigating the molecular basis of skeletal muscle cell specification and development. To examine the biochemical adaptations associated with myocytes formation comprehensively, a liquid chromatography coupled with tandem mass spectrometry label-free semi-quantitative approach was used to analyse the myogenic C_2C_{12} proliferation program. Over 1987 proteins were identified in C_2C_{12} cells. The MFG-E8 (200 μg/mL) and MFG-E8 (500 μg/mL) with significant differences were compared based on the relative abundance. The result profiles of regulation of MFG-E8 to the expression of proteins in C_2C_{12} cells revealed that differential waves of expression of proteins linked to intracellular signaling, transcription, cytoarchitecture, adhesion, metabolism, and muscle contraction across during the C_2C_{12} cell proliferation process. Based on the analysis of KEGG and STRING database, further to verification the expression of PI3K and ERK phosphorylation levels by *Western blot*. This study found that the data of proteomic was complementary to recent MFG-E8 studies of protein expression patterns in developing myotubes and provided a holistic framework for understanding how diverse biochemical processes are coordinated at the cellular level during skeletal muscle development.

Keyword: LC-MS/MS, label-free, MFG-E8, *Western blot*, biological activity.

Introduction.

Sarcopenia is a frequent disease in the elderly populations and is related to metabolic diseases, such as diabetes and obesity [1]. The age-related process is associated with losses of muscle mass, bone mass, strength and function, which pose significant threats to physical performance and quality of life [2].

The milk fat globule membrane (MFGM) has gained much attention as a kind of nutritional supplementation due to its positively influence aspects of sarcopenia and thereby prevention of skeletal muscle protein degradation [3]. Milk fat globule-EGF factor 8 (MFG-E8), known as lactadherin, is a highly abundance glycoprotein in MFGM [4]. It is also expressed in a range of tissues by a variety of cells including macrophages, fibroblasts, dendritic, and epithelial cells.

PI3K/Akt signaling pathway plays an essential role in a wide range of biological functions, including metabolism, cell growth, proliferation, differentiation and intracellular trafficking [5]. Our previous studies reported that MFG-E8 can promote C_2C_{12} cell proliferation via the PI3K/Akt/mTOR/P70S6K signal pathway. MAPK signal pathway is known to regulate cell proliferation, differentiation and cell survival. Three subsets of Mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK (p38) and c-Jun N-terminal kinase (JNK), are vital pathways that transfer extracellular signals into cells and closely associate with the proliferation and differentiation of cells. Growing evidences point to the important role of MAPKs on mechanical stretch regulated proliferation, differentiation and protein synthesis of skeletal muscle cells.

Cell metabolomic analysis has been developed as a powerful, convenient and efficient platform to systematically clarify the molecular mechanisms underlying nutritional supplementation [6]. Metabolites, as the downstream products of gene transcription, its alternations may reflect overall metabolic changes of cells intuitively. Therefore, it is convenient to study the effects and molecular mechanisms of nutritional interventions on muscle cells. In vivo animal and human experiments have been conducted to study the effect and mechanism of MFGM supplementation. Our previous work has shown that MFG-E8 was isolated from MFGM and purified by an

ion exchange column [7], LC-MS/MS was used to identify MFG-E8 as the main components in MFGM P2 (82.4%). MFG-E8 can promote C_2C_{12} cell proliferation and differentiation based on the results of flow cytometry, laser confocal microscopy and transmission electron microscopy analyses. The key regulatory factors, eg., PI3K P85α, p-pI3K p85α (Tyr 508), Akt, p-Akt (Ser 473), mTOR and p-mTOR (Ser 2448), in cells incubated with MFG-E8 with or without wortmannin, were measured by qRT-PCR and *Western blot* assay. These result were indicated MFG-E8 could promote C_2C_{12} cell proliferation and differentiation via PI3K/Akt signal pathway [8]. However, MFG-E8 has been reported to promote the invasion, migration and proliferation of cancer cells via the multiple-signaling pathways, and there are rarely performed in vitro cell experiments by using metabonomic analysis.

In this study, we performed lable-free-based metabolomics analysis to explore metabolic responses of MFG-E8 supplementation on C_2C_{12} mouse myoblast cell line, which can promote C_2C_{12} cells proliferation in vitro, and a systematic investigation on the differences in proteomic was induced by different concentration of MFG-E8. The differences in proteomic profiles of myoblast C_2C_{12} cell induced by MFG-E8 were investigated by label-free semiquantitative approach. Related signal pathways were analyzed by KEGG analysis and phosphoprotein interacting-networks were generated by the STRING database. Further to verification of phosphoproteins by *Western blot*. The purpose of this study is to better understand how diverse biochemical processes are coordinated at the cellular level during MFG-E8 regulated skeletal muscle development.

2 Materials and Methods

2.1 Materials

C_2C_{12} cell was supplied by Chinese Academy of Agricultural Sciences (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and Trypsin-EDTA solution (approximately 0.25% trypsin and 0.02% EDTA) were purchased from gibco. All other chemicals and reagents used were of analytical grade.

2.2 Preparation of MFG-E8

The MFG-E8 protein was extracted according to the method reported

previously[7].Briefly, a fresh milk sample (100 mL) containing sucrose (5 g) was centrifuged using an automatic cream separator, at 3500g at 38 °C for 30 min. The buttermilk was initially adjusted to pH 4.8 using 0.01 mol L^{-1} HCl in order to allow the MFGM to precipitate, and then neutralized with 0.01 mol L^{-1} NaOH. Finally, the MFGM pellet was resuspended and its supernatant was freeze dried and stored at −20 °C before analysis.

MFGM (0.2 g) was dissolved in PBS buffer (pH 8.0, 20 mg mL^{-1}) and applied onto a DEAE Cellulose DE-52 (2.6 × 60 cm) column (Biotopped, China). The protein fractions were eluted using a linear gradient elution of NaCl (0.2 M~0.8 M) in PBS buffer (pH 8.0) at a flow rate of 1 mL min^{-1}. The MFGM protein fraction 2 were collected, dialysed, concentrated and freeze-dried. The molecular weight and purity of the MFG-E8 protein was confirmed using LC-MS/MS.

2.3 Cell Culture

The mouse skeletal myoblast C_2C_{12} cells were grown in DEME medium (10 % FBS, 90 % DEME). The C_2C_{12} cells were treated with 200 μg/mL or 500 μg/mL MFG-E8 for 48 h at 37 ℃ in a 95 % humidified atmosphere filtered air and 5% CO_2 in a CO_2 incubator (HF90/HF240, Shanghai, China).

2.4 The identification of protein

2.4.1 protein Preparation

C_2C_{12} cells were treated with 200 μg/mL of either MFGM or MFG-E8 for 48 h, washed twice with PBS buffer. SDT buffer (4% sodium dodecyl sulfate, 100 mM dithiothreitol, 150 mM Tris–HCl and pH 8.0) was added to the protein sample. The lysate was sonicated and then boiled for 15 min. After centrifuged at 14000 g for 40 min, the supernatant was quantified with the BCA Protein Assay Kit (Bio-Rad, USA). The sample was stored at -80 °C.

2.4.2 SDS-PAGE

The protein concentration was quantified using the BCA kit. 100 μg protein was loaded onto a 1-D SDS gel (12% polyacrylamide) [9]. The gel was stained with Coomassie brilliant blue R-250. The gel was destained with a solution of methanol and glacial acetic acid (9:2, V/V). The image of the gel was taken using LKB 2400

GelSCan XL gel imaging system (Bio-Rad, USA).

2.4.3 Liquid chromatography (LC)-electrospray ionization (ESI) Tandem MS (MS/MS) analysis by Q exactive

The protein in C_2C_{12} cell components were further analysed with capillary LC-MS/MS based on our previous research [9]. The identification and quantification of protein components in C_2C_{12} cells was confirmed using LC-MS/MS.

2.4.4 Sequence database searching and data analysis

MASCOT engine and Proteome Discoverer 1.3 were applied to search and analysis of MS/MS spectra based on the uniprot_cetartiodactyla. fasta database (download on uniprot_mouse_84433_20180105.fasta, http://www.uniprot.org/). The search parameters of Mascot and the analysis parameters of protein identification were carried out according to the method in Ji et al [4].

2.5 Western blot

C_2C_{12} cells were treated with 200 μg/mL of either MFGM or MFG-E8 for 48 h, washed twice with PBS buffer and homogenized in lysis buffer. Total protein was extracted and harvested by scraping with a modified radioimmunoprecipitation assay buffer containing 100 nM phenylmethylsulfonyl fluoride for 30 min. Following centrifugation at 10000 rpm for 15 min at 4℃, the supernatant was sonicated. The protein concentration was quantified using a BCA kit (Solarbio, China). Proteins (100 μg) were loaded onto a 1-D SDS gel (12 % polyacrylamide). Next, proteins were transferred onto a nitrocellulose filter membrane (PPLYGEN, China) using a wet electrotransfer system (Bio-Rad, USA） for 1 h 30 min 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 Cruz，USA) for each protein, for 1 h at 37 ℃, or overnight at 4 ℃, according to the requirements. The concentrations of antibodies were as follows: GAPDH (1:500), PI3K (1:500), p-PI3K (1:300), ERK (1:600) and p-ERK (1:600). The membrane was washed for 5 minutes three times with TBST, followed by incubation with anti-mouse or anti-rabbit secondary antibody (1:2000; Santa Cruz Biotechnology, Inc.) at 37 ℃ for 1 h. The membrane was washed with TBST twice and with TBS once, 5 min each,

and 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).

2.6 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 Result and discussion

3.1 The identification of protein in C_2C_{12} cells

The differences in proteomic profiles of myoblast C_2C_{12} cell induced by MFG-E8 were analyzed by the LC-MS/MS, tryptic peptide sequence was matched with MASCOT and Uniport. 1987 proteins in C_2C_{12} cells were identified, Histone H4 (5.82 %), Histone H3 (3.97 %), Histone H2A (3.84 %) and Histone H2B (3.28 %) were the higher abundant protein components in C_2C_{12} cells (Table 1). Previous research was found that MFG-E8 induced C_2C_{12} cell proliferation and apoptosis in a dose-dependent manner and 200 μg mL^{-1} MFG-E8 was an optimal dosage for cell growth, the proliferation rate reached a maximum (35.8%), however, over 300 $\mu g/mL^{-1}$, the cell proliferation activity decreased. Therefore, further to clarify the effects of optimal concentration and high concentration of MFG-E8 on intracellular protein metabolites, 200 $\mu g/mL^{-1}$ and 500 $\mu g/mL^{-1}$ MFG-E8 were selected for experimental analysis. Compared with 500 ug/ml MFG-E8 group, 200 ug/ml MFG-E8 up-regulated the expression of proteins, such as Histone H3, Histone H2A and Protein S100 ($p<0.05$), two of the nucleosomal histone families, H3 and H2A, have highly conserved variants with specialized functions, which are essential for development in higher eukaryotes [10, 11]. It was indicated that MFG-E8 induced C_2C_{12} cell proliferation in a dose-dependent manner, high concentration of MFG-E8 appeared an inhibition activity on the expression of these main proteins in C_2C_{12} cell; Enolase 1, an important protein in regulating the differentiation and functions of bone

marrow-derived mast cells and playing a crucial role in innate and adaptive immune responses [12], was down-regulated compared with 500 ug/ml MFG-E8; And the expression of other 14 proteins were not shown the significant difference ($p>0.05$) (Table 1).

3.2 Gene onology (GO) analysis of the identified proteins

There were 415 of total proteins having significant differences in MFG-E8 group compared with control group (>1.5 fold), including 123 up-regulation proteins and 293 down-regulation proteins. Among them, 15 proteins were statistically significant of up-regulation (MFG-E8/Control>3fold) and 74 proteins were down-regulation (MFG-E8/Control<0.29 fold) (table 2 and table 3).

GO analysis was carried out to obtain biological processes and molecular functions of proteins (89 of the total identified significant differential proteins in the C_2C_{12} cell) were categorized into biological process, molecular function and cellular components according to their annotation (Fig. 1). The main molecular functions were protein binding, peptidase activity and ATP binding (Fig. 1A). The annotation of cell components showed that these differential proteins were mainly in cytosol, nucleus, membrane, nucleoplasm and cytoskeleton, etc (Fig. 1B). The most common biological process was the catabolic process, followed by translation, metabolic process, cellular response and apoptotic process (Fig. 1 C).

3.3 Differences in proteomic profiles of myoblast C_2C_{12} cell

We futher to investigate the MFG-E8-mediated signaling pathways through KEGG and interaction network analyses of all regulated phosphoproteins. An interaction network of all MFG-E8-regulated protein-protein interaction was constructed by using the program String (Fig. 2). From this complex network, we extracted subnetworks based on KEGG analysis. We identified that MFG-E8 can regulate the expression of protein in C_2C_{12} cell associated with cell growth, metabolism pathway, immune response pathway, respectively.

3.3.1 The up-regulation of myoblast C_2C_{12} cell protein

In order to gain a better knowledge of the potential biological functions of MFG-E8 proteins, the differences in proteomic profiles of C_2C_{12} myoblast cell

induced by MFG-E8 were analyzed by LC-MS/MS (Table 1~3). Proteins displaying a 3 fold between MFG-E8 and control were considered to be at differential levels if $p<0.05$. According to the statistical analysis, 6.19% of 1987 proteins showed $p<0.05$. In total, 15 proteins were considered as major differential proteins (Table 2).

Table 2 listed that 15 proteins significant up-regulated in MFG-E8 group compared with control group (MFG-E8/Control>3 fold). In addition, compared with 500 μg/mL MFG-E8, Acylamino acid releasing enzyme, SWI/SNF-related matrix associated actin dependent regulator of chromatin subfamily A member 5 (SWI/SNF -5) and RNA-binding protein 25 (RP25) were significantly up-regulated (1.57-7.68 fold) in the 200 μg/mL, it was indicated that the high concentration MFG-E8 can negatively regulated the expression of these up-regulated protein compared with 200 μg/mL, MFG-E8 induced C_2C_{12} cell proliferation in a dose-dependent manner.

The KEGG analysis demonstrated that up-regulation of proteins are mainly involved in PI3K-Akt, MAPK, Focal adhesion, ECM-receptor interaction, Phagosome. Cushing syndrome and Oxidative phosphorylation signaling pathways to regulate cell activity. The KEGG analysis revealed that Vesicle transport through interaction (VTTI), AH receptor-interacting protein (AHRIP) and Acylamino acid releasing enzyme (AARE) participateed in the MFG-E8 dependent immune response (table. 2). AARE is a high abundance protein in C_2C_{12} cell, and the activity of AARE was highly elevated, apparently reflecting the increased population of immune cells in the inflamed lung [13]. Toru et al [13] found that Acylamino Acid Releasing Enzyme is a novel biomarker candidate for lung inflammation, which can be detected in bronchoalveolar lavage fluid in a mix-and-read manner, down-regulation of AARE activity could lead to more prolonged inflammation[14] .

The KEGG and subnetwork analysis demonstrated that Integrin alpha-V, 60S ribosomal protein L29 and NADH proteins were identified as target molecules of MFG-E8-dependent cell growth and metabolism signaling pathway. NADH dehydrogenase is the major electron entry site for the mitochondrial electron transport chain and therefore of great significance for mitochondrial ATP generation [15]. The complex dysfunction causes reorganization of cellular respiration and affects

metabolic processes in mitochondria with drastic consequences for growth and development [16]. Autophagy is induced in NADH dehydrogenase knockdown cells to remove the damaged mitochondria in order to generate more functional mitochondria. Mitochondrial function is directly involved in both cellular differentiation and apoptotic cell death [17]. These results were consistent with our previous research, which was found that MFG-E8 can increase the quantity of mitochondrion in C_2C_{12} cells [7] .

Integrin alpha-V is necessary for gastrulation movements that regulate vertebrate body asymmetry [18]. Integrin alpha-v mediated through regulation of Toll-like receptors signaling exists to limit excessive B cell responses to self-antigens. In addition, the regulation of Integrin alpha-V mediated TLR signaling not only regulates B cell responses to self-antigens but important for regulating germinal center B cell responses and production of high-affinity antibodies.

60S ribosomal protein L29 plays important regulatory functions in the ribosome and helps maintain a normal protein synthetic rate [19]. Several mutations in genes encoding ribosomal proteins or proteins involved in ribosome biogenesis have been associated with human birth defects with clinical features that include skeletal growth deficiencies and/or deformities. These observations strongly link the fundamental processes of ribosome synthesis, growth, and skeletogenesis.

Our previous research found that MFG-E8 promoted C_2C_{12} cells proliferation via PI3K/Akt signal pathway, which can activate P70S6k through phosphorylation of Akt. Therefore, the results was further to prove that MFG-E8 promote C_2C_{12} cell proliferation mainly via PI3K/Akt signal pathway [9].

3.3.2 The down-regulation of myoblast C_2C_{12} cell protein

Proteins displaying a 0.29 fold between MFG-E8 and control were considered to be at differential levels if $p<0.05$. According to the statistical analysis, 14.75% of total proteins showed $p<0.05$. In total, 74 of 293 down-regulated proteins were considered as differential proteins. Among them, 32 proteins have significant down-regulated in MFG-E8 compared with 500 μg/ml MFG-E8 and control (table 3). In addition, compared with 500 μg/mL MFG-E8, Acylamino acid releasing enzyme, SWI/SNF-5

and RNA-binding protein, etc. 12 of 32 proteins were significantly down-regulation (0.02-0.06 fold) in the 200 μg/mL; Meanwhile, Protein YIPF5, Serine/threonine protein phosphatase PGAM5 and CCA tRNA nucleotidyltransferase 1 were significantly up-regulated (1.83-2.62 fold) (table 3).

The KEGG and subnetwork analysis suggusted that down-regulation of protein mainly mediated the signaling pathway of metabolism process, immunoregulation process (such as insulin resistance, transcriptional misregulation in cancer and Alzheimer disease) and transcriptional process.

Insulin-degrading enzyme (IDE) was 0.13 fold lower in MFG-E8 200 group compared to MFG-E8 500 group (Table 3). Insulin-degrading enzyme (IDE) is responsible for the cellular degradation of insulin. In particular, the inhibition of IDE activity results in increased extracellular and intracellular insulin concentration which might be crucial for insulin-dependent regulation of gene expression and cell proliferation [20]. IDE can regulate insulin-like peptide 2 levels, which is crucial for restricting the activation of the PI3K pathway and promoting activation of Forkhead box.

The serine/threonine protein phosphatase 2A is a major intracellular protein phosphatase that plays crucial roles in hormone signal transduction and abiotic stress response, and it is involved in cell growth inhibition and antitumor activity [21].

Histones H3 is now regarded as integral and dynamic components of the machinery responsible for regulating gene transcription [22]. Many types of cancer and other diseases are associated with translocations or mutations in chromatin-modifying enzymes and regulatory proteins. Epidermal growth factor receptor (EFGR)-independent p38 MAPK and EGFR-dependent ERK1/2 activation by 2,3,5-Tris-(glutathion-S-yl) hydroquinone induce the activation of two downstream signaling factors, histone H3 and Hsp27 phosphorylation, and finally lead to renal cell death [23]. Histone H2AX plays a critical role in regulation of tumor cell apoptosis and acts as a novel human tumor suppressor protein [24].

Phosphoglycerate mutase 5 (PGAM5) is an atypical mitochondrial Ser/Thr phosphatase that modulates mitochondrial dynamics and participates in both apoptotic

and necrotic cell death. Serine/threonine-protein phosphatase PGAM5 plays a central Role to negatively regulate $CD4^+$ T cells by inhibiting NDPK-B-mediated histidine phosphorylation and activation of the K^+ channel KCa3.1, which is required for TCR-stimulated Ca^{2+} influx and cytokine production [25].

In summary, the biological functions of both up-regulation and down-regulation proteins which related to C_2C_{12} cell proliferation mainly includes metabolic process, regulation of biological process and transport. However, there are some main differences in the relative abundance of cell in the different MFG-E8 concentration, specifically for 60S ribosomal protein L29, NADH dehydrogenase, Integrin alpha-V, Serine/threonine-protein phosphatase 2A activator, Insulin-degrading enzyme and Podoplanin and Guanine nucleotide-binding protein-like 3-like protein. The abundance of these intracellular proteins in the MFG-E8 group was considerably higher than control group, and the high concentration of MFG-E8 (500 μg/mL) can significant inhibit expression of Protein YIPF5, Serine/threonine-protein phosphatase PGAM5 and CCA tRNA nucleotidyltransferase, which lead to metabolic disorder and negative regulation of cell growth. Further detail characterization of the molecular structures of these proteins and their associated biological functions in C_2C_{12} cell were needed to further research in order to take advantage of MFG-E8 protein for human skeletal muscle development and health.

3.4 Verification of phosphoproteins by Western Blotting

ERK is essential for cell proliferation in general, ERK activation plays a fundamental role for G1/S transition since its activation regulates the assembly of cyclin/CDK complex and is required for the induction of the cyclin D1 protein. The Ras/ERK signaling cascade is classically linked to cell proliferation and survival [26]. PI3K also plays a central role in cell signaling and leads to cell proliferation, survival, motility, secretion, and specialized cell responses, such as the respiratory burst of granulocytes [9, 27]. Based on the KEGG analysis, the mechanism of MFG-E8 and the relationship between the PI3K/Akt and MAPK/ERK signal pathway. Fig. 7 was shown that MFG-E8 can up-regulate the expression of phosphorylation of PI3K and ERK. Wortmannin plays a central role to negatively regulate C_2C_{12} cell by inhibiting

PI3K/Akt-mediated PI3 phosphorylation and activation of the PI3K/Akt pathway [9, 27]. Furthermore, PI3K and ERK phosphorylation was reduced by combination treatment with wortmannin and U0126, U0126 exerted effects similar to wortmannin on ERK phosphorylation either alone or in combination with wortmannin ($p<0.05$). It is worth noting that p-PI3K (1.52) and p-PI3K/PI3K (1.40) was significant higher than p-ERK (1.25) and p-ERK/ERK (1.26), respectively ($p<0.05$). The result showed that PI3K and ERK phosphorylation is induced by MFG-E8 during activation of reserve cells. Moreover, the inhibitors of the PI3K and ERK cascades suppressed reserve cell activation [27]. Therefore, the result indicated that reserve cell activation required phosphorylation of both pathways, PI3K and ERK. Although, MFG-E8 regulate C_2C_{12} cell proliferation mainly through PI3K/Akt signal pathway.

Based on the results, we proposed that the mechanism of MFG-E8 on C_2C_{12} cell proliferation might be described as follows (Fig.4): i) Previous studies have been reported that MFG-E8 promotes the regulatory and catalytic subunit of PI3K and promote C_2C_{12} cell proliferation via PI3K/Akt/mTOR/P70S6K singnal pathway [9]; ii) MFG-E8 bind to cell surface receptors including G-protein coupled receptors and receptor tyrosine kinases resulting in the activation of the GTPase Ras. Raf activity was increased by Ras, and MEK was activated. The downstream target MEK was phosphorylated by the RAS protein kinase and its activity was promoted, resulting in activation of ERK, which ultimately catalyzes phosphorylation of a wide variety of downstream effector proteins, *eg:* phosphatases and transcription factors.

Conclusion

This study provides a quantitative proteomic method was used to investigate the differences in proteomic profiles of myoblast C_2C_{12} cell induced by MFG-E8. The effect of MFG-E8 on the improvement of sarcopenia and its potential mechanisms underlying the PI3K/Akt and ERK signaling pathway in C_2C_{12} cells. Further detail characterization of the molecular structures of these proteins and their associated biological functions in C_2C_{12} cell were needed to further research in order to take advantage of MFG-E8 protein for human skeletal muscle development and health.

Acknowledgments

Conflict of interest statement

The authors declare no conflicts of interest

Reference

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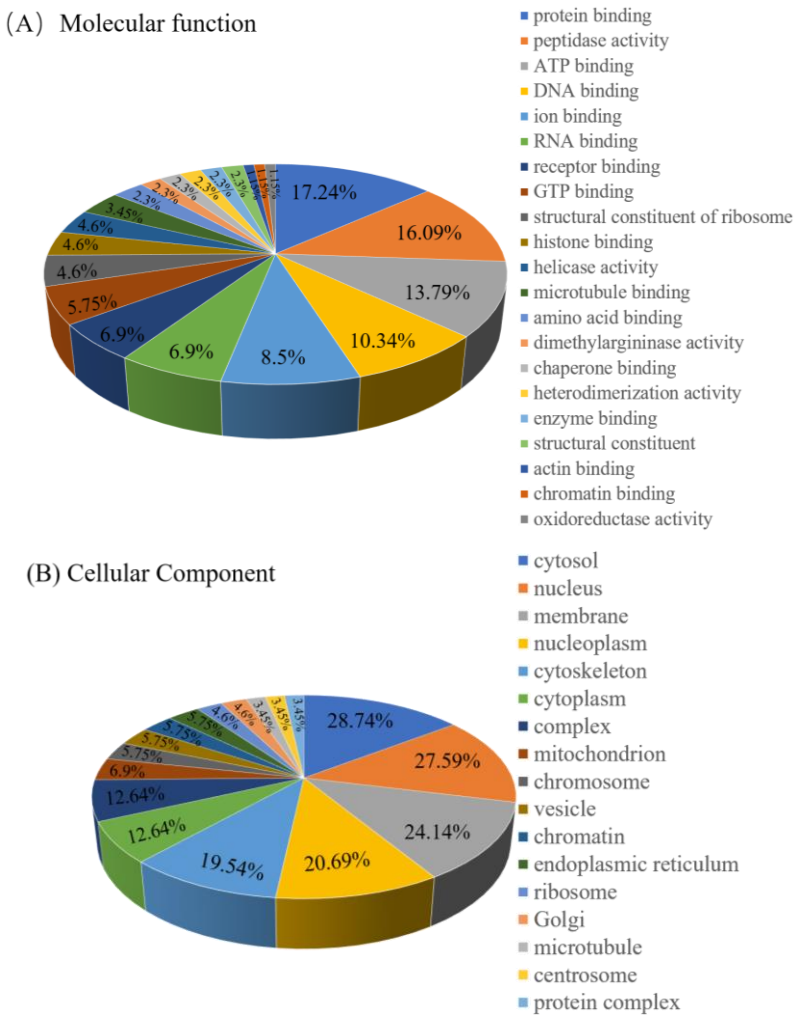

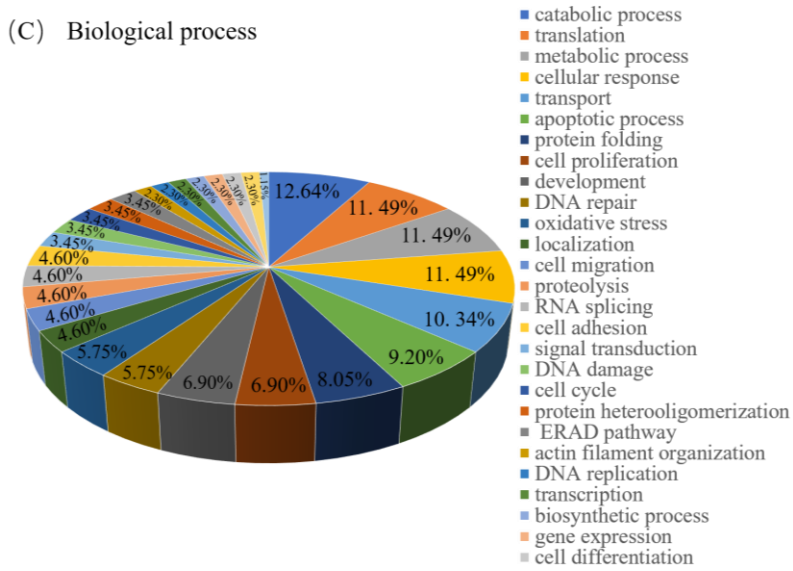

Figure. 1 GO classification results of up-regulation and down-regulation protein of C_2C_{12} cell induced by MFG-E8. (A) Annotation of molecular function; (B) annotation of cellular component; (C) annotation of biological process. Note: 89 proteins were analysed by GO classification.

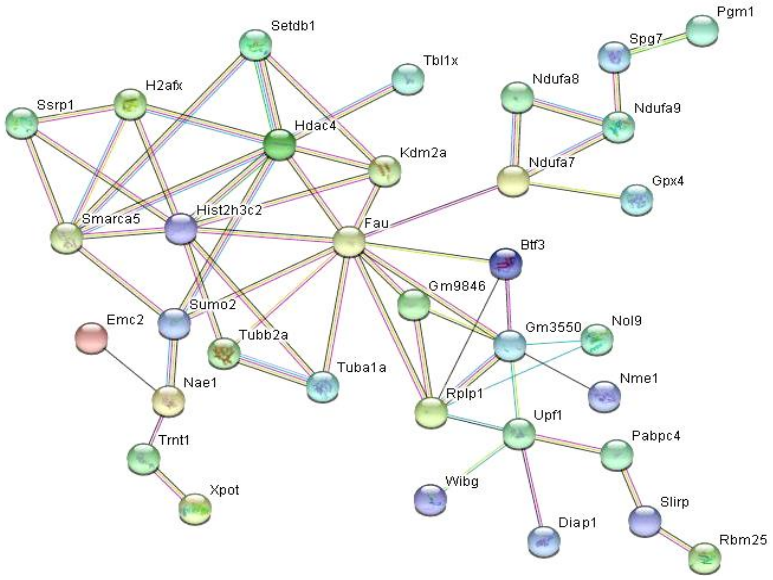

Figure. 2 The network of protein-protein ineraction mediated by MFG-E8.

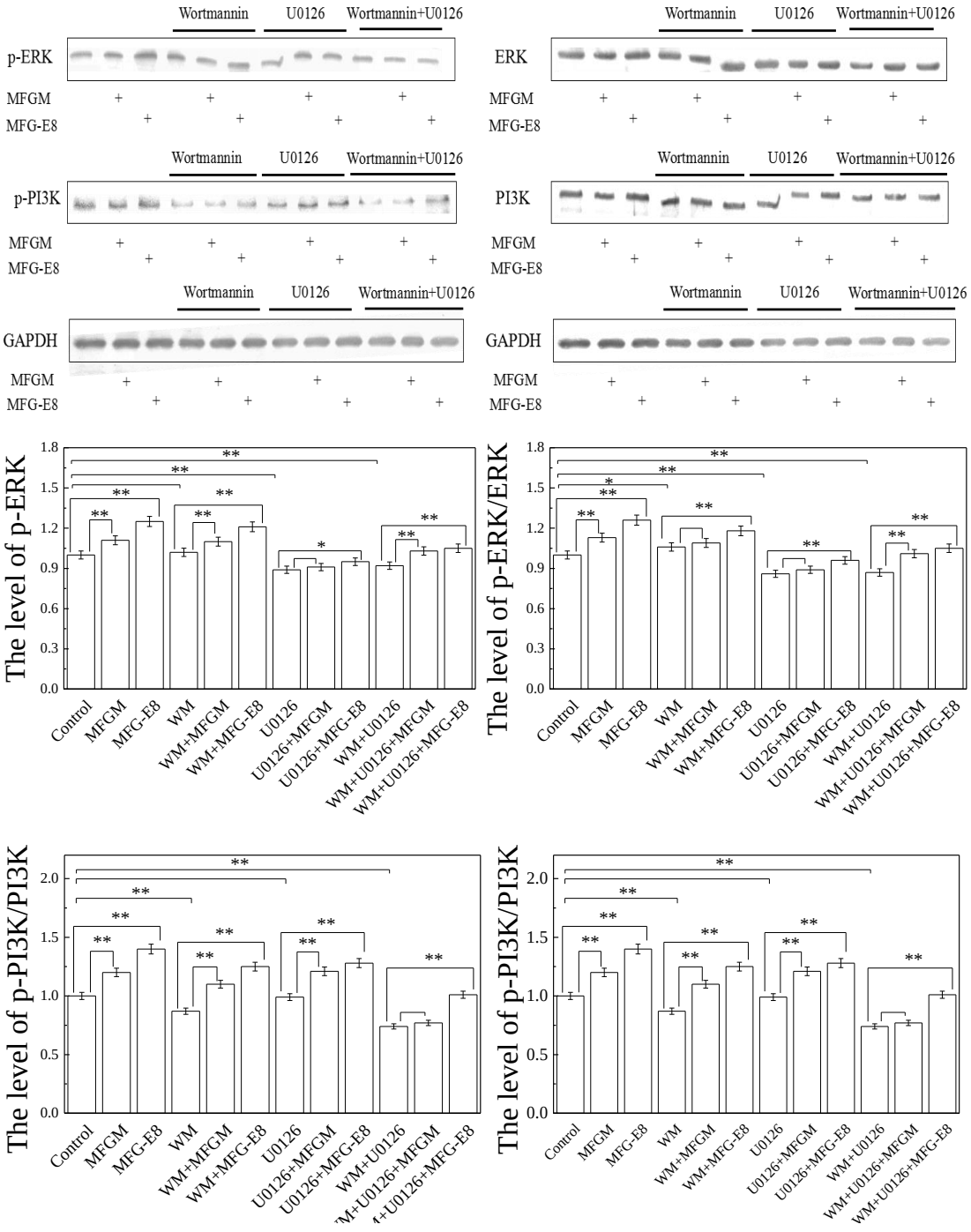

Figure. 3 The effect of MFG-E8 on PI3K, p-PI3K, ERK and p-ERK expression at 48h. The results are expressed as mean±SEM, *p<0.05, **p<0.01 vs. Control or MFGM (n=3).

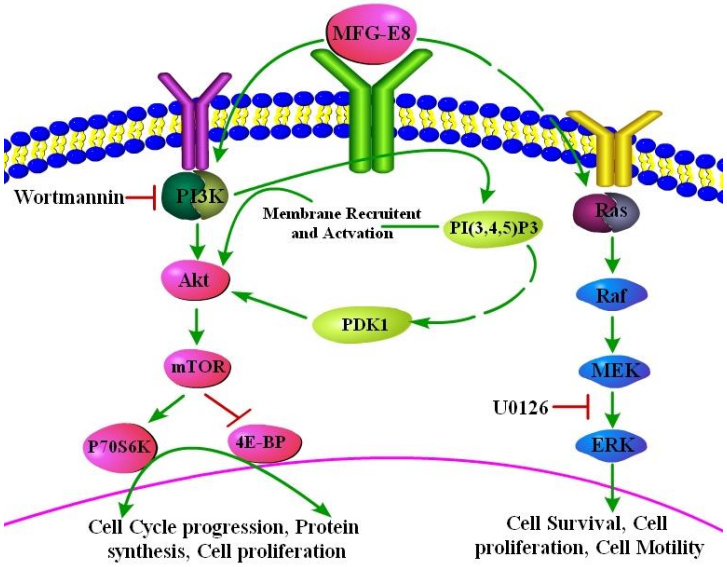

Figure. 4 The role of MFG-E8 in C_2C_{12} cell proliferation via PI3K/Akt and ERK signal pathway.

Based on the results, we proposed that the mechanism of MFG-E8 on C_2C_{12} cell proliferation might be described as follows: i) MFG-E8 promotes the regulatory and catalytic subunit of PI3K and promote C_2C_{12} cell proliferation via PI3K/Akt/mTOR/P70S6K singnal pathway [9]; ii) MFG-E8 might be activate Ras, which stimulates the protein kinase activity of Raf, resulting in the phosphorylation of MEK, and finally ERK, which ultimately results in phosphorylation of a wide variety of downstream effector proteins [28, 29]. The diagram of the mechanism of MFG-E8 on cell proliferation was modified from Li and Huang et al [9, 30]. .

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518 Table 1. Higher abundant protein in C_2C_{12} cell

NO	Protein IDs	Fasta headers	E1/C	E2/C	E1/E2
1	tr|B2RTM0|	Histone H4	0.80	0.81	0.99
2	tr|E0CZ27|	Histone H3	0.96	0.78	1.23
3	tr|B2RWH3|	Histone H2A	0.87	0.63	1.38
4	tr|A0JLV3|	Histone H2B	0.54	0.64	0.84
5	tr|Q4KL81|	Actin	0.82	0.88	0.93
6	sp|P16045|	Galectin-1	0.83	0.82	1.01
7	tr|Q5FWJ3|	Vimentin	0.72	0.80	0.90
8	tr|Q545I9|	Protein S100	0.96	0.75	1.28
9	tr|A0A0G2JGD2|	Protein S100-A4	0.70	0.73	0.96
10	tr|D2KHZ9|	Glyceraldehyde-3-phosphate dehydrogenase	0.79	0.99	0.80
11	tr|Q58E64|	Elongation factor 1-alpha	0.92	0.83	1.11
12	tr|Q3V471|	Galectin	0.62	0.76	0.82
13	tr|Q542G9|	Annexin	0.86	1.00	0.86
14	tr|Q5FW97|	Enolase 1	0.80	1.05	0.76
15	tr|Q642L7|	MCG13441	0.56	0.58	0.97
16	tr|Q545F4|	Hspb1 protein	0.75	0.84	0.89
17	tr|Q3U292|	Uncharacterized protein	0.72	0.74	0.97

519 Note: C: Control, E1: MFG-E8: 200 ug/ml, E2: MFG-E8: 500 ug/ml.

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Table 2. The up-regulation of myoblast C_2C_{12} cell induced by MFG-E8

NO	Protein IDs	Fasta headers	E1/C	E2/C	E1/E2
1	tr|Q9Z2P7|	Vesicle transport through interaction	9.69	7.58	1.28
2	sp|Q8CHP5|	Protein wibg homolog	9.45	9.80	0.96
3	tr|A0A0R4J107|	Acylamino-acid-releasing enzyme	9.22	5.88	1.57
4	tr|A0A1L1SUN1|	60S ribosomal protein L29	7.04	9.98	0.71
5	sp|Q91ZW3|	SWI/SNF-related matrix-associated	6.43	0.84	7.68
6	tr|F6Q750|	Microtubule-actin cross-linking factor 1	4.90	4.31	1.14
7	tr|A0A068BGR9|	NADH dehydrogenase	4.30	4.33	0.99
8	sp|B2RY56|	RNA-binding protein 25	4.01	2.54	1.58
9	tr|Q4VAA9|	Methionine aminopeptidase	3.93	4.24	0.93
10	tr|F7C9F6|	Aryl hydrocarbon receptor nuclear	3.63	6.94	0.52
11	tr|A2AKI5|	Integrin alpha-V	3.58	3.31	1.08
12	sp|O08915|	AH receptor-interacting protein	3.50	3.68	0.95
13	sp|Q99LD8|	N(G),N(G)-dimethylarginine	3.44	4.31	0.80
14	tr|G3UYF9|	Prefoldin subunit 6	3.27	2.22	1.47
15	tr|A0A0R3P9C8|	NADH dehydrogenase	3.23	3.02	1.07

Note: C: Control, E1: MFG-E8: 200 ug/ml, E2: MFG-E8: 500 ug/ml.

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539 Table 3. The down-regulation of myoblast C_2C_{12} cell induced by MFG-E8

	Protein IDs	Fasta headers	E1/C	E2/C	E1/E2
1	tr|Q543N6|	Serine/threonine-protein phosphatase 2A	0.02	0.24	0.08
2	tr|D3Z3F1|	Pre-mRNA 3-end-processing factor FIP1	0.03	0.09	0.40
3	tr|A1L0V4|	Histone H3	0.04	0.32	0.14
4	tr|Q58E35|	MCG10168	0.05	0.27	0.18
5	tr|G5E8N3|	Histone-lysine N-methyltransferase	0.05	0.28	0.17
6	tr|F6RPJ9|	Insulin-degrading enzyme	0.05	0.40	0.13
7	tr|B7ZCU2|	Abl interactor 1	0.06	0.18	0.32
8	sp|Q6PGG6|	Guanine nucleotide-binding protein-like 3-like protein	0.11	0.22	0.49
9	tr|A3KFU5|	Polyadenylate-binding protein	0.13	0.26	0.48
10	sp|P68369|	Tubulin alpha-1A chain	0.13	0.96	0.14
11	sp|D3YYU8|	Obscurin-like protein 1	0.14	0.29	0.49
12	sp|Q9QZD8|	Mitochondrial dicarboxylate carrier	0.15	0.43	0.35
13	sp|Q3TZX8|	Polynucleotide 5-hydroxyl-kinase NOL9	0.18	0.48	0.38
14	tr|E9Q9X1|	Dystonin	0.18	0.58	0.31
15	tr|Q8K0M3|	Sorbs3 protein	0.19	0.56	0.34
16	r|G3UXX3|	Sepiapterin reductase	0.19	0.85	0.23
17	tr|F6XC54|	Protein diaphanous homolog 1	0.20	0.41	0.48
18	tr|F6SS03|	Centromere protein V	0.20	0.35	0.57
19	tr|A2AW05|	FACT complex subunit SSRP1	0.21	0.95	0.22
20	tr|S4R1M2|	Scaffold attachment factor B1	0.21	0.48	0.43
21	tr|Q14BZ3|	Latexin	0.21	0.86	0.24

22	tr|A0A0R4J1H0|	E3 ubiquitin-protein ligase MARCH5	0.22	0.87	0.25
23	tr|D3YZN4|	Paraplegin	0.23	0.56	0.42
24	sp|Q9EPU0|	Regulator of nonsense transcripts 1	0.24	0.96	0.25
25	tr|F8WHU8|	SRA stem-loop-interacting RNA-binding protein	0.24	0.49	0.49
26	sp|Q9D1D4|	Transmembrane emp24 domain-containing protein 10	0.24	0.55	0.44
27	sp|Q921T2|	Torsin-1A-interacting protein 1	0.25	0.98	0.26
28	tr|Q544R7|	Heme oxygenase	0.26	0.72	0.36
29	sp|Q9EQQ2|	Protein YIPF5	0.28	0.11	2.62
30	tr|E0CZE0|	NEDD8-activating enzyme E1 regulatory subunit	0.28	0.66	0.43
31	tr|A0A0G2JG95|	Serine/threonine-protein phosphatase PGAM5	0.29	0.16	1.83
32	sp|Q8K1J6|	CCA tRNA nucleotidyltransferase 1	0.29	0.15	1.92

Note: C: Control, E1: MFG-E8 200 ug/ml, E2: MFG-E8: 500 ug/ml