

Ezrin regulated myoblast differentiation/fusion and muscle fiber specialization through PKA-NFAT-MyoD/MEF2c signalling pathway

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Research

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Abstract

Background: Neuromuscular diseases are a kind of nervous system diseases that have a high disability rate. Ezrin's role in skeletal muscle has not been identified. This study aims to confirm the effect and mechanism of Ezrin on myoblast differentiation and fusion, myotube size, and myofiber type.

Method: By using immunoassaying and western blot analyses, Ezrin, MyHC, MEF2c, MyoG, PKA $\alpha/\beta/\gamma$, PKA reg I α , PKA reg II β and NFATc1-c4 were detected in myoblast cells treated with Ad-Ezrin or Ad-shEzrin. Real-time PCR were used to evaluate MyoD, Myf5, MyHC-I, MyHC-IIa/b and MyHC-IIx in myoblast cells. PKA inhibitor H-89 or PKA reg I activator N⁶-Bz-cAMP were added into medium to confirm their relationship between Ezrin and PKA during myoblast differentiation/fusion. *In vitro*, Ad-NFATc1/c2 or Ad-shNFATc3/c4 were respectively transfected into C2C12 cells, myoblast differentiation/fusion, myotube size and myofiber type were assessed by using immunostaining of MyHC, MEF2c and MyoG. *In vivo*, transfection of Ad-Ezrin into gastrocnemius and soleus muscles for 7 days, the numbers of MyHC-1 positive myofibers were analyzed after immunostaining of MyHC-1.

Results: Ezrin expression were time-dependently increased during myoblast differentiation/fusion. Knockdown of Ezrin by shRNA delayed myoblast differentiation and fusion in a time dose-dependent pattern, as shown by immunostaining of MyHC. Conversely, over-expression of Ezrin by adenovirus time- and dosage-dependently promoted myoblast differentiation/fusion, and muscle fiber specialization characterized by increased MyHC I and MyHCIIa/b. Forced expression of Ezrin did not alter PKA, and PKA reg II α levels, but altered the levels of PKA reg I α/β , Myf5 and MyoD, and leading to the accumulation of MyoG⁺/MEF2c⁺ nuclei. By contrast, Ezrin knockdown significantly decreased the PKA reg I/II ratio and MyoG⁺/MEF2c⁺ nuclei. The PKA inhibitor H-89 remarkably abolished the beneficial effect of over-expressing Ezrin on the numbers of MyHC⁺ myotubes and MyoG⁺/MEF2c nuclei. These opposite changes mediated by knocking down Ezrin were almost eliminated by PKA reg I activator N⁶-Bz-cAMP. Furthermore, over-expression of NFATc2 or knockdown of NFATc4 reversed the inhibitory effect of Ezrin knockdown on myoblast differentiation/fusion, resulting in the recovery of the numbers of MyoG⁺/MEF2c⁺ nuclei in 3-nuclei⁺ myotubes. Meanwhile, overexpression of Ezrin specifically induced type I muscle fiber specialization, which was associated with increased levels of NFATc1/c2. Furthermore, *in vivo* transfection of Ad-Ezrin into gastrocnemius and soleus muscles increased the numbers of MyHC-1 positive myofibers. By contrast, knockdown of NFATc4 resulted in the recovery to normal levels of MyHC-2b in Ezrin-knockdown myoblast cells, attributing to regaining MyoD and MEF2c expression.

Conclusions: Ezrin trigger myoblast differentiation and fusion, myotube size, and alters muscle fiber specialization through PKA-NFAT-MyoD/MEF2C signalling pathway.

Introduction

Neuromuscular diseases are a kind of nervous system diseases that have a high disability rate, causing long-term mental and economic burden to the society, family, and the individual¹. Muscle biopsy

pathology is the most effective for the diagnosis of myopathy. Studies found that the pathological basis of muscular atrophy and muscle weakness is the degeneration and necrosis of muscle fibers. Skeletal muscles have the ability to regenerate to prevent the loss of muscle mass and maintain normal shape and function². It is, therefore, crucial to study the characteristics of muscle fiber regeneration and their associated factors for the prevention and cure of neuromuscular diseases³.

Muscle satellite cells conduce to the need for physiological self-renewal and the repair of pathological injury⁴. Inflammatory reactions are one of the pathologies that result in myopathy, including polymyositis and dermatomyositis⁵. A recent study has shown that a more robust inflammatory area within the skeletal muscle demonstrated features of high Ezrin expression, a member of the Ezrin/radixin/moesin (ERM) proteins family. Published data supplied evidence that Ezrin could play a crucial role in transferring extracellular signal molecules into the skeletal proteins, activating the corresponding signal pathways, regulating the cell morphology, adhesion, phagocytosis, movement, and angiogenesis⁶⁻¹⁰. However, the role and mechanism of Ezrin in muscle satellite cells are still unclear.

Traditionally, the activated protein kinase A (PKA) has been linked to the unique phenomenon of myoblast differentiation/fusion and myotube formation, ascribing to the alteration in PKA regulatory subunit I (PKA RI) under normal differentiation condition¹¹. Our previous study has shown that it abated the ratio of PKA RI/RII in myoblast cells, resulting in the postponement of myoblast differentiation and fusion¹². Further evidence shows that ERM proteins act as PKA-anchoring proteins and sequester PKA close to its target proteins for their effective phosphorylation and functional regulation¹³. NFATs (Nuclear factor of activated T cells) activation mediated by PKA plays crucial role in myoblast differentiation and fusion, myotube size, and altered muscle fiber specialization^{4,13}. In this study, knockdown of Ezrin by shRNA reduced the numbers of MyoG/MEF2C-positive cells and myotube number/size while decreasing the ratio of PKA RI/RII, causing the increased expression of NFATc2/c3/c4, suggesting that Ezrin triggered myoblast differentiation and fusion, myotube size, and altered muscle fiber specialization through PKA-NFAT-MEF2C signalling pathway.

Method

C2C12 myoblast culture and differentiation induction

C2C12 myoblast cells were inoculated in 75-cm² culture dishes and cultured with proliferation medium (PM) containing high glucose DMEM (HG-DMEM) supplemented with 10% FBS at 37°C and 5% CO₂. When the confluence of the cells reached 75%, the PM was replaced with differentiation medium (MD) containing HG-DMEM supplemented with 2% horse serum (HS) to induce C2C12 myoblast cell differentiation. Traits of myotube formation from myoblast differentiation were observed daily under a microscope¹⁴.

Overexpression or Knockdown of Ezrin in vitro

Construction of Ezrin overexpression and short hairpin RNA (shRNA) adenoviral vector were prepared as previously described¹⁵. To confirm the role of Ezrin on myoblast cells, Ad-Null, Ad-shEzrin, or Ad-Ezrin (1×10^9 pfu) were added into the corresponding culture dishes a day before adding the differentiation medium. These cells were then replaced with the differentiation medium for further observation.

Overexpression or Knockdown of NFATs in vitro

Construction of NFATc1/c2 overexpression adenoviral vector were prepared as previously described¹⁵. The gene accession number of overexpressing-NFATc1/c2 is NM_172390 and NM_173091, respectively. Construction of NFATc3/c4 short hairpin RNA (shRNA) adenoviral vector were prepared as previously described¹⁵. These overexpression adenoviral vectors containing Ad-NFATc1, Ad-NFATc2, Ad-shNFATc3 and Ad-shNFATc4 were obtained from Vigenebio. To confirm the role of NFATc3 or NFATc4 on myoblast cells, the addition of Ad-shCtrl, Ad-shNFATc3, or Ad-shNFATc4 (1×10^9 pfu) into corresponding culture dish one day before the ISO was performed. And then these cells were replaced with differentiation medium for further observation.

In vivo transfection

For the transfection of adult muscles, C57BL/6 mice were anesthetized by using an isoflurane vaporizer maintained at 2% isoflurane, 1 L/min oxygen. Gastrocnemius and soleus muscles were exposed and injected with Ad-Ezrin (1×10^{10} pfu, two points, 50 μ m/each)¹⁵. Muscles were removed 7 days after transfection, frozen in isopentane cooled in liquid nitrogen, and stored at -80 °C.

Immunofluorescence Staining

C2C12 myoblast differentiation was determined by immunofluorescence staining. Primary monoclonal and polyclonal antibodies against MEF2C (#5030s, 1:200, CST), MyHC (sc-20641, 1:150, Santa Cruz) were added into each well in every group and incubated for 12 h at 4 °C. The cells were washed with PBS 3 times for 15 min and incubated with appropriate fluorescent dye-labeled secondary antibodies (Jackson Lab, 1:500, USA) at 25 °C for 2 h. The nuclei were stained with DAPI (Molecular Probes). The images for each group were photographed under Nikon 80i fluorescence microscope¹⁶.

Myoblast Differentiation

After myoblast cells were treated under DM for the indicated time, the differentiated myoblast cells were stained for MyoG or MEF2C using the primary polyclonal antibody MyoG (sc-12732, 1:150, Santa Cruz) or MEF2C (5030S, 1:400, CST) and appropriate TRITC-labeled secondary antibody (Jackson Lab, 1:500, USA). The nuclei were stained with DAPI. C2C12 myoblast cells with only 1–2 nuclei within a cellular structure were evaluated with MyoG or MEF2C staining. The MyoG + or MEF2C + cells were defined as the differentiated cells that did not fuse to form myotubes. Myoblast cells with 3 or more nuclei in the structure of a cell were defined as myotubes. The number of double-positive nuclei under high power field (HPF, 50 μ m) were analyzed after double staining of MyoG/DAPI or MEF2C/DAPI. Two individuals who

did not know the results evaluated the images using Image J (Java) software (National Institutes of Health, USA).

Myoblast Fusion And Myotube Morphology

The differentiated myoblast cells were stained for MyHC with the primary polyclonal antibody MyHC (rabbit anti-mice antibody, sc-20641, 1:150, Santa Cruze) and appropriative TRITC or FITC-labeled secondary antibody (Jackson Lab, 1:500, USA). C2C12 myoblast cells with only 1–2 nucleuses within a cellular structure were evaluated by MyHC staining, indicating that the MyHC + cells were defined as the differentiated cells without mutually fusing to myotube. Myoblast cells with 3 or more nucleuses in the structure of a cell were defined as myotube. The nuclei were stained with DAPI.

To analyze myotube size, we divided the cells into 2 groups, including short myotube with 3 ~ 5 myoblast fusion and long myotube with more than 5 myoblast fusions. Morphology was assessed by myotube length, area (grouped less than 200 μm and more than 200 μm), and the number of myotubes (grouped 3 ~ 5 nuclei or more than 5 nuclei myoblast fusion) under high-power magnification^{15,17}. Two individuals who did not know the results evaluated the images using Image J (Java) software (National Institutes of Health, USA).

Quantitative RT-PCR

Total RNA from C2C12 myoblast cells was obtained using TRIzol (Invitrogen, Life Technologies) and transcribed into cDNA using the SuperScript II cDNA kit (Invitrogen, Life Technologies). Quantitative PCR was carried out using SYBR green PCR master mix (Thermo Fisher Scientific, Applied Biosystems, CN) in Real-Time PCR System (RotorGene 6000, Qiagen, Germany). The transcript levels of the gene of interest in each group were normalized to GAPDH levels¹⁸. The primers used are listed in Table 1.

Table 1
The sequences of primers of qPCR.

Gene	Forward	Reverse
MyoG	5'-GAGACATCCCCCTATTTCTACCA-3'	5'-GCTCAGTCCGCTCATAGCC-3'
MyoD1	5'-CCACTCCGGGACATAGACTTG-3'	5'-AAAAGCGCAGGTCTGGTGAG-3'
MyHC1	5'-CAAGCAGCAGTTGGATGAGCGACT-3'	5'-TCCTCCAGCTCCTCGATGCGT-3'
MyHC2a	5'-AGAGGACGACTGCAGACCGAAT-3'	5'-GAGTGAATGCTTGCTTCCCCCTTG-3'
MyHC2b	5'-ACGCTTGCACACAGAGTCAG-3'	5'-CTTGGACTCTTCCTCTAGCTGCC-3'
MyHC2x	5'-ACCAAGGAGGAGGAACAGCAGC-3'	5'-GAATGCCTGTTTGCCCCTGGAG-3'
GAPDH	5'-ATGACTCCACTCACGGCAAA-3'	5'-ATGATGACCCTTTTGGCTCC-3'
qPCRs were performed to identified satellite cell differentiation and muscle fibers traits by using the specific primers of satellite cell differentiation markers including MyoD and MyoG, type I muscle fiber makers like MyHC1, and type II muscle fiber makers such as MyHC2a, MyHC2b, and MyHC2X.		

Western Blot

C2C12 myoblast cells were homogenized on ice in 0.1% Tween-20 homogenization buffer containing protease inhibitors. Nuclear and cytosolic protein were separated and collected using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacture's instruction (78835, Thermo Fisher Scientific, USA). 20 µg of protein in each well were separated by 7 or 10% SDS-PAGE gel electrophoresis and transferred onto PVDF membrane (Millipore). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies, including α-tubulin (T9026, 1:5000, Sigma), Histone H3 (ab6002, 1:500, ABCAM), NFATc1 (ab2796, 1:500, Abcam), NFATc2 (ab2722, 1:500, Abcam), NFATc3(ab83832, 1:500, Abcam), NFATc4 (SAB4501982, 1:1000, Sigma) and MyHC (sc-20641, sc-376157, 1:500, Santa Cruze) overnight at 4 °C, respectively. Thereafter, the blots were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, anti-goat IgG, 1:10000; Santa Cruz) for 90 min. Protein expression was detected by enhanced chemiluminescence method, and the Image J software was used for gray value analysis¹⁹.

Statistical analysis

Data of quantitative and semi-quantitative analysis presented are mean ± SD. Paired or unpaired Student's t-test determined statistical significance between the two groups. One-way ANOVA was used to compare the results for more than two experimental groups to specify the differences between groups. $P < 0.05$ is considered meaningful.

Results

Ezrin is expressed in C2C12 cells during myoblast differentiation and fusion

To confirm the role of Ezrin in C2C12 myoblast cells, we determined the changes of Ezrin expression during myoblast differentiation and fusion by western blot. We found that the expression of Ezrin gradually increased, reaching its peak on day 4 of myoblast differentiation (Fig. 1). This expression is accompanied by C2C12 myoblast cell alterations, that time-dependently differentiated into mature muscle cells, forming myotubes characterized by MyHC positive staining under differentiation medium containing 2% HS-DMEM. These results indicated that Ezrin could play an essential role in myoblast differentiation and fusion.

Our results further showed that the number of MyHC + myotubes with either 3–5 or 5⁺ nuclei increased upon treatment with Ad-Ezrin in a time-dependent manner (Fig. 1E-1H). Conversely, knockdown of Ezrin by shRNA did not only obviously reduce MyHC + cell number, but it also dramatically decreased myotube numbers with either 3–5 or 5⁺ nuclei (Fig. Figure 1E-1H). This indicates that knockdown of Ezrin can almost effectively block myoblast differentiation and fusion. Combined with Fig. 1A-1D, these results demonstrated that Ezrin is involved in early differentiation and fusion of myoblast.

Transfection efficiency of overexpression or knockdown of Ezrin into myoblast cell

To further determine the effect of Ezrin in C2C12 myoblast differentiation and fusion, we transfected the C2C12 cells with an adenovirus-mediated overexpression of Ezrin, or knockdown of Ezrin by shRNA. Assessment of the transfection efficiency revealed that following the application of 100 optimal multiplication of infection (MOI) with the adenovirus, C2C12 myoblast cells almost reached a confluence of 95% (sfig. 1A-1C). His-tag was used to confirm the successful expression of the exogenous genes and their respective functions (sfig. 1C-1E).

Ezrin Altered The Muscle Fiber Types

Indeed, there are different types of muscle fibers formed by MyHC-1, MyHC-2a, MyHC-2b, or MyHC-2X. MyHC-1-positive type I fiber shows a slim-long feature. MyHC-2a, MyHC-2b, and MyHC-2X positive type II fiber has thick-short traits¹⁴. In line with the increased myotube formation following forced-expression of Ezrin, MyHC-1 expressions were markedly increased (Fig. 2C–2F). By contrast, MyHC-2a and MyHC-2b expressions were evidently increased in knock-downing-Ezrin myoblast cells. However, MyHC-2X expressions were not obviously altered in either overexpression or knockdown of Ezrin (Fig. 2C–2F). More importantly, overexpression of Ezrin in gastrocnemius (GA) and musculus soleus (SL) increased numbers of MyHC-1 positive muscle fibers (Fig. 2G–2J). Nevertheless, these results suggested that Ezrin regulated the expressions of all MyHC isoforms.

MYOG/MEF2C Involved Ezrin-mediated Myoblast Differentiation And Fusion

Since MyoG and MEF2C play a crucial role in the initiation and later of myoblast cells differentiation²⁰, we used MyoG and MEF2C staining to confirm the relationship between Ezrin and myoblast differentiation. Our results showed that following treatment of Ad-Ezrin, the number and percentage of MyoG⁺ nuclei were higher than that of MEF2C + nuclei within the myoblast during C2C12 myoblast

differentiation(Fig. 3A–2G). Knockdown of Ezrin by shRNA markedly reduced the percentage of MyoG + and MEF2C + nuclei cells in C2C12 myoblast cells(Fig. 3A–2G).Moreover, the percentage of MEF2C⁺ nuclei was lower than that of MyoG + nuclei in Ezrin-knockdownC2C12 myoblast cells(Fig. 3C–2G).These results indicated thattheknockdown of Ezrincouldinhibit the initiation and late phase of myoblast differentiation through MyoG and MEF2C, especially MyoG.

Ezrin regulated myoblast differentiation and fusion through PKA signalling pathway

Previous results have shown that PKA and PKAreg I/II ratio play crucial roles in controlling myoblast differentiation and fusion^{11,12}. To confirm if Ezrin's role in myoblast differentiation and fusion was involved in PKA signalling pathway, we used western blot to detect PKA signaling(Fig. 4A-4G). Our results revealed that, the overexpression of Ezrin did not alter PKAreg II levels, but it significantly increased the levels of PKA α ,PKAreg Ia,and PKAreg I β , resulting in an increased PKAreg I/II ratio. By contrast, knockdown of Ezrin by shRNA significantly reduced PKAreg Iaand PKAreg I β levels, but it did not alterPKAreg II levels, resulting in a decreased PKAreg I/II ratio (Fig. 4A-4G).

PKA Involved Ezrin-mediated Myoblast Differentiation And Fusion

PKA activity is found to have effects on myoblast differentiation and fusion^{11,12}. Combining with the above results that the overexpression or knockdown of Ezrinaffected myoblast differentiation and fusion by altering PKA activity, we treated C2C12 cells with PKA inhibitor (H-89). We found that PKA inhibitor (H-89)abolished the beneficial role of Ezrin-mediated C2C12 myoblast differentiation and fusion. By contrast, both PKA activator and cAMP analogues reversed the inhibitory effects of Ezrin knockdown onC2C12 myoblast differentiation and fusion (Fig. 5A-5D). These results indicated that the effect of Ezrinduring myoblast differentiation and fusion could be associated with PKA signalling.

Ezrin regulated myoblast differentiation and fusion through PKA-MyoG/MEF2C signalling pathway

Indeed, myotube is formed by the fusion of differentiated myoblasts, which is characterized by three (3⁺) or morenuclei in the structure of a cell^{15,17}. As shown in Fig. 6A-6J, we found that knockdown of Ezrin by shRNA markedly decreased the percentage of MyoG + and MEF2C + nuclei in less than 3 cells and 3⁺myotubes, and these effects could be abolished by PKA activator. By contrast, overexpression of Ezrin substantially increased the number of MyoG + or MEF2C + nucleus in less than 3 and 3⁺myotubes. However, the specific changes were almost entirely cancelled by the PKA inhibitor (Fig. 6C-6J). These results indicated that Ezrin participated in C2C12 myoblast differentiation and fusion with coordination of MyoG and MEF2C.

NFATs signaling involved in the regulation of myotube types mediated by Ezrin

Previous studies have reported on two types of muscle fibers, including slow (slim-long) and fast (thick-short) myofibers, MyHC-1 forms the former, and MyHC-2a, MyHC-2b or MyHC-2X the latter^{24,25}. Corresponding with myoblast differentiation and fusion, especially myofiber specification mediated by

NFATs²⁶⁻³². We firstly found that overexpressing Ezrin increased nucleus NFATc1/c2 levels while decreasing NFATc3/c4 levels. Conversely, knocking down Ezrin increased NFATc3/c4 levels while decreasing NFATc1/c2 levels (Fig. 7A-7E). Secondly, either NFATc2 overexpression or NFATc4 knockdown almost completely reversed the inhibitory effects of knocking-down Ezrin on myoblast differentiation and fusion, resulting in the recovery of numbers of slim-long myofibers (nuclei numbers more than 5) (Fig. 8A). Eventually, NFATc1/c3 knockdown did not significantly recuperate the inhibitory role of knocking-down Ezrin in myoblast differentiation/fusion (sFigure2). Meanwhile, we found that knockdown of Ezrin increased MyoD expressions, the specific effects could be abolished by Ad-NFATc1/c2 or Ad-shNFATc3/c4. More importantly, the specific increase in MyHC-2a and MyHC-2b mediated by knockdown of Ezrin could obviously be reversed by Ad-NFATc1/c2 or Ad-shNFATc4 (Fig. 8B-7D), respectively. Thus, Ezrin mainly regulated myoblast differentiation/fusion, and myofiber specification through the integrative role of NFATc2/c4 signaling pathway.

Ezrin regulated myoblast differentiation and fusion through NFATc2/c4-MyoG/MEF2C signaling pathway

Since MyoG and MEF2C are involved in the control of the initiation and later stage of myoblast differentiation, respectively²⁶. As shown in Fig. 8A and sFigure3A-3F, we found that knockdown of Ezrin by shRNA markedly decreased the numbers and percentage of MyoG⁺ and MEF2C⁺ nuclei in less than 3-nuclei cells and 3-nuclei⁺ myotubes, and these effects could be abolished by Ad-NFATc2 or Ad-shNFATc4. In addition, Ad-NFATc1 or Ad-shNFATc3 reversed the numbers of MEF2C⁺ nuclei in 3-nuclei⁺ myotubes. These results indicated that Ezrin participated in C2C12 myoblast differentiation and fusion with coordination of MyoG and MEF2C, which were associated with NFATc2/c4, at least in part.

Discussion

In this study, we made three novel observations. Firstly, we found that the Ezrin expression has a time-dynamic characteristic during myoblast differentiation and fusion. Secondly, Ezrin significantly controlled myoblast differentiation and fusion. And lastly, Ezrin regulated C2C12 myoblast differentiation, fusion, and myotube type formation via PKA/Ri-NFAT-MyoD/MEF2C signaling pathway.

Ezrin belongs to the ERM family of proteins that play structural and regulatory roles in the assembly and stabilization of plasma membrane interactions through their ability to interact with transmembrane proteins and the cytoskeleton¹³. As one of ERM proteins, Ezrin activated the intracellular signal pathways through transferring extracellular signal molecules into the actin cytoskeleton, affecting several key cellular processes, including membrane dynamics, cell adhesion, cell survival, motility, and determination of cell shape⁶⁻¹⁰. Indeed, these cellular processes were associated with myoblast differentiation and fusion²¹. For the first time, we found that Ezrin gradually increased during myoblast differentiation/fusion, and rapidly decreased when formed myotubes approached and/or reached maturity. Furthermore, overexpression of Ezrin significantly accelerated the process of myoblast differentiation and myotube formation. Conversely, this process was delayed by the knockdown of Ezrin. These results suggest that

Ezrin could act as a crucial factor during skeletal muscle regeneration and restoration in physiological and pathological conditions.

Previous studies have shown that the overexpression of PKA inhibited myogenic differentiation, contributing to HDAC4 phosphorylation and the transcriptional repression of muscle-specific genes by the myogenic regulators Myf-5 and MyoD^{22,23,24,25}. Furthermore, ERM proteins, including Ezrin, are reported to act as PKA-anchoring proteins and sequester PKA close to its target proteins for their effective phosphorylation and functional regulation¹³. However, our results did not show any apparent changes in expressions of Myf-5 and MyoD in overexpressing Ezrin in myoblast cells, but showed increased numbers of MyoG and MEF2C-positive myoblast cells and myotubes. Actually, MyoG and MEF2C play an essential role in the initiation and later stage of myoblast differentiation, respectively²⁶. Recent reports have also associated PKA RI and RII to myoblast differentiation and myotube formation^{11,12}. Of interest, we found that overexpressing Ezrin markedly increased PKA RI levels, leading to an increased PKA RI/RII ratio, accompanied by an acceleration of myoblast differentiation and fusion. Furthermore, the knockdown of Ezrin, resulting in a lower PKA RI/RII ratio, inhibitory effects observed during myoblast differentiation/fusion could be reversed by the PKA RI activator. More importantly, PKA RI activator almost completely recovered the numbers of MyoG or MEF2C positive myotubes. Therefore, Ezrin could promote myoblast differentiation/fusion via PKA RI/RII-MyoG/MEF2C signalling pathway.

Existing data have shown that NFATs act as a crucial player in myoblast differentiation and fusion, especially myofiber specification²⁶⁻³². Moreover, NFATs activities are frequently regulated by the PKA-calcineurin signalling pathway during cell differentiation^{33,34,35,36}. Indeed, NFATc2 primarily controlled myoblast recruitment and myoblast fusion^{37,38,39,40}. Similarly, we found that the inhibitory effect of myoblast fusion by knockdown of Ezrin could be restored by the overexpression of NFATc2, but not NFATc1. In line with stimulatory effect of constitutively active NFATc1 (caNFATc1) on MyHC-1 expression⁴¹, the increased NFATc1 levels by overexpression of Ezrin induced MyHC-1 expression. However, enhanced NFATc1 levels by Ad-Ezrin did not decrease MyHC-2b as caNFATc1 did, but keeping its normal levels. Meanwhile, Ad-Ezrin reduced NFATc3/c4 levels in myoblast cells, but maintaining MyHC-2a expression, which was different from the inhibitory role of knockdown for NFATc3 and NFATc4 in the expression of MyHC-2a³⁰. These different changes could be related with the increased levels of NFATc2 by Ad-Ezrin, because NFATc2 acted as important role in MyHC-2a and MyHC-2X expressions^{37,40,41}. In a word, altered NFATs signaling by Ezrin affected myoblast differentiation/fusion, especially type-1 and type-2 muscle fiber specialization.

In relation to muscle fiber type specialization, slow muscle specialization and fast to slow myofiber-type switch need the coordination of MEF2C and MyoD^{39,40,41,42,43} respectively. In this study, the overexpression of Ezrin increased the MEF2C levels within the nucleus while decreasing the levels of MyoD in myoblast cells, resulting in the increased MyHC-1 expressions, accompanied by normal levels of MyHC-2a, MyHC-2b and MyHC-2X. The knockdown of Ezrin reduced MEF2C + myoblast cells numbers while increasing MyoD expressions, leading to special changes that the levels MyHC-2a and MyHC-2b were

obviously increased. Previous reports have shown that NFATc2 could perform specific role in MyHC-2a and MyHC-2X expression with synergistic effect of MyoD while NFATc1 promoted MyHC-1 expression through inhibiting MyoD signaling^{37,40}. More, NFATc4 has shown the inhibitory role in myoblast fusion³⁸, and appears to mostly contribute to fast muscle fiber formation, especially MyHC-2b³⁰. The overexpression of NFATc4 affects the differentiation process by decreasing the expression of late differentiation markers, including MEF2c, and impairs myotube formation^{30,38}. We found that these similar effects mediated by the knockdown of Ezrin could be reversed by knockdown of NFATc4. Thus, Ezrin could regulate myoblast fusion and type IIb muscle fiber specialization through NFATc4-MEF2C signalling pathway.

Conclusion

Ezrin affects myoblast differentiation, fusion, and muscle fiber specialization via PKA-NFAT-MyoD/MEF2C signalling pathway.

Abbreviations

DAPI: 4',6-Diamidino-2-Phenylindole; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; HF: heart failure; HRP: horseradish peroxidase; HS: horse serum; IF: Immunofluorescence; MD: Muscular dystrophy; MyHC: myosin heavy chain; NFAT: nuclear factor of activated T cells; MEF2C: Myocyte-specific enhancer factor 2C; MyoD: myogenic differentiation 1; M: Mol/L; PKA: protein kinase A; PKA R1α: PKA regulatory subunit 1α; PVDF: polyvinylidene fluoride.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of Data and material

Please contact corresponding author for data requests.

Consent for publication

Not applicable.

Author contributions

R.L.Zmainly performed cells experimental and prepared the first draft; L.X and C.Q.H fulfilled qPCR; H.T.Z and X.Bperformed protein detection; Y.W and Z.F.Zparticipated in the immunostaining; S. L, Z.J.Rand X.W participated in myotube analysis; L.Y.Y andL.Cparticipated in Adenovirus preparation; J.X.Z and Y.Whad a hand in the study design; M.N.M andS.Y.C partook in revising the manuscript;L.L.S and J.M.T composed the study, partook in the design and coordination of the whole study, and was conducive to revising the manuscript. All authors have read and agreed to the final draft.

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Figures

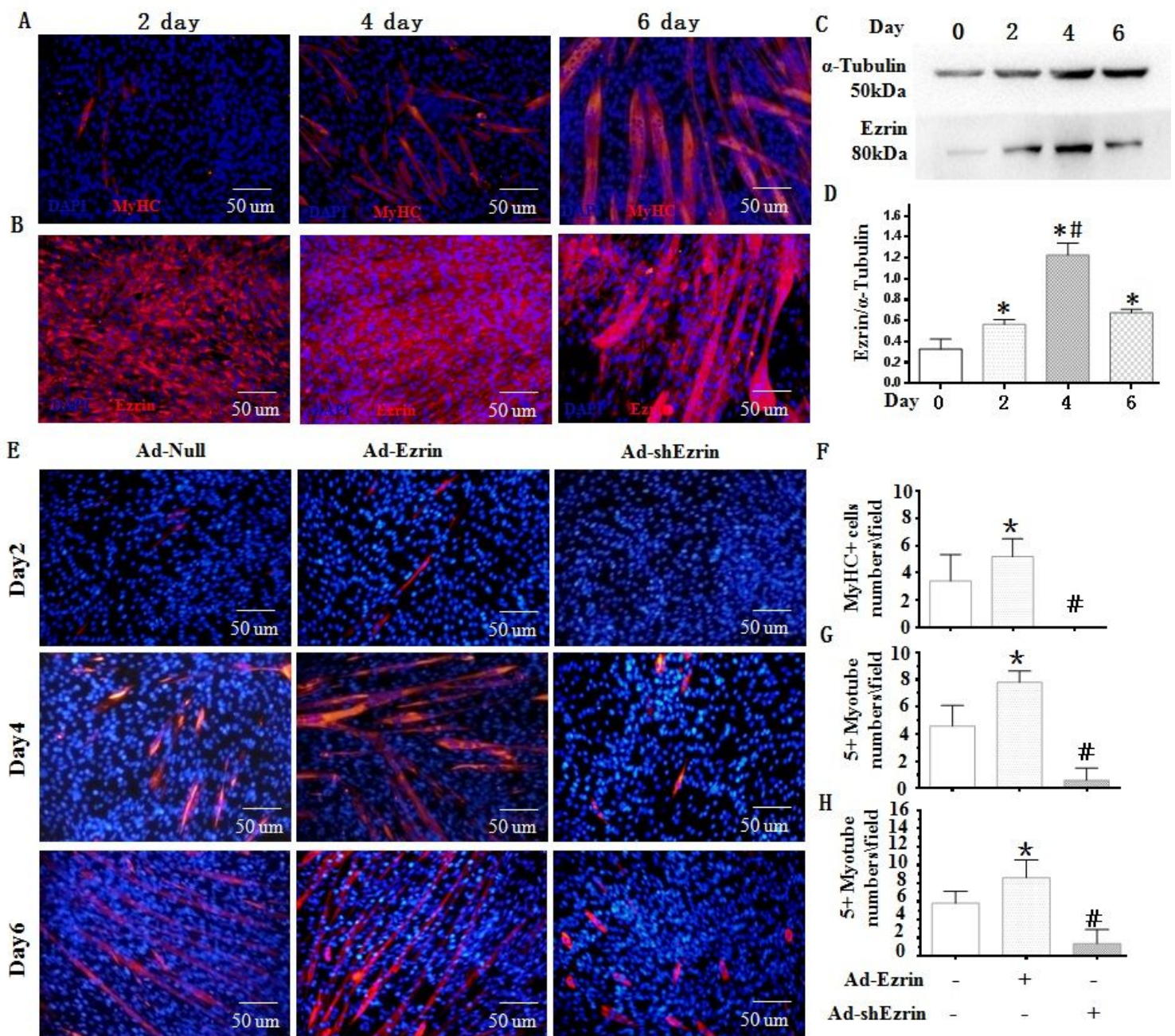


Figure 1

Time course of Ezrin during myoblast differentiation and fusion. Typical image for MyHC during myoblast differentiation and fusion. (B) Western blot for Ezrin expressions at 0, 2, 4, 6 days after myoblast differentiation. (C) Quantitative assay for Ezrin expressions was analyzed 2, 4 and 6 days after myoblast differentiation. $n=3$, * $P \leq 0.05$ vs. 0 day; # $P \leq 0.05$ vs. 2 day; & $P \leq 0.05$ vs. 4 day. (E) Typical image of MyHC staining in differentiated C2C12 myoblast cells treated by overexpression or knockdown of Ezrin for 2, 4, 6 days. (F-H) Quantitative assay for the number of MyHC+ myotubes with more than 5 nuclei was analyzed 2, 4 and 6 days after myoblast differentiation treated by overexpression or knockdown of Ezrin, respectively. $n=3$, * $P \leq 0.05$ vs. Ctrl; # $P \leq 0.05$ vs. Ctrl.

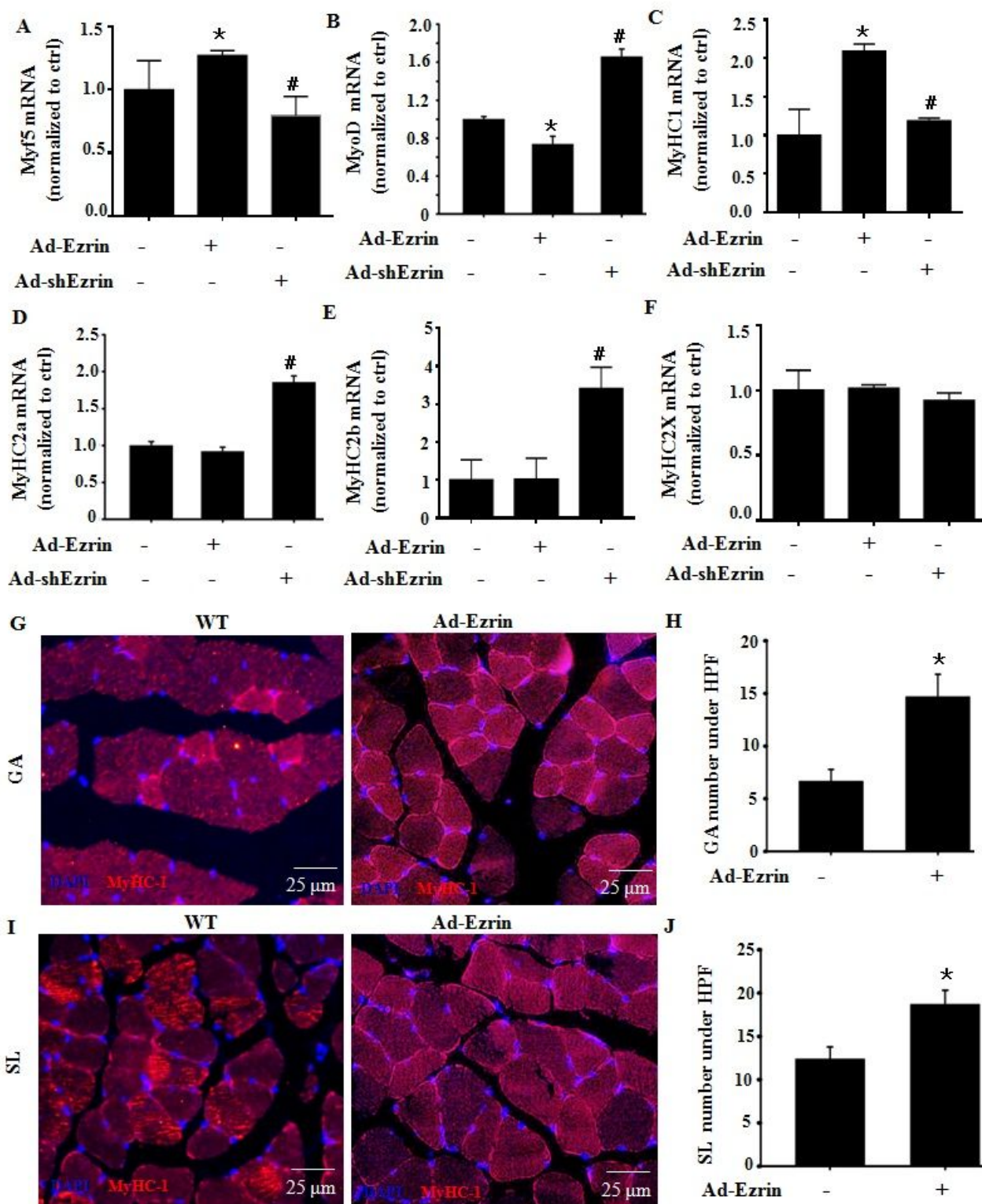


Figure 2

Ezrin altered the muscle fiber types. (A-B) Myf5 and MyoD, myoblast cells differentiation regulatory factors, were altered in differentiated C2C12 cells pretreated with Ad-AdEzrin or Ad-shEzrin. (C) MyHC1, as one of type I muscle fiber maker, were repressed in differentiated C2C12 cells exposed to overexpression or knockdown of Ezrin by detecting the levels of mRNA using Real-time PCR. (D-F) Overexpression of Ezrin promoted MyHC-1 expressions while knockdown of Ezrin increased the levels of type II muscle fiber

makers such as MyHC-2a and MyHC-2b in differentiated C2C12 cells pretreated with Ad-AdEzrin or Ad-shEzrin by detecting the levels of mRNA using Real-time PCR. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ctrl. (G-J) *lv viv* assay for MyHC-1 expressions in gastrocnemius (GA) and muscoli soleus (SL) following the local injection of Ad-Ezrin. n=6, *P<0.05 vs. WT.

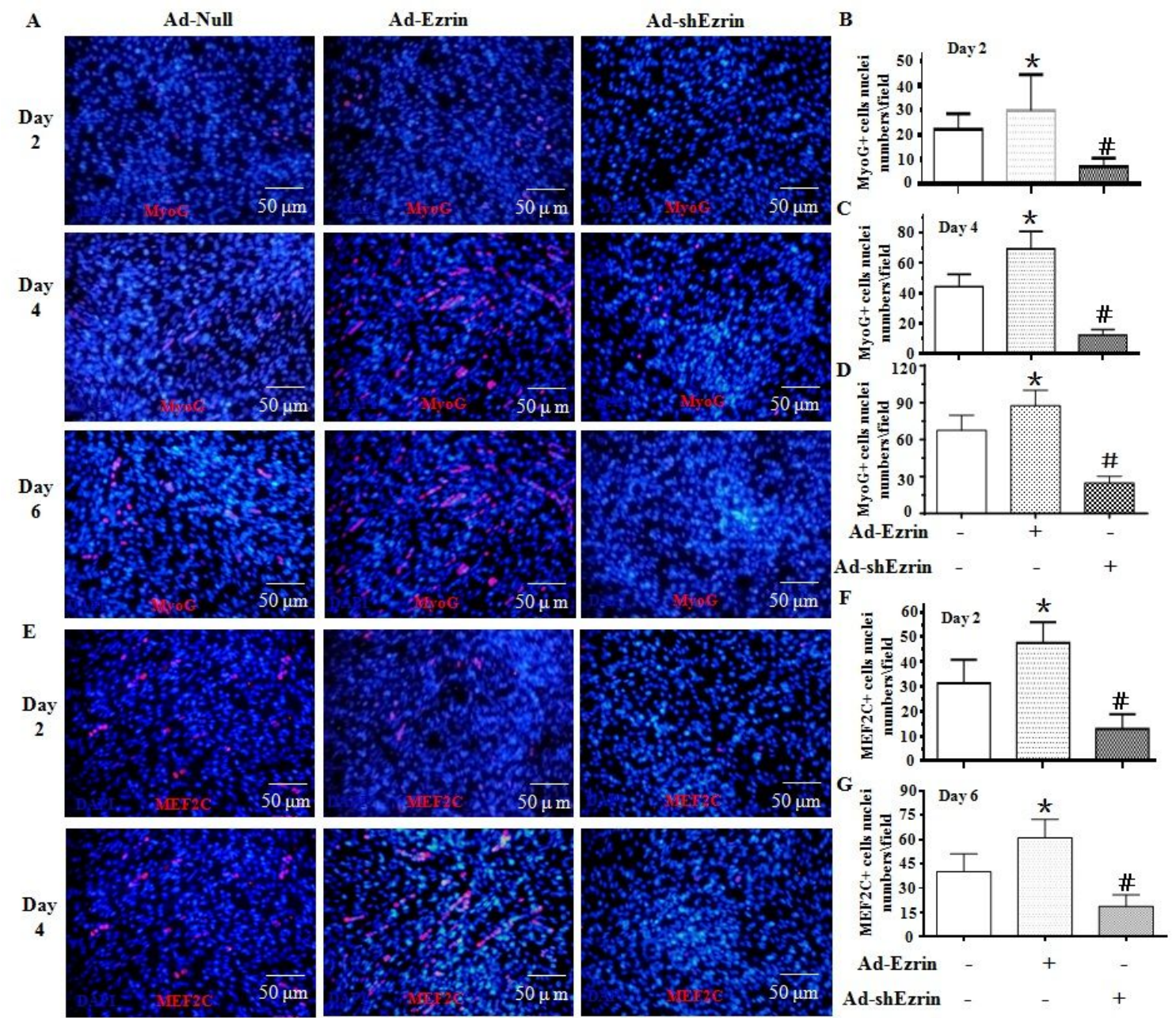


Figure 3

MyoG/MEF2C involved Ezrin-mediated myoblast differentiation and fusion. (A) Typical image of MyoG staining in differentiated C2C12 myoblast cells treated by overexpression or knockdown of Ezrin for 2, 4 6 days. (B-D) Quantitative assay for the number of MyoG+ nuclei was analyzed 2, 4 and 6 days after myoblast differentiation in treated by overexpression or knockdown of Ezrin, respectively. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ctrl. (E-G) Typical image of MEF2c staining in differentiated C2C12 myoblast cells treated by overexpression or knockdown of Ezrin for 2, 4 6 days. Quantitative assay for the number of MEF2c+

nuclei was analyzed 2, 4 and 6 days after myoblast differentiation in treated by overexpression or knockdown of Ezrin, respectively. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ctrl.

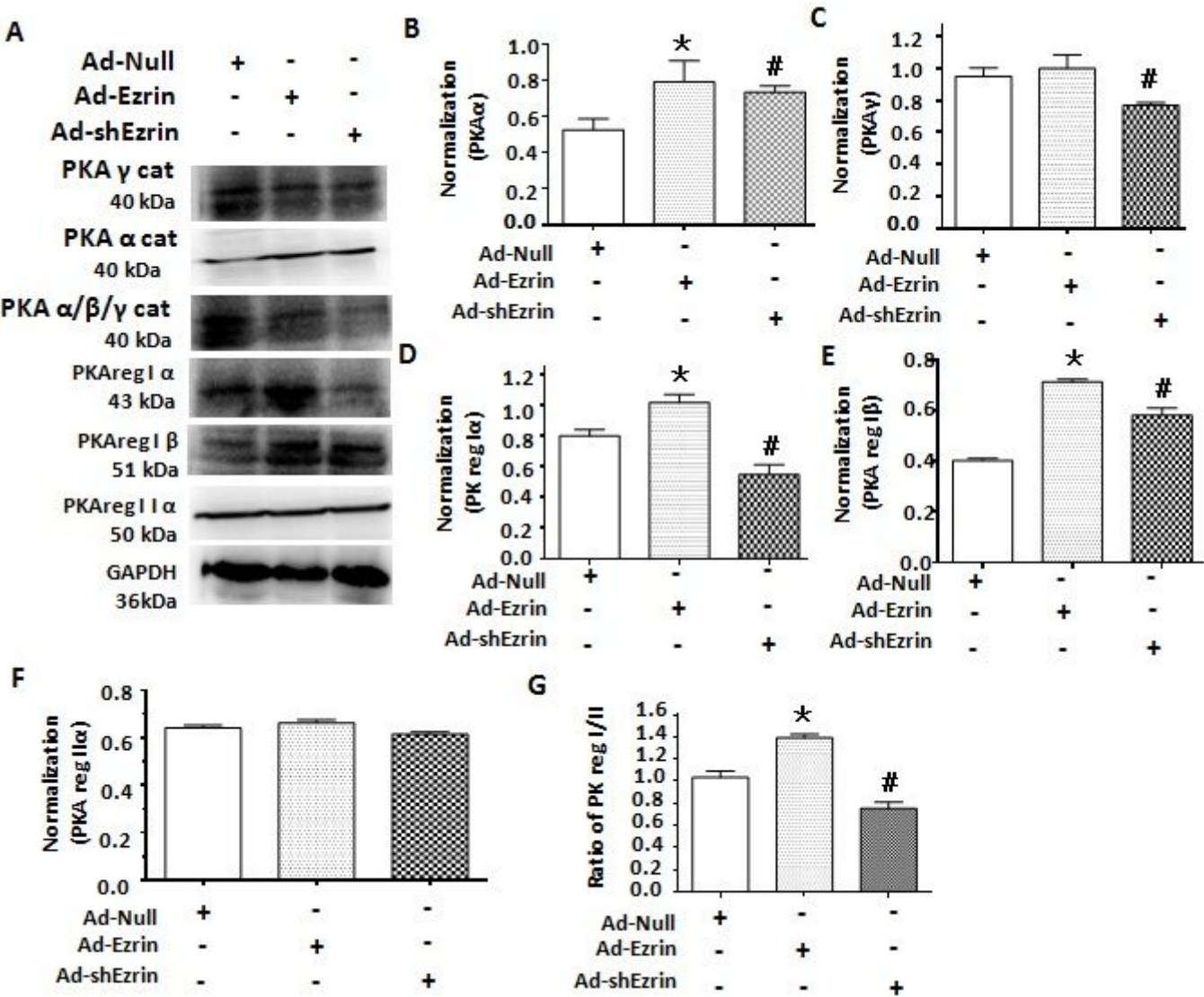


Figure 4

Ezrin regulated myoblast differentiation and fusion through PKA signaling pathway. (A) Western blot for indicated proteins in myoblast cells treated by over-expression or knockdown of Ezrin for 4 days. (B-G) Quantitative assay for indicated proteins was analyzed 4 days after myoblast differentiation following the treatment of over-expression or knockdown of Ezrin. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ctrl.

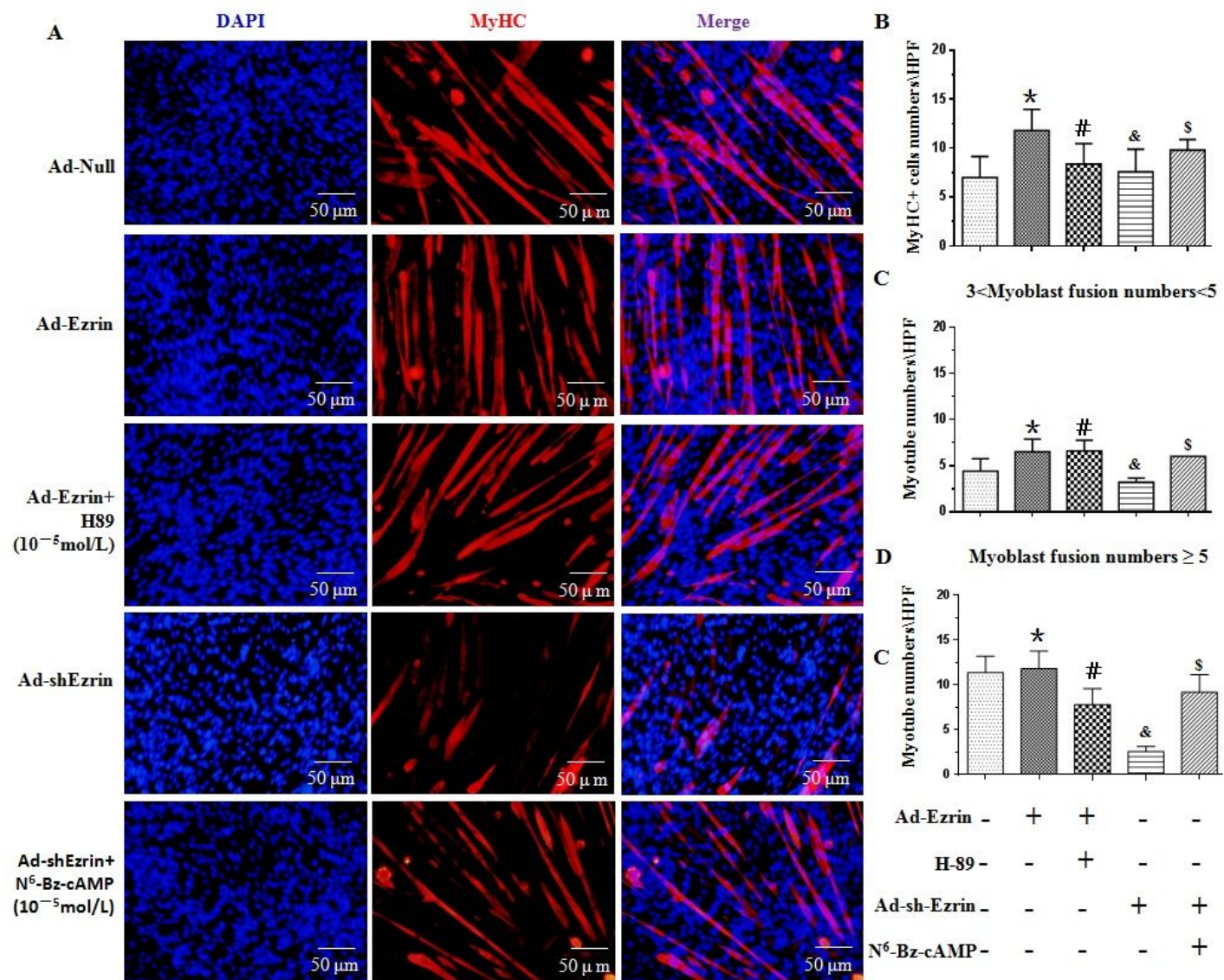


Figure 5

PKA involved Ezrin-mediated myoblast differentiation and fusion. (A) Typical image of MyHC staining in differentiated C2C12 myoblast cells treated by Ad-Ezrin with or without H-89(10^{-5} mol/L), or Ad-shEzrin with or without N⁶-Bz-cAMP(10^{-5} mol/L). (B-D) Quantitative assay for the number of MyHC⁺ myotubes with 3-5 or more than 5 nuclei were analyzed six days after myoblast differentiation. $n=3$, * $P \leq 0.05$ vs. Ctrl; # $P \leq 0.05$ vs. Ad-Ezrin; & $P \leq 0.05$ vs. Ctrl; \$ $P \leq 0.05$ vs. Ad-shEzrin.

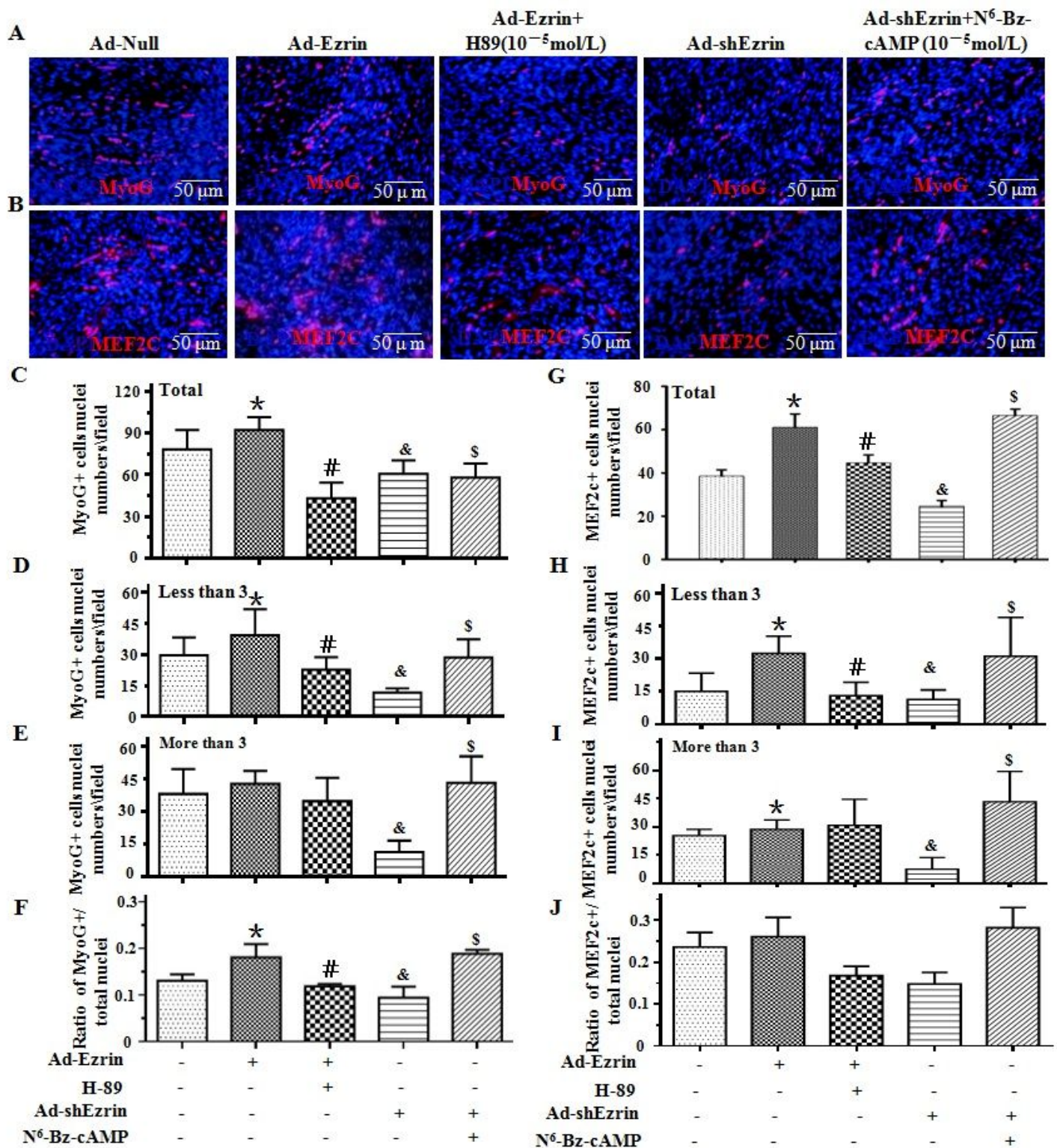


Figure 6

Ezrin regulated myoblast differentiation and fusion through PKA-MyoG/MEF2C signaling pathway. (A-B) Typical image of MyoG and MEF2c staining in differentiated C2C12 myoblast cells treated by Ad-Ezrin with or without H-89(10^{-5} mol/L), or Ad-shEzrin with or without N⁶-Bz-cAMP(10^{-5} mol/L), respectively. (C-J) Quantitative assay for the number of MyoG+ or MEF2c+ nuclei was analyzed 6 days after myoblast

differentiation treated by Ad-Ezrin with or without H-89, or Ad-shEzrin with or without N6-Bz-cAMP(10-5mol/L. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ad-Ezrin; &P<0.05 vs. Ctrl; \$P<0.05 vs. Ad-shEzrin.

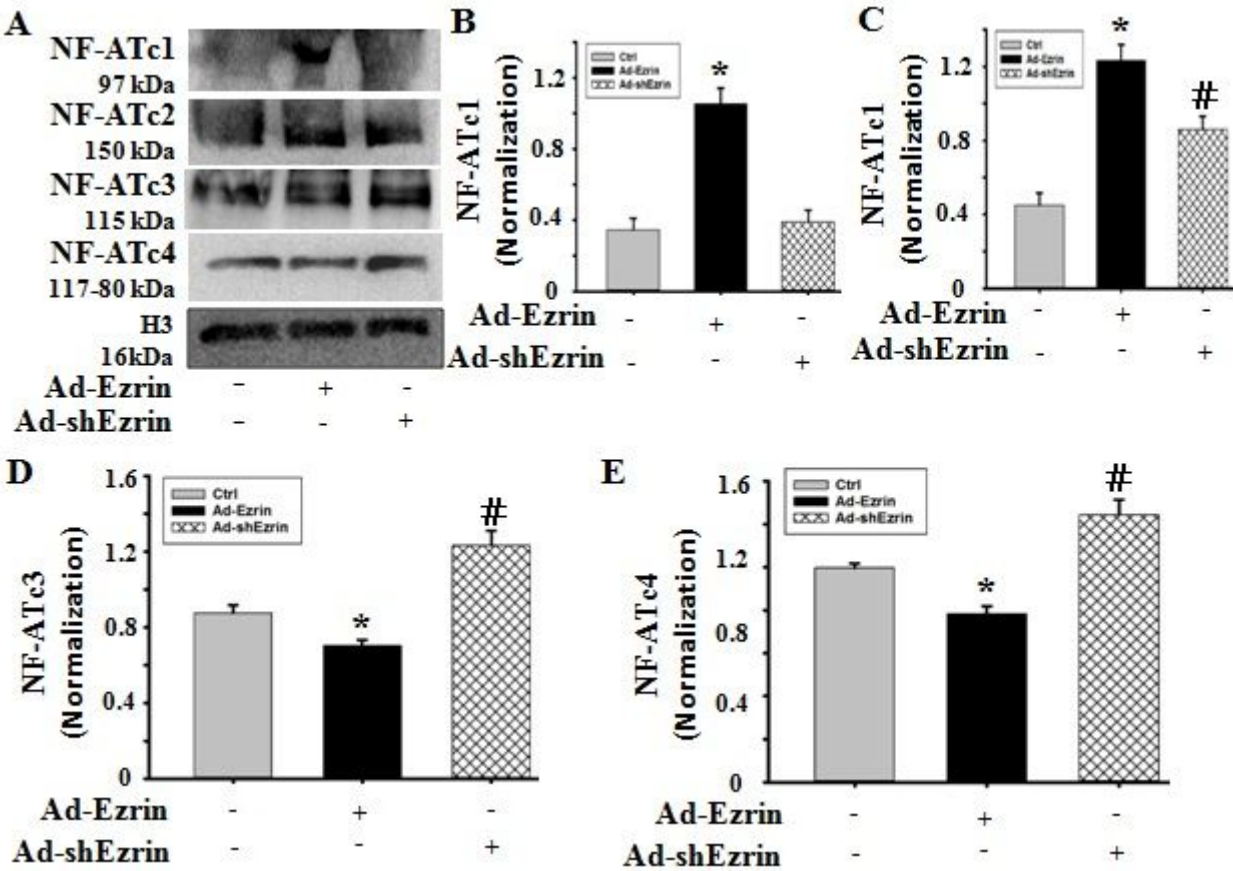


Figure 7

NFATs involved in Ezrin-mediated myoblast differentiation and fusion. (A)Western blot for indicated proteins in myoblast cells treated by over-expression or knockdown of Ezrin for 4 days. (B-E) Quantitative assay for indicated proteins was analyzed 4 days after myoblast differentiation following the treatment of over-expression or knockdown of Ezrin. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ctrl.

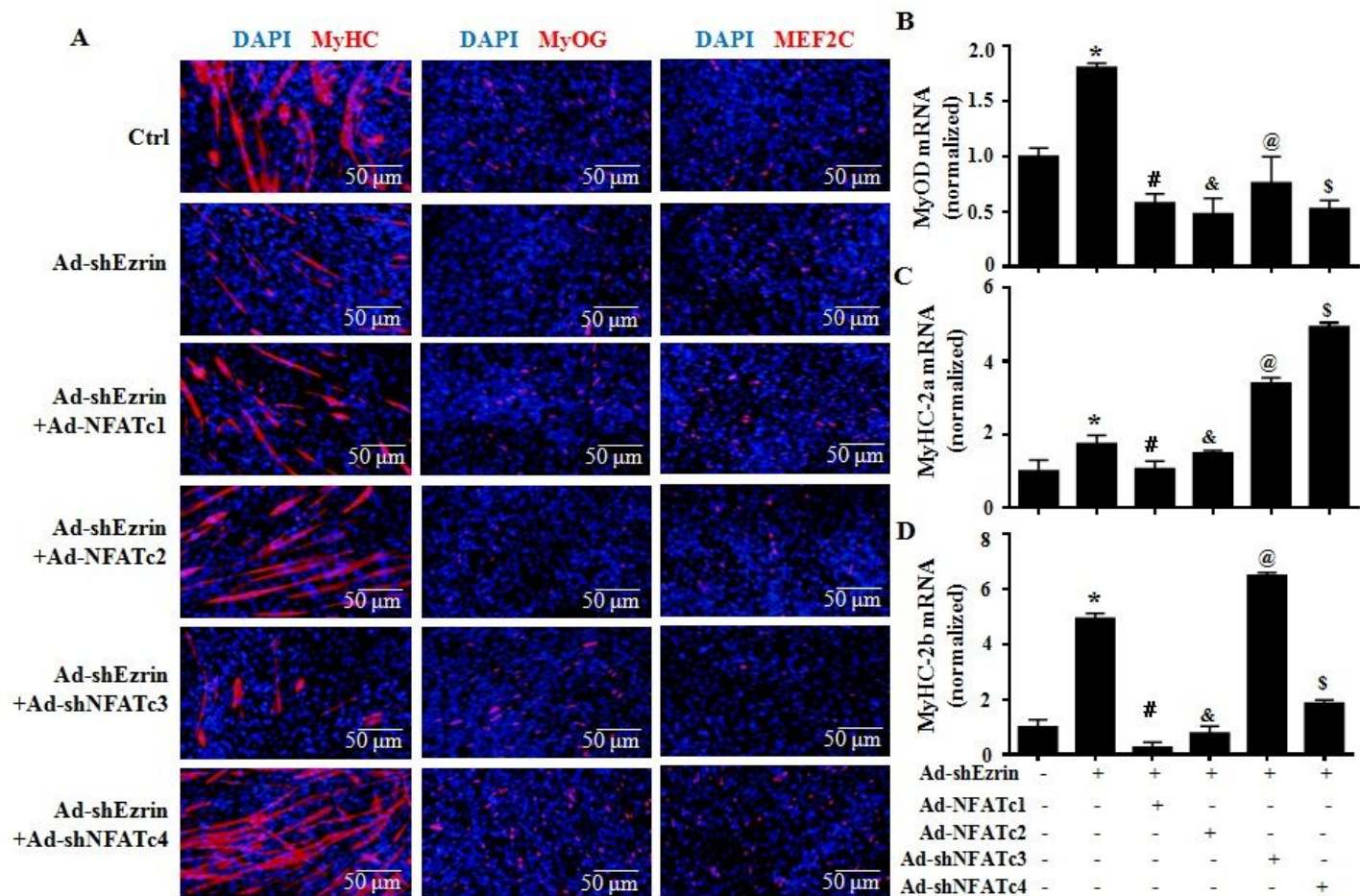


Figure 8

Ezrin regulated myoblast differentiation and fusion through NFATs-MyoD/MEF2C signaling pathway. (A) Typical image of MyHC, MyoG or MEF2cstaining in differentiated C2C12 myoblast cells treated by Ad-shEzrin with or without Ad-NFATc1/c2, Ad-shNFATc3.c4, respectively. (B-D) Real-time PCR for MyoD, MyHC-2a and MyHC-2b in Ezrin-knockdown myobalst cells pretreated with Ad-NFATc1/c2 or Ad-shNFATc3/c4. n=3, *P<0.05 vs. Ctrl; #P<0.05 vs. Ad-shEzrin; &P<0.05 vs. Ad-shEzrin; \$P<0.05 vs. Ad-shEzrin; @P<0.05 vs. Ad-shEzrin.

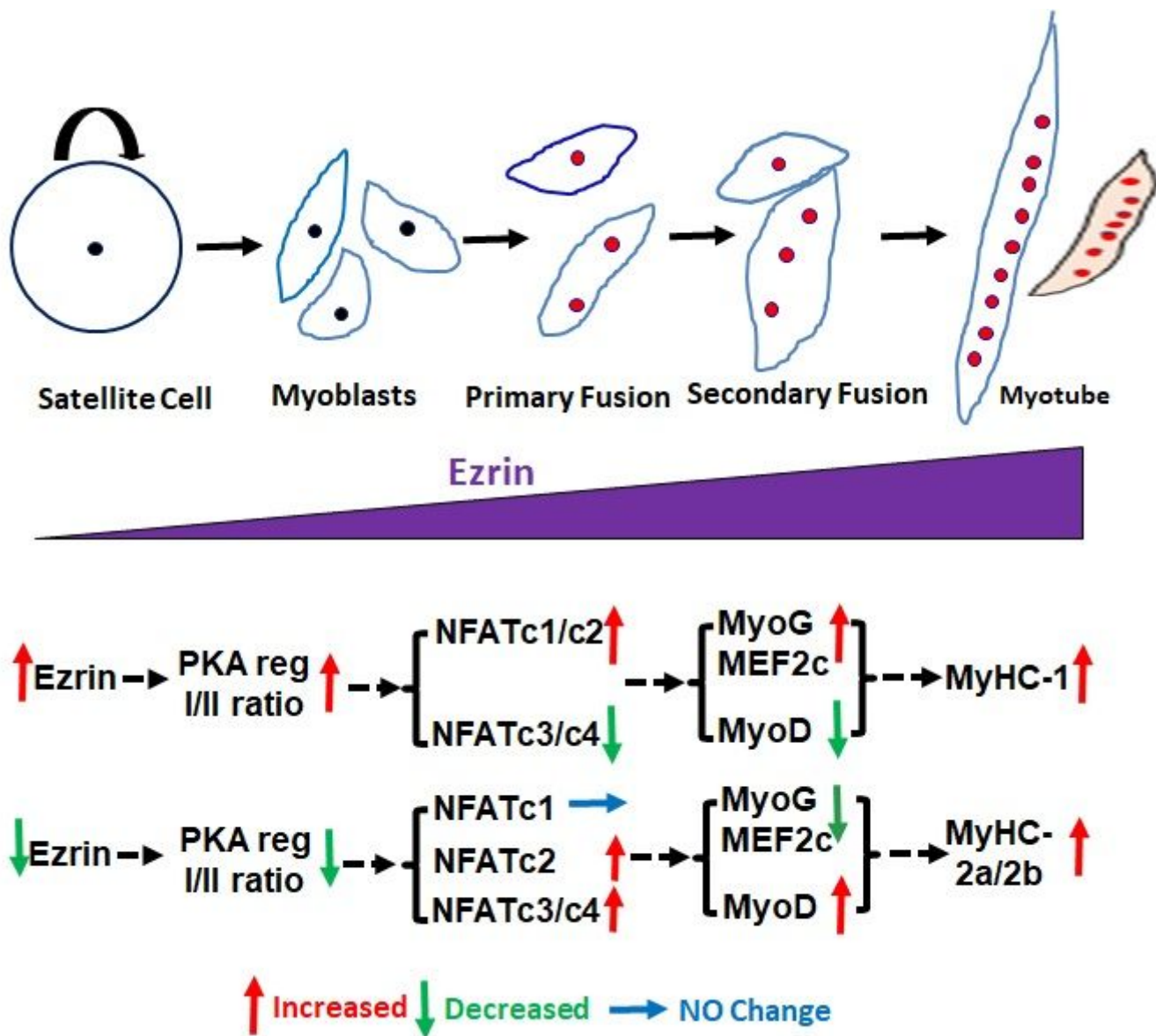


Figure 9

Working Model: Ezrin regulated myoblast differentiation/fusion and muscle fiber specialization through PKA-NFAT-MyoD/MEF2c signalling pathway. Ezrin expression show a time-dynamic characteristic during myoblast differentiation and fusion. Ezrin promoted myoblast differentiation and fusion through altering PKA RI/RII ratio, triggering type I muscle fiber specialization through NFATc2-MEF2C signaling pathway. Knockdown of Ezrin inhibited myoblast differentiation/fusion, especially type IIb muscle fiber specialization through NFATc4-MyoD/MEF2C signaling pathway.

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