Fibromodulin and regulation of the intricate balance between myoblast differentiation to myocytes or adipocyte-like cells

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ABSTRACT: Interactions between myoblasts and the surrounding microenvironment led us to explore the role of fibromodulin (FMOD), an extracellular matrix protein, in the maintenance of myoblast stemness and function. Microarray analysis of FMODkd myoblasts and in silico studies were used to identify the top most differentially expressed genes in FMODkd, and helped establish that FMOD-based regulations of integral membrane protein 2a and clusterin are essential components of the myogenic program. Studies in knockout, obese, and diabetic mouse models helped characterize the operation of a novel FMOD-based regulatory circuit that controls myoblast switching from a myogenic to a lipid accumulation fate. FMOD regulation of myoblasts is an essential part of the myogenic program, and it offers opportunities for the development of therapeutics for the treatment of different muscle diseases.—Lee, E. J., Jan, A. T., Baig, M. H., Ahmad, K., Malik, A., Rabbani, G., Kim, T., Lee, I.-K., Lee, Y. H., Park, S.-Y., Choi, I. Fibromodulin and regulation of the intricate balance between myoblast differentiation to myocytes or adipocyte-like cells. FASEB J. 32, 768–781 (2018). www.fasebj.org

KEY WORDS: extracellular matrix · adipogenesis · myogenesis · muscle disease

Skeletal muscle accounts for nearly half of body mass and represents the largest protein reservoir in the human body (1, 2). Because muscle is endowed with the features of contractility and extensibility (3), excessive loss in different myopathies reflects its ability to deal with increasing incidences of diseases. Skeletal muscle is enriched with a heterogeneous cell population in a multipotent state that are referred to as muscle satellite cells (MSCs), which reside between sarcolemma and the basal lamina of muscle fibers (4, 5). By exhibiting a high degree of sensitivity and strong migration abilities, MSCs play critical roles in maintaining the structural and functional integrity of skeletal muscle (6, 7). MSCs complement damaged myofibrils by promoting the synthesis of new muscle fibers, and their determination of the success or failure of regeneration thus plays a key role in postnatal growth and repair (3, 8, 9). The orchestrated myogenic program that drives the transformation of proliferating myogenic cells to syncytial contractile myofiber dictates the balance between proliferation and differentiation events.

Extracellular matrix (ECM) is credited with the maintenance of skeletal muscle architecture (10–12). Study of MSCs and muscle tissues has revealed substantial changes in the expression of ECM proteins, such as fibromodulin (FMODE) and matrix gla protein, during transition from proliferation to differentiation. MSC differentiation involves interaction of the proteins present in the ECM with MSCs to regulate their activity, and therefore their phenotype. We found that FMODE mediates the expression of myogenic marker genes and as such participates in the assembly of ECM to regulate myogenesis (13, 14) and matrix gla protein (15). The cytosolic localization of FMODE is correlated with the structural organization of collagens in connective tissue (16), whereas matrix gla protein functions as a calcification inhibitor (17, 18). The roles of

ABBREVIATIONS: Clu, clusterin; CP, ceruloplasmin; CTX, cardiotoxin; DEG, differentially expressed gene; DPT, dermatopontin; ECM, extracellular matrix; FMODE, fibromodulin; GO, Gene Ontology; HFD, high-fat diet; Itm2a, integral membrane protein 2a; kd, knockdown; KO, knockout; MSC, muscle satellite cell; MYL2, myosin light chain 2; MYOD, myogenic differentiation; MYOG, myogenin; ND, normal diet; OGN, mimecan; PPARγ, peroxisome proliferator-activated receptor γ; qPCR, quantitative PCR; SERPING1, plasma protease C1 inhibitor; shRNA, short hairpin RNA; WT, wild type

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FMOD (a member of the class 2 leucine-rich proteoglycan family) in tissue morphogenesis, cellular adhesion, cytokine modulation, and tumor suppression, as well as the role in overcoming the apoptotic cascade have been well established (14). The present study was performed to explore the possibility of FMOD involvement in the regulation of MSC function during the myogenic program.

To increase our understanding of the physiological processes of muscle development, regeneration, and repair, the study of MSC function in the surrounding milieu seemed essential. To gain an insight into the role of FMOD in MSC in the myogenic program, we studied its involvement in the differentiation of MSCs. Study of its role in the regulation of MSCs revealed that it interfered with the interaction between the negative muscle regulator myostatin and its receptor, activin receptor type IIB (13). In addition, binding of curcumin or gingerol with myostatin decreased the binding affinity of myostatin to activin receptor type IIB (19). The involvement of FMOD in different cellular processes (14) made us study its regulatory function on MSC fate in the myogenic program. Because MSCs have the ability to differentiate into muscle cells or transdifferentiate into adipocyte-like cells (20, 21), substantial expression of FMOD in muscle tissue led us to explore the possibility of FMOD involvement in shaping the fate of MSC at the differentiation stage to the myogenic or lipid accumulation paths.

ECM regulation of MSC function was studied by microarray analysis of normal [wild-type (WT)] (FMODwt) and FMOD knockout (FMODkd) murine myoblast C2C12 cells. This identified a regulatory circuit that included the involvement of integral membrane protein 2a (Itm2a) and clusterin (Clu). Subsequent studies performed on Clu knockout (KO) (CluKO) and on obese and diabetic mouse models helped to establish the functions of FMOD, Itm2a, and Clu in the myogenic program. The study revealed a novel mechanism whereby FMOD controls the expression of myogenic and adipogenic markers, and thus controls myoblast fate during the differentiation stage of the myogenic program. This investigation of the role played by FMOD in the regulation of MSC function helped in identifying a novel regulatory circuit that controls the myoblast continuum to myogenesis or switches their fate to lipid accumulation.

MATERIALS AND METHODS

Mouse studies

Male mice (C57BL/6, 6–8 wk old) were housed in a temperature-controlled room in an animal facility with free access to water and food [normal diet (ND) containing 4.0% (w/w) total fat or high-fat diet (HFD) containing 45% fat]. C57BL/6 treatment-naive control animals and HFD mice (3–4 mo old) were used for the muscle studies. Diabetes was induced using the protocol devised by the Animal Models of Diabetic Complications and Consortium (22). Mice were administered intraperitoneal injections of streptozocin at 50 mg/kg [0.75% streptozocin in 0.1 M sodium citrate buffer (pH 4.5); Sigma-Aldrich, St. Louis, MO, USA] once daily for 5 d. All experiments were conducted 1 mo after final injection. Clu KO mice were generated in the Swiss Black outbred genetic background and backcrossed for at least 8 generations into C57BL/6 mice (23). All experimental procedures were performed in accordance with the guidelines issued by the Institutional Animal Care and Use Committees of the Catholic University of Daegu (IACUC-2014-035), and by Yeungnam University (AEC2012-004).

The muscle injury model was produced as described by Kim et al. (24). Briefly, mice were anesthetized with avertin, and 100 µl of 10 µM cardiotoxin (CTX) was injected into the gastrocnemius muscle. PBS-injected gastrocnemius muscles were used as controls. All experiments were conducted 3 d after final injection. Animals were humanely killed and samples collected following a standard protocol approved by the Institutional Animal Care and Use Committee of Yeungnam University (AEC2015-006) at the time points indicated.

Antibodies and short hairpin RNA constructs

Antibodies against FMOD (sc-33772), Itm2a (sc-134811), Clu (sc-8354), myogenic differentiation (MYOD, sc-760), myogenin (MYOG, sc-12732), CD36 (sc-9154), peroxisome proliferator-activated receptor γ (PPARγ, sc-7273), dermatopontin (DPT, sc-143012-SH), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Myosin light chain 2 (MYL2, ab79935) antibody was obtained from Abcam (Cambridge, MA, USA). Short hairpin RNA (shRNA) construct for FMOD (sc-44823-SH), Itm2a (sc-60868-SH), Clu (sc-43689-SH), DPT (sc-143012-SH), and scrambled vector were purchased from Santa Cruz Biotechnology. Sequences of the shRNA constructs are provided in Supplemental Table S1.

DNA microarray

An Agilent Technologies mouse GE 4 × 44K (V2) chip was used for DNA hybridization (Agilent Technologies, Santa Clara, CA, USA). DNA microarray analyses for FMODwt vs. FMODkd C2C12 cells were performed to identify differentially expressed genes (DEGs). DNA microarray analysis was performed as previously described (25). Briefly, FMODwt and FMODkd C2C12 cells were grown in differentiation medium (DMEM supplemented with 2% fetal bovine serum) for 2 d and then used to synthesize cDNA probes using a Low RNA Input Linear Amplification kit (Agilent Technologies).

Cell culture

Murine myoblast C2C12 cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and cultured in DMEM (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified 5% CO2 incubator at 37°C. For differentiation, cells (70% confluent) were cultured for 2 or 4 d in differentiation medium; media were changed every 2 d.

Fusion index

Fusion indices were calculated as described previously (23). Briefly, cell nuclei were stained with Giemsa G250 (Sigma-Aldrich), and images were captured randomly at 3 different regions per slide. Numbers of nuclei in myotubes and total numbers of nuclei in cells were counted in each field. Fusion indices were calculated by expressing numbers of nuclei in myotubes as percentages of total numbers of nuclei.
Gene knockdown

C2C12 cells (30% confluent) were transfected with FMOD, Itm2a, Clu, or DPT shRNA (1 ng) or scrambled vector construct using transfection reagent (Santa Cruz Biotechnology), according to the manufacturer’s instructions. shRNA transfected cells were incubated in a 5% CO2 humidified incubator at 37°C. Cells selected with puromycin (2 μg/ml; Santa Cruz Biotechnology) were allowed to grow to 70% confluence before switching to differentiation medium. FMOD, Itm2a, Clu, or DPT knockdown cells were subjected to real-time quantitative RT-PCR (qPCR), Western blot analysis, and immunocytochemistry. Differences between gene expression in control (WT) and knockdown cells were expressed as percentages of gene expression in the WT cells and used to quantify transfection efficiencies of shRNA knockdown constructs.

RNA isolation and qPCR

Total RNAs from cultured cells or muscle tissues were extracted using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions, and then stored in diethylpyrocarbonate-treated H2O at −80°C until use. Two micrograms of RNA in 20 μl of standard reaction mixture was primed with random hexamer (Thermo Fisher Scientific), then reverse transcribed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. First-strand cDNA product (2 μl) and 10 pM of gene-specific primers were used for PCR, which was conducted using a 7500 real-time PCR system (Thermo Fisher Scientific) and powerSYBR Green PCR Master Mix (Thermo Fisher Scientific) as the fluorescence source. All reactions were performed in triplicate, and relative amounts of gene expressions normalized vs. controls were calculated using 2^−ΔΔCt, where ΔCt = Ct gene − Ct control. Primer details are provided in Supplemental Table S2.

Western blot analysis

Cell or muscle tissues were lysed with RIPA buffer containing protease inhibitor cocktail (Thermo Fisher Scientific). Whole cell or muscle tissue lysates in RIPA buffer were quantified using the Bradford assay. Total protein extract (40 μg) was resolved in 8 or 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA) using the Bio-Rad mini protein transfer system (Bio-Rad, Hercules, CA, USA). Membranes were rinsed in Tris-buffered saline, blocked for 1 h at room temperature in 3% skim milk or bovine serum albumin in Tris-buffered saline containing Tween 20, and then incubated with primary antibodies in Tris-buffered saline overnight at 4°C. Primary antibodies were used at the following dilutions: FMOD (1:400), Itm2a (1:400), Clu (1:400), MYOD (1:400), MYOG (1:1000), CD36 (1:400), PPARY (1:400), β-actin (1:2000), MYL2 (1:2500), and DPT (1:400). After washing, blots were incubated with horseradish peroxidase–conjugated secondary antibodies (goat anti-mouse or anti-rabbit; Santa Cruz Biotechnology) for 1 h at room temperature and developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Immunocytochemistry

Immunocytochemistry was performed using cells grown in differentiation medium and then fixed with 4% formaldehyde (Sigma-Aldrich). After permeabilization with 0.2% Triton X-100 (Sigma-Aldrich), cells were incubated with primary antibody [FMOD (1:50), MYOD (1:50), MYOG (1:50), Itm2a (1:50), Clu (1:50), or MYL2 (1:50)] at 4°C in a humid environment overnight. Secondary antibody (1:100; Alexa Fluor 594 goat anti-rabbit and goat anti-mouse; Thermo Fisher Scientific) was then applied for 1 h at room temperature. Cells were counterstained with DAPI (Sigma-Aldrich) and imaged under a fluorescence microscope equipped with a digital camera (Nikon, Tokyo, Japan).

Oil Red O staining

Oil Red O staining was performed as previously described (20). Briefly, cells were fixed with 10% formaldehyde (Sigma-Aldrich) for 20 min and incubated for 1 h with Oil Red O solution (6:4 dilution of stock: 3.5 mg/ml Oil Red O powder in 100% isopropanol). After washing with PBS, cells were imaged under a light microscope equipped with a digital camera (Nikon). To quantify Oil Red O staining in cells, 500 μl of 100% isopropanol was added to plates to elute the dye, and optical density was measured at 510 nm using a Versa Max microplate reader (Tecan, Männedorf, Switzerland).

Gene Ontology Consortium and hub gene selection

The GeneMania server (26) was used to predict functional interactions between selected DEGs. An additional set of 50 genes from Mus musculus was added to create the network using the Gene Ontology (GO) Consortium term “biological process.” Predicted interactions were filtered by removing interactions with weights of <0.01. Finally, a filtered network consisting of up- and down-regulated genes was created and analyzed in Cytoscape 2.8.2 (27). Genes (nodes) with most interacting partners were considered as hubs. The Network Analyzer (28) plugin of Cytoscape was used to calculate node degree distributions.

Community analysis

Relationships between genes in the network were evaluated in terms of expression, physical and genetic interactions, pathways, colocalization, protein domain similarity, and predicted interactions. Communities (clusters) in the network were detected by using the GLay (29) community detection module of Cytoscape. Functional enrichment analysis of each identified cluster was conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp). Clusters with fewer than 10 nodes were excluded from the functional enrichment analysis.

Interaction network of FMOD using the Search Tool for the Retrieval of Interacting Genes/Proteins

A protein–protein interaction network was constructed and analyzed using the Search Tool for the Retrieval of Interacting
TABLE 1. Microarray analysis of FMOD knockdown cells

| FMOD<sub>wt</sub> vs. FMOD<sub>kd</sub> | Genes (4-fold/2-fold) |
|----------------------------------|----------------------|
| Down-regulated                  | 13/119               |
| Up-regulated                    | 36/263               |

Microarray analysis was performed on FMOD knockdown C2C12 cells. FMOD<sub>wt</sub> indicates cells transfected with scrambled vector. Means ± s.d. (n = 3).

Genes/Proteins (STRING; http://string-db.org) (30). Stepwise interaction analysis of 2-fold up- and down-regulated genes was performed (Supplemental Fig. S1). Interactions were predicted from physical or functional associations resulting from genomic context, literature mining, high-throughput experiments, and coexpression using a set of prediction algorithms. The parameters used in the analysis were maintained at default values (except confidence score was set as 0.150, low confidence, to obtain the maximum possible number of interactions among query proteins).

**Statistical analysis**

Mean values of normalized expressions were compared by Tukey’s Studentized range test (honest significant difference) to identify significant gene expressional differences. Nominal values of *P* < 0.05 were considered statistically significant. qPCR data was normalized using glyceraldehyde 3-phosphate dehydrogenase as the internal standard and analyzed by 1-way ANOVA using PROC GLM in SAS 9.0 (SAS Institute, Cary, NC, USA).

**RESULTS**

**FMOD and regulation of myoblast fate**

To define the function of FMOD in myoblast differentiation, we performed microarray analysis on normal (FMOD<sub>wt</sub>) and FMOD knockdown (FMOD<sub>kd</sub>) cells (Supplemental Fig. S2). Using 4- or 2-fold cutoffs for down- and up-regulated genes, analysis of the effects of FMOD knockdown on myoblast functioning revealed marked up and down regulatory effects on muscle-related genes. Compared to FMOD<sub>wt</sub>, FMOD<sub>kd</sub> showed down-regulation for 13 of 119 up-regulation for 36 of 263 genes (Table 1). Subsequent *in silico* analysis of these 49 genes was conducted to establish their functions. The 10 top statistically significant enriched GO Consortium terms for FMOD<sub>kd</sub> are summarized in Tables 2 and 3, and most entries were related to ECM.

An examination of the top 9 down-regulated and 8 up-regulated genes (Table 4) was performed in C2C12 cells. qPCR study of the effects of FMOD knockdown in FMOD<sub>wt</sub> and FMOD<sub>kd</sub> cells confirmed expression changes observed in the microarray study (Fig. 1A, B). To check the expression patterns of the affected genes, a day point study of the top most down- and up-regulated genes was performed (Supplemental Fig. S3). The expression of Itm2a, GDPD2, and Clu were found to be higher than those of other genes during myogenic differentiation. GDPD2 is a member of glycerophosphodiester phosphodiesterase enzyme family. It is a surface-localized protein expressed in bone and spleen. Its expression in bone promotes osteoblast differentiation, while in spleen its function is correlated with growth (31, 32). To our knowledge, there is no study that reports the function of GDPD2 in muscle. However, on observing that it underwent a significant change in the microarray, we performed a validation study using C2C12 cells (Supplemental Fig. S3). The expression of GDPD2 was found to be decreased in differentiated cells compared to undifferentiated cells during cell transition from the proliferation to the differentiation stage. Furthermore, Clu expression was significantly higher than GDPD2 during myoblast differentiation. Henceforth, Clu and Itm2a were studied for their effects toward elucidating role of FMOD during differentiation and lipid accumulation in the myoblasts. Western blot analysis and immunocytochemistry revealed down-regulation of Itm2a and up-regulation for Clu, as was

**TABLE 2. GO Consortium processes for down- or up-regulated genes observed in FMOD knockdown cells**

| Category | Term                        | Count | *P*       |
|----------|-----------------------------|-------|-----------|
| GOTERM_CC_FAT | GO:0005576 ~ extracellular region | 23 | 0.000299  |
| GOTERM_BP_FAT | GO:0016055 ~ Wnt receptor signaling pathway | 6 | 0.000622  |
| GOTERM_BP_FAT | GO:0001558 ~ regulation of cell growth | 5 | 0.001425  |
| GOTERM_CC_FAT | GO:0005578 ~ proteaceous extracellular matrix | 8 | 0.001984  |
| GOTERM_CC_FAT | GO:0031012 ~ extracellular matrix | 8 | 0.002480  |
| GOTERM_MF_FAT | GO:0030247 ~ polysaccharide binding | 5 | 0.003515  |
| GOTERM_MF_FAT | GO:0001871 ~ pattern binding | 5 | 0.003515  |
| GOTERM_CC_FAT | GO:0044421 ~ extracellular region part | 12 | 0.006167  |
| GOTERM_BP_FAT | GO:0043062 ~ extracellular structure organization | 5 | 0.008095  |
| GOTERM_BP_FAT | GO:0007155 ~ cell adhesion | 9 | 0.009427  |
| GOTERM_CC_FAT | GO:0005576 ~ extracellular region | 60 | 9.85E-17  |
| GOTERM_CC_FAT | GO:0044421 ~ extracellular region part | 39 | 6.79E-15  |
| GOTERM_CC_FAT | GO:0005615 ~ extracellular space | 28 | 2.05E-11  |
| GOTERM_BP_FAT | GO:0009611 ~ response to wounding | 19 | 3.73E-08  |
| GOTERM_BP_FAT | GO:0006952 ~ defense response | 21 | 7.46E-08  |
| GOTERM_MF_FAT | GO:008009 ~ chemokine activity | 8 | 1.25E-07  |
| GOTERM_MF_FAT | GO:0042379 ~ chemokine receptor binding | 8 | 1.51E-07  |
| GOTERM_BP_FAT | GO:0006935 ~ chemotaxis | 11 | 2.76E-07  |
| GOTERM_BP_FAT | GO:0042330 ~ taxis | 11 | 2.76E-07  |
| GOTERM_BP_FAT | GO:0006955 ~ immune response | 20 | 7.66E-07  |
observed in the microarray study (Fig. 1C). As a result of these marked changes in the expression of \textit{Itm2a} and \textit{Clu}, we sought to elucidate their roles during differentiation in the myogenic program.

**Itm2a expression and myogenic differentiation**

A day point study of \textit{Itm2a} in normal C2C12 cells was performed to determine its function during myoblast differentiation. \textit{Itm2a} expression at mRNA and protein levels showed a progressive increase during transition from the proliferative (d 0) to the differentiation (d 4) stage, and a slight decrease at terminal differentiation (d 6) (Fig. 2A). Western blot analysis and immunocytochemistry revealed an increase in \textit{Itm2a} expression on d 4 vs. 0. Growth of \textit{Itm2a} knockdown cells (\textit{Itm2a}kd) under differentiation conditions for 4 d was monitored by checking myotube formation, suggesting a distinct role in the differentiation process (Fig. 2B). Confirmation of decrease in fusion indices on d 4 was performed by expression checking of scrambled vector transfected cells (\textit{Itm2awt}) and \textit{Itm2a}kd cells by qPCR and Western blot analysis (Fig. 2C). \textit{Itm2a} expression was found to be lower in \textit{Itm2a}kd cells. Furthermore, the expression of myogenic marker genes (\textit{MYOD}, \textit{MYOG}, and \textit{MYL2}) revealed that they were down-regulated at both the RNA and protein levels (Fig. 2D, E). These findings suggest that \textit{Itm2a} predominantly drives differentiation of myoblasts and is required to maintain muscle tone in the myogenic program.

**Clu expression in myogenic program**

Day point study of Clu expression in normal C2C12 cells by qPCR, Western blot analysis, and immunocytochemistry revealed a peak on d 4 (Fig. 3A). To determine Clu function in myoblast differentiation, we proceeded to check the effect of \textit{Clu}kd in C2C12 cells (Fig. 3B). \textit{Clu}kd was found to have a pronounced inhibitory effect on myotube formation as revealed by fusion indices on differentiation d4. Compared to \textit{Clu}wt, Western blot analysis showed Clu levels were lower in \textit{Clu}kd cells (Fig. 3C). Furthermore, qPCR, Western blot analysis, and immunocytochemistry

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**TABLE 3. Functional enrichment of DEGs and 50 related genes in the networks as reported by GeneMania**

| Category       | Description                                      | \(P\)   |
|----------------|--------------------------------------------------|---------|
| GO:0031012     | Extracellular matrix                             | 6.40E-17|
| GO:0051272     | Positive regulation of cellular component movement| 9.77E-10|
| GO:0030335     | Positive regulation of cell migration             | 1.37E-09|
| GO:2000147     | Positive regulation of cell motility              | 1.71E-09|
| GO:0005578     | Proteinaceous extracellular matrix                | 7.99E-09|
| GO:0050795     | Regulation of behavior                            | 2.15E-08|
| GO:2000027     | Regulation of organ morphogenesis                 | 2.69E-08|
| GO:0060070     | Canonical Wnt signaling pathway                   | 3.54E-08|
| GO:0060828     | Regulation of canonical Wnt signaling pathway     | 5.31E-08|
| GO:0060326     | Cell chemotaxis                                   | 1.64E-07|

FMODwt indicates cells transfected with scrambled vector. Means ± sd (\(n = 3\)).

**TABLE 4. List of top down- or up-regulated genes in FMODkd cells**

| Regulation | Gene   | Normalized fold difference | \(P\)  | GenBank accession no. | Description                                                                 |
|------------|--------|---------------------------|-------|-----------------------|-----------------------------------------------------------------------------|
| Down-regulated | Itm2a  | 0.02                      | 0.000396 | NM_008409            | Integral membrane protein 2A                                                |
|            | Fmod   | 0.10                      | 0.000436 | NM_021355            | Fibromodulin                                                                |
|            | Ncf4   | 0.10                      | 0.002020734 | NM_008677         | Neutrophil cytosolic factor 4                                               |
|            | Dpyd   | 0.15                      | 0.001461221 | NM_170778          | Dihydropyrimidine dehydrogenase                                             |
|            | Kif1a  | 0.19                      | 0.000978  | NM_008440            | Kinesin family member 1A, transcript variant 1                             |
|            | Str6   | 0.21                      | 0.001023538 | NM_001162476       | Stimulated by retinoic acid gene 6, transcript variant 1                   |
|            | Iglon5  | 0.23                      | 0.009962117 | NM_001164518       | IgLON family member 5                                                      |
|            | Aec2   | 0.23                      | 0.000356  | NM_009597            | Anisloride-sensitive cation channel 2, neuronal                             |
|            | Htr7   | 0.24                      | 0.000231  | NM_008315            | 5-Hydroxytryptamine (serotonin) receptor 7                                  |
| Up-regulated | Gdpd2  | 18.47                     | 0.00047   | NM_029608            | Glycerophosphodiesterase domain containing 2                               |
|            | Clu     | 11.35                     | 0.000177  | NM_013492            | Clusterin                                                                   |
|            | Aox3    | 9.23                      | 0.00059   | NM_009675            | Amine oxidase, copper containing 3                                          |
|            | Cyp2f2  | 8.30                      | 0.0043458 | NM_007817            | Cytochrome P450, family 2, subfamily f, polypeptide 2                       |
|            | Phex    | 7.17                      | 0.000726  | NM_011077            | Phosphate-regulating gene with homologies to endopeptidases on the X chromosome |
|            | Esm1    | 6.88                      | 0.044852  | NM_023612            | Endothelial cell–specific molecule 1                                        |
|            | Plac8   | 6.78                      | 0.0244181 | NM_199198            | Placenta-specific 8                                                        |
|            | Fbln7   | 6.39                      | 0.000691  | NM_024237            | Fibulin 7                                                                   |
for MYOD, MYOG, and MYL2 showed that their expression was also lower in Cluko cells (Fig. 3D, E).

**FMOD, Clu, and lipid accumulation during myogenic differentiation**

Lipid accumulation in CluBWT and Clukd C2C12 cells was assessed by Oil Red O staining and the expression of CD36 and PPARγ (adipogenic marker genes) by real-time RT-PCR. Less Oil Red O staining was observed in Clukd cells than in controls during myoblast differentiation (Fig. 4A). Furthermore, the expression of CD36 and PPARγ in Clukd cells gradually decreased as determined by qPCR, Western blot analysis, and immunocytochemistry (Fig. 4B). To investigate the effect of FMODkd on lipid accumulation, FMODBWT and FMODkd cells were subjected to Oil Red O staining during myoblast differentiation. Unlike that observed in Clukd cells, an increase in lipid accumulation was observed in FMODkd cells (Fig. 4C), and the mRNA and protein expression of CD36 and PPARγ in FMODkd cells were higher than in controls (Fig. 4D).

**In vivo study using CluKO mice**

Marked reductions in the expression of CD36 and PPARγ in Clukd C2C12 cells led us to perform functional studies on Cluko mice muscle tissues. CD36 and PPARγ expression in Cluko mice were lower than in controls, similar to what was observed in Clukd cells (Fig. 5A). Furthermore, decreases in the expression of CD36 and PPARγ were accompanied by decreases in the expression of myogenic marker proteins at the RNA and protein levels. Immunohistochemistry was...
performed to establish differences in the expression of adipogenic and myogenic marker gene expression between Clu<sub>wt</sub> and Clu<sub>kd</sub> muscle tissues. The results showed a similar decreasing trend for all proteins as that found by Western blot analysis (Fig. 5B). These results demonstrated that Clu plays a vital role in the myogenic program by regulating the expression of adipogenic and myogenic marker genes.

**Muscle disease and regeneration**

The effect of diabetes on muscle mass led us to assess the effect of an HFD on the expression of FMOD, Clu, and adipogenic markers in normal, obese (mice fed an HFD), and insulin-depleted C57BL/6 mice. Though FMOD expression showed a slight decrease in the muscle tissue of obese mice (Fig. 6A), it was up-regulated in diabetic C57BL/6 mice fed an HFD (Fig. 6B). Furthermore, the expression of Clu, CD36, and PPARγ were higher in obese mice and diabetic C57BL/6 mice fed an HFD. These results highlight the importance of FMOD and Clu homeostasis in the maintenance of myoblast function and muscle mass. Regenerative studies were performed 3 d after injecting CTX (100 μl of 10 μM) into the gastrocnemius muscle. Immunohistochemical studies revealed up-regulation of Itm2a and Clu in injected...
muscles (Fig. 7), indicating their importance in muscle repair.

**Network analysis of DEGs**

The 263 up-regulated and 119 down-regulated genes identified by FMO

The interaction network of DEGs revealed the highest node degree for 5 genes (mimcan, OGN; plasma protease C1 inhibitor, SERPING1; DPT; collagen, type I, α1 (COL1A1); ceruloplasmin, CP) (Fig. 8A). OGN exhibited the highest node degree and was considered as the hub in the network (Supplemental Table S3). OGN and its interacting partners are primarily involved in functions related to ECM, calcium ion binding, and adhesion (Supplemental Table S4). Protein–protein interaction network analysis of genes up- or down-regulated by ≥2-fold was performed using STRING to explore functional associations between DEGs (Supplemental Fig. S4A). Interestingly, network analysis revealed that

**Figure 3.** Clu expression and its knockdown. C2C12 cells were incubated with differentiation media for 0, 2, 4, or 6 d. A) Clu expression by qPCR, Western blot analysis, and immunocytochemistry. Clu shRNA-transfected cells were incubated with differentiation medium for 4 d. B) Myotube formation and fusion indices Cluwt and Clukd cells. C) Clu mRNA and protein expression by qPCR and Western blot analysis. D, E) MYOD, MYOG, and MYL2 expression at mRNA and protein levels by qPCR, Western blot analysis, and immunocytochemistry. Day 0 cell or Cluwt indicate controls, which were transfected with scrambled vector. Results are presented as means ± SD of 3 independent experiments. *P ≤ 0.05, **P ≤ 0.001.
FMOD exhibited direct interactions with 30 different proteins (Supplemental Fig. S4B and Supplemental Table S5).

Point studies on hub genes (Fig. 8B) showed that DPT expression gradually increased from d 0 to d 4, whereas OGN, SERPING1 and CP showed decreasing trends. Study of the hub genes in FMODkd cells showed increases in the expression of CP and SERPING1 and decreases in the expression of DPT and OGN. Decrease in the expression of MYOD and MYOG in DPTkd cells showed that DPT regulates the expression of myogenic marker genes (Fig. 8C).

Community analysis using the clustering method

Six functional clusters or modules were identified in the network using the greedy algorithm (GLay), and all 6 were subjected to functional analysis to identify enriched GO Consortium terms. The DAVID functional analysis tool was used to assign functional category to genes in each cluster and to detect over represented GO Consortium terms. A total of 70, 73, 12, and 1 statistically significant (P ≤ 0.05 and gene count ≥ 5) GO Consortium terms were found to be over represented in clusters 1, 2, 3, and 5. No enriched

Figure 4. Clu, FMOD, and lipid accumulation. Clu or FMOD knockdown cells were incubated with differentiation medium for 4 d. A) Oil Red O staining and staining intensities were measured in Clu kd cells. B) CD36 and PPARY expression by PCR, Western blot analysis, and immunocytochemistry in Clu kd cells. C) Oil Red O staining and staining intensity results. D) FMOD, CD36, and PPARY mRNA and protein expression as determined by qPCR, Western blot analysis, or immunocytochemistry. Clu wt and FMOD wt indicate controls, which were transfected with scrambled vector. Results are presented as means ± sd of 3 independent experiments. * P ≤ 0.05, ** P ≤ 0.001.
DISCUSSION

Population aging in developed and developing nations means that age-related muscle loss will become an issue of considerable concern because of its relation to disease progression. MSC enrichment in skeletal muscle confers strong regenerative potential on muscle (33, 34). MSC maintenance of muscle architecture during repair and postnatal growth helps in restoring muscle contractile function. Orchestrated by a series of transcription factors (35, 36), the expansion of quiescent cellular machinery dictates between proliferation and differentiation events in the myogenic program. Though FMOD expression was found to be significant in young muscle tissues compared to old muscle tissues (13, 37), no direct correlation of FMOD effect on muscle has been reported in in vivo models. However, indirect evidences of FMOD’s effect on ECM component assembly such as collagens and its involvement in wound healing is well reported in the literature (16, 38–40). In addition, its involvement in cellular reprogramming has also been recently reported by Zheng et al. (41). Furthermore, one study has indicated that myogenic differentiation and muscle generation of FMOD reprogramming cells were better than normal cells (42). To enhance our understanding of the regulatory mechanisms that govern the activation and progression of proliferating myogenic cells to syncytial myofibers, we performed microarray analysis of FMODkd myoblasts. High-throughput analysis was used to identify DEGs, and their functional enrichments were investigated using in silico approaches and validated experimentally by determining expressional fold changes of selected genes.

Microarray studies of FMODwt and FMODkd cells revealed crucial information regarding genes differentially expressed during the myogenic program. Use of in silico approaches helped us gain an insight into the core regulatory network that directs myogenic differentiation from multipotent MSCs. Interaction networks were constructed for DEGs (both up- and down-regulated) observed for FMODkd in C2C12 cells and to explore relationships among DEGs. In addition, node degrees were calculated for genes in the network. OGN was found to have the highest node degree, followed by SERPING1, DPT, COL1A1, and CP. Considering these to be main hub genes, their functional significances in skeletal muscle development was then explored.
Functional enrichment and pathway analysis of the DEGs revealed the importance of the processes related to ECM and inflammatory response. Statistically significant GO Consortium terms for down-regulated genes showed that they were enriched with genes related to ECM, whereas up-regulated genes were enriched with genes related to inflammatory response. Clustering analysis of DEGs and their functional annotation for GO Consortium term suggested ECM-related gene enrichment, particularly for the "cellular component" GO Consortium term. Cluster 1 was the largest cluster, with 102 nodes and 755 interactions, and was enriched in processes related to immune/inflammatory response in the extracellular region. In addition to ion binding and adhesion, cluster 2 was enriched with terms related to ECM, whereas cluster 3 represented processes related to cell differentiation. Cluster 5 was the smallest and consisted of only a single significant GO Consortium term (Supplemental Table S6). Interaction analysis of genes up- or down-regulated by ≥2-fold showed coexpression, neighborhood, gene co-occurrence, and gene fusion interactions between DEGs with respect to their association with FMOD. To obtain an insight into the regulatory mechanism of FMOD, we studied the expression pattern of Itm2a and Clu genes during differentiation.

Microarray analysis of FMOD knockdown cells showed that Itm2a was the top down-regulated gene. Itm2a is a type II integral membrane protein and has been reported to be a candidate marker for chondro-osteogenic differentiation (43). In the present study, progressive increases in the expression of Itm2a indicated its importance in the differentiation process. Although role of Itm2a in skeletal muscle involves enhancement of the cellular differentiation, little is known of the mechanism that regulates the myogenic program. Our study of Itm2a

Figure 6. FMOD or adipogenic marker expression in ND, HFD, and diabetic muscle tissues. FMOD, Clu, CD36, and PPARγ mRNA and protein expression were analyzed by qPCR and Western blot analysis in ND, HFD, and diabetic muscle tissues. A) FMOD, Clu, CD36, and PPARγ expression in ND and HFD C57BL/6 mouse muscle. B) FMOD, Clu, CD36, and PPARγ expression in ND and diabetic C57BL/6 muscle tissue. ND was used as control. Results are presented as means ± SD of 3 independent experiments (n = 3). *P ≤ 0.05, **P ≤ 0.001, ***P ≤ 0.0001.

Figure 7. Itm2a and Clu expression after CTX-induced muscle injury. Immunohistochemistry and Western blot results of CTX-injected muscles demonstrate involvement of Itm2a and Clu in muscle regeneration.
expression in FMOD\textsubscript{kd} cells revealed it to be down-regulated at the RNA and protein levels. Furthermore, knockdown of Itm2a in C2C12 cells showed down-regulation of the expression of myogenic marker genes, which confirmed its importance in the differentiation process of myogenesis. Namkoong et al. (44) reported a decrease in autophagic machinery through regulation of the PKA-CREB pathway. In the present study, Itm2a was found to regulate myogenesis by checking the expression of myogenic marker genes.

The majority of the genes up-regulated by FMOD\textsubscript{kd} were associated with adipogenesis. Clu (also known as apolipoprotein J) is a ubiquitous protein that is well expressed in almost all cells. Clu is a secreted glycoprotein with roles in lipid transport, tissue remodeling, apoptosis, and complement-mediated cell lysis, and it acts as an extracellular chaperone (45, 46). In the present study, we found that Clu regulated myogenic marker genes during the differentiation stage in C2C12 cells. In addition, the expression of Itm2a and Clu were up-regulated in CTX-injected C57BL/6 mice, suggesting their involvements in the regeneration process.

Advancements in high-throughput sequencing and systemic biology have established the involvement of MSCs in the myogenic program. In the present study (Fig. 9), we found the following: FMOD predominantly controls a wide range of myogenesis-related genes; FMOD regulation of Itm2a controls myoblast progression to the differentiation stage; control of myogenic and lipid marker...
genes by Clu suggests that it plays a dual role during myogenesis; control of Clu by FMOD maintains a balance between the myogenic and adipogenic fates of MSCs under normal and diseased conditions; and Itm2a and Clu play active roles in the muscle regeneration process and thus present a potential route to the development of interventions to combat muscle diseases. Taken together, our results suggest that FMOD constitutes a regulatory switch that determines myogenic or adipogenic fates of myoblasts.

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AUTHOR CONTRIBUTIONS

E. J. Lee and I. Choi conceived and designed the experiments; E. J. Lee, A. T. Jan, M. H. Baig, K. Ahmad, and A. Malik were involved in experimental operation; E. J. Lee, A. T. Jan, M. H. Baig, K. Ahmad, and I. Choi performed the analysis and interpreted data; E. J. Lee, A. T. Jan, and I. Choi drafted the article; G. Rabbani, T. Kim, I.-K. Lee, Y. H. Lee, and S.-Y. Park contributed reagents, materials, and/or analysis tools; and E. J. Lee and I. Choi were involved in obtaining funds for the study and critically reviewed the article.

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