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# Fibromodulin modulates myoblast differentiation by controlling calcium channel

Eun Ju Lee [a], Joo Hyun Nam [b], Inho Choi [a, *]

[a] Department of Medical Biotechnology, Yeungnam University, Gyeongsan, 38541, Republic of Korea
[b] Department of Physiology, Dongguk University, College of Medicine, Gyeongju, 38067, Republic of Korea

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ABSTRACT

Fibromodulin (FMOD) is a proteoglycan present in extracellular matrix (ECM). Based on our previous findings that FMOD controls myoblast differentiation by regulating the gene expressions of collagen type I alpha 1 (COL1α1) and integral membrane protein 2 A (Itm2a), we undertook this study to investigate relationships between FMOD and calcium channels and to understand further the mechanism by which they control myoblast differentiation. Gene expression studies and luciferase reporter assays showed FMOD affected calcium channel gene expressions by regulating calcium channel gene promoter, and patch-clamp experiments showed both L- and T-type calcium channel currents were almost undetectable in FMOD knocked down cells. In addition, gene knock-down studies demonstrated the COL1α1 and Itm2a genes both regulate the expressions of calcium channel genes. Studies using a cardiotoxin-induced mouse muscle injury model demonstrated calcium channels play important roles in the regeneration of muscle tissue, possibly by promoting the differentiation of muscle stem cells (MSCs). Summarizing, the study demonstrates ECM components secreted by myoblasts during differentiation provide an essential environment for muscle differentiation and regeneration.



## 1. Introduction

Skeletal muscles provide mobility, but they also perform many other functions essential for maintaining normal physiological functions, such as, body temperature control. Recent findings that myokines can be produced and secreted by muscles and affect numerous tissues, including muscles, serve to demonstrate the importance of muscles [1]. Myofibers are a basic unit of skeletal muscles and are multinucleated cells formed by the fusion of myoblast cells during differentiation [2]. As myofibers are completely differentiated cells that are unable to proliferate, it would appear myogenic stem cells (MSCs) present in muscle tissues must be activated to undergo further cell division and differentiation to form additional myofibers. MSCs are located between the sarcolemma of myofibers and basal lamina, a layer of extracellular matrix (ECM) [3].

Various factors have been reported to affect MSC proliferation and differentiation. Muscle regulatory factors (MRFs; MEF2, MyoD, myogenin; MYOG, Myf5, etc.) share a helix loop helix (HLH) domain, which recognizes a specific nucleotide sequence present in the promoter regions of genes that regulate the expression of genes involved in the proliferation and differentiation of MSCs [4]. Calcium acts as an intracellular messenger and is known to control the expression of MRFs [5], and L-/T-type calcium channels enable intracellular calcium flow and facilitate the differentiation of MSCs [6]. Furthermore, ECM has been reported to have crucial effects on the activation, division, and differentiation of MSCs [7]. Together with lumican, decorin, and biglycan, fibromodulin (FMOD) is a member of the small leucine-rich repeat proteoglycan (SLRP) family found in ECM and is known to be involved in fibrillogenesis and collagen assembly [8]. The SLRP family can be classified according to the type of glycosaminoglycan (GAG) attached to the serine residue of the core protein, for example, chondroitin sulfate and dermatan sulfate are attached to biglycan and decorin, respectively, and keratan sulfate is attached to FMOD or lumican [9].

We previously found FMOD is involved in the expressions of COL1α1, integral membrane protein 2a (Itm2a), MYOG, and myosin light chain 2 (MYL2) genes during MSC differentiation [10,11]. Considering that MYOG is expressed during the later stage when

* Corresponding author. Department of Medical Biotechnology Yeungnam University Gyeongsan, 38541, Republic of Korea.
*E-mail address:* inhochoi@ynu.ac.kr (I. Choi).

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myoblasts are differentiated and that MYL2 is one of the constituent proteins of myofibrils produced during myotube maturation, this result suggests FMOD is an important factor of myofibril formation. Interestingly, two genes of voltage-dependent calcium channels (Cav1.1; L-type, alpha 1 S subunit, and Cav3.1; voltage-dependent T-type calcium channel) are expressed during the differentiation of myoblasts to myotubes, and it is known myotube formation does not occur when this channel protein is blocked [12,13]. Therefore, we undertook this study to determine how FMOD and calcium channels are involved in myoblast differentiation and to identify the link between FMOD and calcium during myoblast differentiation.

## 2. Materials and methods

### 2.1. Mouse tissues

Muscle injury experiments were performed as described previously [10]. Briefly, 100 μl of 10 μM cardiotoxin (CTX) was injected into left gastrocnemius muscles of C57BL/6 mouse and phosphate buffered saline (PBS) was injected into contralateral right gastrocnemius muscles as a control. Tissue samples were collected 3 days after injection. All treatments were administered while the animals were under avertin (i.p.) anesthesia, and animals were sacrificed using the standard protocol approved by the Institutional Animal Care and Use Committee, Yeungnam University (permit number: YUMC-AEC2015-006).

### 2.2. C2C12 cell culture

C2C12 cells (an immortalized mouse myoblast cell line) were obtained from the Korean Cell Line Bank and cultured in DMEM (Dulbecco's Modified Eagle's Medium; HyClone Laboratories) supplemented with 10% FBS (HyClone Laboratories) and 1% penicillin/streptomycin (Invitrogen, CA, USA-) at 37 °C in a 5% $CO_2$ atmosphere. For studies of myoblast differentiation, cells were grown to 70% confluence and then switched to DMEM containing 2% FBS (differentiation medium; DM) and cultured for 0 or 4 days.

### 2.3. Gene knock-down

To induce gene knock-down, we adopted the procedure described by Lee et al. [10]. Briefly, FMOD, Itm2a, or COL1α1 knock-down constructs ($FMOD_{kd}$, $ITM2a_{kd}$ and $COL1\ \alpha\ 1_{kd}$, respectively) or scrambled vector were transfected using transfection reagent and transfection medium (Santa Cruz Biotechnology, CA, USA) into C2C12 cells. Cells were treated 2 μg/mL puromycin (Santa Cruz Biotechnology) to select transfected cells and media was switched to DM when cells reached 70% confluency. Knock-down efficiencies were checked by assessing mRNA and protein expressions by RT-PCR and Western blot. Knock-down construct information is provided in Supplemental Table S1.

### 2.4. FMOD protein neutralization

FMOD protein neutralization was performed with FMOD specific antibodies (Santa Cruz Biotechnology) at a concentration of 2 mg/mL in medium.

### 2.5. RNA extraction and real-time RT-PCR analysis

Total RNA extraction and cDNA synthesis were performed as described previously [14]. Briefly, RNA was extracted using Trizol reagent (Thermo Fisher Scientific) and cDNA was synthesized with 2 μg RNA and random hexamer according to the manufacturer's instructions. cDNA product (2 μl) and 10 pmole of gene-specific primers were used for PCR, which was performed using a 7500 real-time PCR system (Thermo Fisher Scientific) and power SYBR Green PCR Master Mix (Thermo Fisher Scientific) as the fluorescence source. Primer details are provided in Supplemental Table S2.

### 2.6. Western blot analysis

Cell lysis was performed using RIPA buffer containing protease inhibitor (Thermo Scientific), and proteins (40 μg) were electrophoresed in 8 or 10% SDS-polyacrylamide gels and transferred to membranes (Millipore, MA, USA). Blots were blocked with 3% skim milk or BSA for 1 h and incubated overnight with FMOD (1:400), Cav1.1 (1:400), Cav3.1 (1:400), MYOG (1:1000), Itm2a (1:400), or β-actin (1:2000) antibody (Santa Cruz Biotechnology) or COL1α1 antibody (1:2500; Abcam, MA, USA) diluted with 1% skim milk or BSA at 4 °C. After washing, blots were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature and developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

### 2.7. Immunohistochemistry

Paraffin-embedded tissue sections of normal or CTX-injected muscles were deparaffinized and hydrated and then endogenous peroxidase activity was quenched. Sections were blocked with 1% normal goat serum, incubated with Cav1.1 or Cav3.1 antibody (1:50) overnight at 4 °C, and then incubated with the HRP-conjugated secondary antibody (1:100; Santa Cruz Biotechnology). Positive signals were visualized by adding diaminobenzidine and hydrogen peroxide as substrates. Stained sections were examined under a light microscope (Leica, Wetzlar, Germany).

### 2.8. Patch-clamp analysis

To measure L-type and T-type $Ca^{2+}$ channel currents in C2C12 cells, we used the whole-cell patch-clamp technique, as previously described [15]. Briefly, C2C12 cells were bathed in an external recording solution containing 120 mM NaCl, 5 mM CsCl, 10 mM TEA-Cl, 10 mM $BaCl_2$, 10 mM glucose, 0.5 mM $MgCl_2$, and 10 mM HEPES and adjusted to pH 7.4 with 1 M NaOH. The pipette solution contained 100 mM Cs-aspartate, 32 mM CsCl, 10 mM ethylene glycol-bis (2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 mM Mg-ATP, and 10 mM HEPES and was adjusted to pH 7.4 with CsOH. Patch-clamp data were sampled at 10 kHz and filtered using a low pass Bessel filter with a cut-off of 5-kHz. To compare the whole-cell currents of scramble siRNA-transfected ($FMOD_{wt}$) and $FMOD_{kd}$ C2C12 cells, current amplitudes were normalized versus membrane areas as determined using electrical capacitance. All $Ca^{2+}$ current data analysis was performed using the Clampfit 10 (Sunnyvale, CA, USA) and Origin 8.0 (Microcal, Northampton, MA, USA) software packages. Peak $Ca^{2+}$ currents measured during each depolarization were plotted against corresponding voltages.

### 2.9. Construction of Cav1.1 promoter and luciferase assay

As described previously by Zheng et al. [16], the region corresponding to Cav1.1 promoter was amplified using primers (forward 5′ gaattctgagggaggacgag 3'; reverse 5′ggggtgtcagggaagccatg 3′), digested with KpnI and SmaI (Thermo Fisher Scientific), and ligated into pGL-Basic vector (Promega, WI, USA). pGL-Basic vector or Cav1.1 promoter

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