Dynamic membrane proteome of adipogenic and myogenic precursors in skeletal muscle highlights EPHA2 may promote myogenic differentiation through ERK signaling

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ABSTRACT: The balance of myogenic and adipogenic differentiation is crucial for skeletal muscle homeostasis. Given the vital role of membrane proteins (MBPs) in cell signal perception, membrane proteomics was conducted to delineate mechanisms regulating differentiation of adipogenic and myogenic precursors in skeletal muscle. Adipogenic and myogenic precursors with divergent differentiation potential were isolated from the longissimus dorsi muscle of neonatal pigs by the preplate method. A total of 85 differentially expressed MBPs (\( P < 0.05 \) and fold change \( \geq 1.2 \) or \( \leq 0.83 \)) between 2 precursors were detected via isobaric tags for relative and absolute quantitation (iTRAQ) assay, including 67 up-regulated and 18 down-regulated in myogenic precursors. Functional enrichment analysis uncovered that myogenic and adipogenic precursors showed significant differences in cytoskeleton organization, syncytium formation, environmental information processing, and organismal systems. Furthermore, key MBPs in regulating cell differentiation were also characterized, including ITGB3, ITGAV, ITPR3, and EPHA2. Noteworthy, EPHA2 was required for myogenic differentiation, and it may promote myogenic differentiation through ERK signaling. Collectively, our study provided an insight into the distinct MBP profile between myogenic and adipogenic precursors in skeletal muscle and served as a solid basis for supporting the role of MBPs in regulating differentiation.

KEY WORDS: cell membrane proteins · adipogenesis · myogenesis

Skeletal muscle is vital for healthy life considering its function in movement and physical activity. Meanwhile, skeletal muscle is also a site where ectopic adipose tissue occurs (1). Recent studies have shown that myocytes and adipocytes within skeletal muscle are derived from common mesenchymal stem cells, which diverge into myogenic or adipogenic lineage during early embryogenesis (2). Myogenesis and adipogenesis within skeletal muscle proceed competitively in the same environment (3), and skeletal muscle integrity can be debilitated by the formation of intramuscular adipose tissue, typically in advanced cases of Duchenne muscular dystrophy (4) and sarcopenia (5). Thus, the differentiation balance between myogenic and adipogenic precursors is crucial for skeletal muscle homeostasis and considerably significant.

The differentiation of myogenic or adipogenic precursors is complicatedly regulated by many intrinsic and extrinsic factors, such as Wingless/Integrated (Wnt), Sonic hedgehog (Shh) and growth factors, which further activate growth factor–mediated pathways, including the MAPK pathway. As an ancient signaling pathway, Wnt signaling induces myogenic differentiation (6, 7) and inhibits adipogenesis (8). Similarly, Shh promotes differentiation of chicken adult myoblasts and mouse myogenic C2 cells (9). Compared with the well-studied Wnt and Shh signaling, both positive and negative roles of ERK1/2 in myogenic differentiation have been suggested. As a part of MAPK

0892-6638/19/0033-5495 © The Author(s) 2019. www.fasebj.org

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pathway, ERK1/2 is required for the fibroblast growth factor (FGF) 19 forced skeletal muscle mass (10), but in chick embryos, the activation of FGF-ERK signaling pathway diminished myogenic differentiation (11). Similarly, inhibition of ERK1/2 promoted the initiation of differentiation but formed small myotubes in C2C12 cells (12). Given the paradox in previous studies, modulation of myogenic and adipogenic differentiation merits further investigation.

Membrane proteins (MBPs) function as receptors for endogenous ligands, form channels or pores for low MW compounds and other nutrients, and act as recognition or adhesion molecules (13). A role for MBPs in regulating cell differentiation has also been delineated in recent years. For example, receptor tyrosine kinases have been recognized as crucial regulators of essential cellular processes, such as proliferation and differentiation (14). Existing data suggest that Ephrin (Eph) receptors, the largest family of receptor tyrosine kinases, function in myoblast migration into limbs (15), and the blockade of EPHB3 function leads to the enhanced differentiation of isolated myofiber cultures along with the repressed self-renewal (16). Similarly, impairment of EPHB1 function in satellite cells forces differentiation by removing the intracellular domain of this receptor (16). Nevertheless, expression profile of type-A and type-B Eph receptors is distinct during embryonic and postnatal myogenesis (16). Therefore, the role for Eph receptors in myogenic differentiation still remains elusive and requires further investigation. Furthermore, MBPs has been used to identify and isolate myogenic and adipogenic precursors in skeletal muscle. It is noteworthy that studies dealing with identification and isolation of precursors are confused and limited. For example, CD15 (17) and -34 (18) were used to isolate cells with adipogenic potential, but in human skeletal muscle, adipogenic cells were also observed in CD15 or -34 populations (19, 20). Compared with mice, studies coping with the isolation of progenitor cells in humans are limited (21), let alone other mammals. Although MBPs act as key players in cell signal perception and cell identification, relatively few membrane proteins are reported because of low recovery rates, protein hydrophobicity, and several other hurdles.

The pig is an appropriate model in many areas related with human disorders because of their anatomic and physiologic similarities to humans (22). For example, dystrophin-deficient pigs were generated to understand the pathophysiology of Duchenne muscular dystrophy (23). To uncover the distinct mechanism underlying the differentiation of adipogenic and myogenic precursors, adipogenic and myogenic precursors were isolated from the longissimus dorsi muscle of neonatal pigs in the present study and subjected to isobaric tags for relative and absolute quantitation (iTRAQ) assay to obtain a compendium of differentially expressed MBPs. EPB222 emerged from our study as a critical MBP promoting myogenic differentiation through ERK signaling in myogenic precursors.

**MATERIALS AND METHODS**

**Cell isolation and culture**

Adipogenic and myogenic precursors were isolated from the longissimus dorsi muscle of 3-d-old newborn Yorkshire pigs according to the protocol described in our previous study (24, 25) by adapting the previous protocol (26) with some modifications. Briefly, skeletal muscles were minced and digested in 0.17% protease (P8811; MilliporeSigma, Burlington, MA, USA) and 0.15% collagenase-type XI (C9407; MilliporeSigma) solution for 1 h, respectively. The dissociated cells were purified and cultured in growth medium (GM) on collagen-1-coated dishes at 37°C, 5% CO2. GM was supplemented with 10% fetal bovine serum, 2 mM glutamine, antibodies (100 U/ml of penicillin and 100 mg/ml of streptomycin), and 5 ng/ml basic FGF based on DMEM/F12 medium. After 2 h, nonadherent cells were transferred to another dish and further collected after 72-h adhering. The first (0–2 h) and second (2–74 h) sets of adherent cells were adipogenic and myogenic precursors, respectively.

ALW-II-H1-27 has been used as an EPB222 inhibitor that blocks the function of EPB222 (27, 28). To determine the role of EPB222 in myogenic differentiation, myogenic precursors were treated with ALW-II-H1-27 (0.5 μM; MedChem Express, HY-18007, Monmouth Junction, NJ, USA) for 12 h in GM followed by myogenic differentiation.

**Adipogenic differentiation and Oil Red O staining**

To induce adipogenic differentiation, cells were treated in adipogenic differentiation medium (DM): 10% (v/v) fetal bovine serum in DMEM, 1% heat-inactivated horse serum (160–90130; Thermo Fisher Scientific, Waltham, MA, USA) for 4 d. Adipogenic differentiation was characterized by the fusion of myoblasts into multinucleated myotubes.

**Myogenic differentiation and immunofluorescence**

Cells at 80–90% confluence were switched to myogenic DM that contained DMEM and 2% heat-inactivated horse serum (160–50130; Thermo Fisher Scientific, Waltham, MA, USA) for 4 d. Myogenic differentiation was characterized by the fusion of myoblasts into multinucleated myotubes.

After myogenic induction, cells were fixed with 4% paraformaldehyde solution for 2 h. After incubated with 60% isopropanol for 2 min, cells were stained with 1 ml of Oil Red O (O0625; MilliporeSigma) working solution for 10 min. Images were taken by inverted microscope (10 × 10).

**RNA preparation and quantitative RT-PCR**

Total RNA was isolated using HiPure Universal RNA Kit (R4130; Magen, Tel Aviv, Israel) according to the manufacturer’s instructions. cDNA synthesis was performed using PrimeScript RT reagent Kit with gDNA Eraser (RR047A; Takara Bio, Kusatsu, Japan) according to the manufacturer’s instructions. Sybr Green–based quantitative PCR was performed in a qTOWER 2.2 thermocycler (Analytik Jena, Jena, Germany), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal
control. The primers used for quantifying selected genes were listed in Table 1. Relative gene expression level was calculated by $2^{-\Delta\Delta Ct}$ method (29).

**Proliferation assay**

Adipogenic and myogenic precursors were plated into 96-well plates at a density of $8 \times 10^5$ cells. When they reached 70–80% confluency, cells were incubated with 5-ethynyl-2'-deoxyuridine (EdU) for 2 h before staining. Cell proliferation was detected using Cell-Light EdU Cell Proliferation Detection Kit (C10310; RiboBio, Guangzhou, China) following the manufacturer’s protocol. Percentage of proliferative cells was determined by quantification of EdU-positive cells under Olympus fluorescent microscope ($10 \times 10$).

**MBPs extraction, quantitation, and validation**

Undifferentiated adipogenic and myogenic precursors (derived from the 4 pigs) were washed with ice-cold PBS then scraped with PBS containing protease inhibitor and centrifuged at 300 g for 5 min at 4°C and frozen at ~80°C for further usage. MBPs were extracted using Mem-PER Plus Membrane Protein Extraction Kit (89842; Thermo Fisher Scientific) according to the manufacturer’s instruction. Supernatant-containing cytosolic proteins were obtained after permeabilization and centrifugation for 15 min at 4°C and frozen at ~80°C for further usage. MBPs extraction was performed by Western blot. GAPDH was used as a positive control of cytosolic proteins, whereas N-cadherin was used as a positive control of MBPs.

**MBPs digestion and iTRAQ labeling**

An aliquot of 200 µg MBPs of both myogenic and adipogenic precursors isolated from 4 neonatal pig samples was reduced, alkylated, and digested with trypsin according to the manufacturer’s guidelines (Applied Biosystems, Foster City, CA, USA). Protein peptides (100 µg) from each sample were labeled using the iTRAQ Reagent-8Plex Multiplex Kit (4390812; AB Sciex, Framingham, MA, USA) as follows: Myogenic1, 113; Myogenic2, 117; Myogenic3, 119; Myogenic4, 116; Adipogenic1, 117; Adipogenic2, 118; Adipogenic3, 119; Adipogenic4, 121. After incubation with iTRAQ labeling reagents at room temperature for 1 h, a 100-µl aliquot of water was added to stop the labeling reaction. After label efficiency confirmation, differentially labeled peptides were mixed and vacuum dried.

**High pH–reversed-phase liquid chromatography fractionation and liquid chromatography–mass spectrometry/mass spectrometry analysis**

The mixed peptide samples with 8 labels were fractionated using a reversed-phase liquid chromatography system (1260 infinity II; Agilent Technologies, Santa Clara, CA, USA) with a C18 column (xBridge peptide BEH 130 C18 column; Waters, Milford, MA, USA). The mobile phase consisted of 2% ACN and 98% H2O (pH 8.0) as follows: Myogenic1, 113; Myogenic2, 117; Myogenic3, 119; Myogenic4, 116; Adipogenic1, 117; Adipogenic2, 118; Adipogenic3, 119; Adipogenic4, 121. After incubation with iTRAQ labeling reagents at room temperature for 1 h, a 100-µl aliquot of water was added to stop the labeling reaction. After label efficiency confirmation, differentially labeled peptides were mixed and vacuum dried.

**TABLE 1. Primer sequences used in quantitative RT-PCR analysis**

| Gene | Forward | Reverse | Product Size (bp) |
|------|---------|---------|------------------|
| PPARG | GAGGCGGATCTTGGACAGGAA | GCCACCTTCTTTGCTGCTG | 135 |
| CEBPA | GCGGACAGACACACATTAGA | CCCCACAAAGGAAGAAACAG | 71 |
| LPL | AAATATTGGCCATCGAGAACC | TTTGACTCTATAGCCAAAGTTCG | 297 |
| MYOD1 | GGTGGATGCTGGAAGTCCTTCGTGTC | CTCCTACACCTGCTGCCTCCAG | 106 |
| Myogenin | CTCGTCACAGTCGGACCTAC | GGTCCATCTGAGGAAGCCAG | 105 |
| Myomarker | CACGACGAGAAAGAAGAAG | CGCACTGAGGTCGCTGAG | 154 |
| EPHA2 | ATTCGCGGACATCTTGATAGCTC | CCCGCAAGGAAGGTGTTCTTAC | 194 |
| IL6ST | TGCAGTCATCTTCTGAGGCAAG | TACCTGCTTCTGAGCACTCTG | 117 |
| TMBIM1 | TGTGCACACGGCATTGGCTC | TCGGCTTCTGAGAACGAGAC | 107 |
| ATP1B1 | GGCAGCCGCTGGAGAGAAG | GTGAAGCTCGACCAAGGAG | 150 |
| ERM1 | GCGGCGGGTGTGCTGCTCTG | GCTTACACTGCTGGCCGAC | 142 |
| ITAG | CACAGGGCTGCTGCTGCTGCTG | GGCTGACTGTTGGCAGTAG | 83 |
| MTFX1 | GAGGCACAGGAGCCATCGAG | TGAATGAGCAATCGGACG | 107 |
| ANT1R1 | GTAGAAGGCGGGCTGCTGC | AGGACGAGGAAAGAAGAAG | 113 |
| S100A10 | AAGACCGGCTGCTGCTGCTG | ATGGATGGATGGATGGATGAC | 113 |
| ITPR3 | TGTCGATGCGGCTGCTGCTGCTG | GGGCTGAGCCTGCTGCTGCT | 199 |
| GGCG | GTGCCTCACGACGGCGTTCTG | GTTTTGTAGATGGATGGATGAC | 168 |
| ITGB3 | AGCCACAGCGGCTGCTGCTG | CTGTGCTTGGATGGATGGATGAC | 142 |
| VCAM1 | AGGACGAGGAAAGAAGAAG | GACGAGGAGCGGAGGAGG | 185 |
| SEMA3C | TGGAGGGGCTGCTGCTGCTG | AAGACGACGCGGAGGAGG | 183 |
| DERL1 | GCCGCTGCTGCTGCTGCTG | GAGGAGCTGCTGCTGCTG | 107 |
| GALNT1 | ATGGCAACACAGGGGCTGCTG | CACGAGGAGCGGAGGAGG | 183 |
| TTYH3 | CTGTCGAGGAGCTGCTGCTG | CAGAGCTGCTGCTGCTG | 156 |
| JAM3 | ATGGAGGAGCGGAGGAGG | AGGAGCTGCTGCTGCTG | 120 |
| JAM5 | ATGGAGGAGCGGAGGAGG | AGGAGCTGCTGCTGCTG | 123 |
| FADS1 | TGTCGATGCGGCTGCTGCTG | TGAGCTGCTGCTGCTGCTG | 109 |
| TMEM209 | GGCAGCGCTGCTGCTGCTG | TCGAGAGAACTGCTGCTGCTG | 154 |
| GAPDH | TGGGAGGAGGGGCTGCTGCTG | CCTGAGGAGAACTGCTGCTG | 219 |
The fractionation was performed for 60 min at a flow rate of 0.7 ml/min with the following gradients: 0% B for 3 min, 0–5% B for 2 min, 5–35% B for 40 min, 35–90% B for 15 min.

The reconstituted peptides were analyzed with the Q-Exactive High Mass Spectrometer (Thermo Fisher Scientific) coupled with a nano high-performance liquid chromatography (Easy Nlc; Thermo Fisher Scientific) system. The peptides were loaded onto a C18-reversed phase column (C18 3 μm 100 μm x 20 mm) and separated on an analytical column (C18 1.9 μm 150 μm x 120 mm; Thermo Fisher Scientific) using mobile phase A: 0.1% formic acid/H2O and B: 0.1% formic acid/ACN at a flow rate of 0.6 μl/min, using a 78-min gradient: 16 min from 5 to 10% B, 35 min from 10 to 22% B, 20 min to 30% B, 1 min to 95% B, and maintained at 95% B for 6 min. A full mass spectrometry (MS) scan (300–1400 m/z) was acquired in the positive ion mode at a resolution of 120,000, an automatic gain control target value of 3 x 10^6, a maximum peptide ion accumulation time of 80 ms, number of scan ranges of 1, and dynamic exclusion of 12.0 s. Information for peptides and peptide fragments were collected as follows: 10 fragment files collected after every full scan (MS2 scan), higher resolution of 1.6 m/z, full scan at a resolution of 15,000 (at 100 m/z), microscans of 1, maximum ion accumulation time of 45 ms, and normalized collision energy of 30 eV.

**Protein identification and quantification**

Protein identification and quantification for iTRAQ experiments were conducted using Proteome Discoverer 1.4 (v.1.4.0.288; Thermo Fisher Scientific) with a false discovery rate <1% and expected cutoff or ion score <0.05 (with 95% confidence). The liquid chromatography–MS/MS data were searched against the UniProtKB (sus scrofa; https://www.uniprot.org/taxonomy/9823), and at least 1 unique peptide was considered for protein identification. MBPs were annotated in the control target value of 3 x 10^6, a maximum peptide ion accumulation time of 80 ms, number of scan ranges of 1, and dynamic exclusion of 12.0 s. Information for peptides and peptide fragments were collected as follows: 10 fragment files collected after every full scan (MS2 scan), higher collision energy dissociation fragmentation, an isolation window of 1.6 m/z, full scan at a resolution of 15,000 (at 100 m/z), micro-scans of 1, maximum ion accumulation time of 45 ms, and normalized collision energy of 30 eV.

**Bioinformatics and pathway analysis**

Blast2GO (BioBam Bioinformatics, Valencia, Spain) software was used to perform gene ontology (GO) enrichment for differentially expressed MBPs to catalog the molecular functions, cellular components, and biologic processes. A value of P < 0.05 was considered the criteria for significant GO enrichment. Revigo (http://revigo.irb.hr) and Cytoscape (v.3.5.1; Cytoscape Consortium, San Diego, CA, USA) were used to summarize and visualize GO enrichment results to reduce redundancy. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was also performed to annotate pathways based on differentially expressed MBPs; KEGG pathways with values of P < 0.05 were subjected to further analysis.

**Small interfering RNA transfection of myogenic precursors**

Myogenic precursors were plated on collagen I–coated 6-well plates (10^5 cells per well) and transfected with 100 nM scrambled small interfering RNA (siRNA) or EPHA2 siRNA (GenePharm, Pallini, Greece) using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. After transfection of 72 h, cells were collected or stimulated for myogenic differentiation. EPHA2 siRNA No. 1: sense 5'-CCUGCCUCGCCGGGAUUCCU-3', antisense 5'-AAUACUGCGCCGAAGACGTT-3'; EPHA2 siRNA No. 2: sense 5'-CCGGAGUGUCACGUA CAATT-3', antisense 5'-UGUACGUGACAGACGCGTGTT-3'; EPHA2 siRNA No. 3: sense 5'-CCACGGAAGGACUUAGCAATT-3', antisense 5'-UUCUCGAACGUCCUAGTT-3' (sense strand) and 5'-ACGUCAACGUCCUAGATT-3' (antisense strand).

**Western blotting**

Total cell protein lysates were extracted with cell lysis buffer that contained protease inhibitor and protein phosphatase inhibitor cocktail. The relative protein expression of randomly selected MBPs, including VCAM1, EPHA2, NUP210, and SLC9A7, was analyzed with Western blotting to verify the results of proteomics. Approximately 60–100 μg of total cell lysates were loaded per well, and GAPDH was run as a loading control.

**Statistical analysis**

Data organization, scientific graphing, and statistical analyses were performed using Microsoft Excel (Redmond, WA, USA), GraphPad Prism (v.6; La Jolla, CA, USA), and SAS (v.9.2; SAS Institute, Cary, NC, USA). Data were presented as means ± SEM. For comparisons between adipogenic and myogenic precursors and comparison between cells in GM and DM, statistical differences were determined by the paired 2-tailed Student’s t test. Meanwhile, statistical differences in other comparisons were determined by the unpaired 2-tailed Student’s t test. A value of P < 0.05 was considered significant.

**RESULTS**

**Characterization of adipogenic and myogenic precursors**

Skeletal muscle-derived adipogenic and myogenic precursors were harvested via preplate technique based on their adhesion characteristics to collagen I–coated dishes. Upon adipogenic induction, adipogenic precursors differentiated into mature adipocytes. Accumulating lipids (as shown by Oil Red O staining) (Fig. 1A) and high mRNA expression levels of peroxisome proliferator-activated receptor γ (PPARG; P < 0.01) and lipoprotein lipase (LPL; P < 0.01) were observed in adipogenic precursors, although the expression level of CCAAT-enhancer binding protein α (CEBPA) was not significantly different after adipogenic differentiation (Fig. 1B).
Membrane proteome analysis of adipogenic and myogenic precursors

To gain insight into the molecular mechanisms associated with adipogenic and myogenic differentiation, we focused on the MBPs as key sensor board controlling cell differentiation. Validation of MBPs extraction was achieved by SDS-PAGE, and the patterns of each sample line were quite similar (Supplemental Fig. S2A). Western blot was also conducted for the same sample (Supplemental Fig. S2B). We observed that the expression level of N-cadherin was much higher in MBPs than the supernatant containing cytosolic proteins despite the adipogenic or myogenic precursors, whereas the expression level of GAPDH was much lower in MBPs.

As shown in Fig. 2A, a total of 2891 proteins was identified in myogenic and adipogenic precursors by the MS analysis with a false discovery rate <1% at both protein and peptide levels. Of the total identifications, 70.3% were based on at least 2 unique peptides with an average of 5 unique peptides per protein (median = 3). Of the total quantified proteins (2691), 296 proteins with P < 0.05 and FC ≥ 1.20 or ≤ 0.83 between myogenic and adipogenic precursors were taken as differentially expressed proteins (DEPs), with 190 proteins being up-regulated (red nodes shown in Fig. 2B) and 106 being down-regulated (green nodes shown in Fig. 2B) in myogenic precursors.

A total of 762 proteins were defined as MBPs based on the TMHMM model (Fig. 2A). Prediction by TMHMM showed that the majority of these 762 MBPs were single-pass transmembrane proteins (Fig. 2C). Eighty-five MBPs were regarded as differentially expressed based on the criteria of DEPs, with 67 MBPs being up-regulated and 18 being down-regulated in myogenic precursors (Fig. 2D, E). The expression levels of these 85 MBPs in all samples were displayed in the heat map generated by hierarchical cluster analysis and showed a clear difference in adipogenic and myogenic precursors (Fig. 2D). More details of 85 differentially expressed MBPs were listed in Tables 2 and 3.

To validate the results of proteome analysis, the relative abundance of 5 MBPs, including VCAM1 (FC = 2.00), EPHA2 (FC = 1.67), NUP210 (FC = 0.48), TMEM209 (FC = 0.66), and SLC39A7 (FC = 0.69), were selected to be determined by employing Western blot. As shown in Supplemental Fig. S3, despite VCAM1, the trend of 4 other MBPs was consistent with that in proteome analysis, indicating high reliability of our proteomic workflow.

Figure 1. Differentiation of adipogenic and myogenic precursors isolated from the longissimus dorsi muscle of neonatal pigs. A) Oil Red O staining and immunofluorescence of adipogenic and myogenic precursors following 9 d of adipogenic induction and 4 d of myogenic induction, respectively. Oil Red O (red), Myosin (red), and DAPI (blue). B) Quantitative RT-PCR for expression level of adipogenic differentiation–related genes (PPARG, CEBPA, LPL) and myogenic differentiation–related genes (MYOD1, Myogenin, Myomarker) following adipogenic or myogenic induction. n = 4. Data are presented as means ± SEM. A paired 2-tailed Student’s t test was used. Adi, adipogenic precursors; Myo, myogenic precursors. Scale bars, 100 μm. *P < 0.05, **P < 0.01 compared with adipogenic precursors.
We next performed GO annotation and enrichment analysis on differentially expressed MBPs and identified 108 significantly enriched GO terms ($P < 0.05$), including 28 cellular component terms, 14 molecular function terms, and 66 biologic process terms. Significant GO terms were processed via ReviGO and represented using the Interactive Graph function to group GO terms (Fig. 3). As shown in Fig. 3A, ReviGO analysis of cellular component terms produced a cluster related to plasma membrane (surrounded by a blue dotted box), such as “plasma membrane part” and “plasma membrane receptor complex.” In addition to the large subgroup associated with plasma membrane, cellular component terms like “vesicle,” “actin-based cell projection,” “extracellular region,” and “cell junction” were also significantly enriched. As for molecular function, 7 GO terms were significantly enriched, including “transmembrane signaling receptor
| Accession | Gene Name     | Description                                                                 | FC    | TMHMM |
|-----------|---------------|-----------------------------------------------------------------------------|-------|-------|
| Q3ZDR4    | CD70          | CD70 antigen                                                                 | 3.44  | 1     |
| F1RW1     | TRPA1         | Transient receptor potential cation channel, subfamily A, member 1          | 2.20  | 5     |
| F1SC45    | ENTPD1        | Ectonucleoside triphosphate diphosphohydrolase 1                            | 2.15  | 2     |
| F1S567    | VCAM1         | Vascular cell adhesion protein                                              | 2.00  | 1     |
| F1SEQ7    | FAM213A       | Redox-regulatory protein FAM213A                                            | 1.96  | 1     |
| F1RT62    | ITGA3         | Integrin α-3                                                                | 1.92  | 1     |
| P79388    | FOLR2         | Folate binding protein                                                       | 1.76  | 0     |
| F1RYT3    | SCARB2        | Scavenger receptor class B member 2                                          | 1.73  | 2     |
| F1SU72    | EPHA2         | Ephrin type-A receptor                                                       | 1.67  | 1     |
| F1LT41    | TMEM234       | Transmembrane protein 234                                                   | 1.61  | 3     |
| F1SL11    | IL6ST         | IL-6 receptor subunit β                                                     | 1.61  | 1     |
| Q4U1U4    | CTS5A         | Cathepsin D                                                                 | 1.60  | 1     |
| F1RQV6    | TMEM63B       | Transmembrane protein 63B                                                   | 1.58  | 11    |
| I3LE23    | LSAMP         | Limbic system associated membrane protein precursor                          | 1.57  | 1     |
| F1SP5M    | ANTXR1        | Anthrax toxin receptor 1                                                     | 1.56  | 1     |
| F1RN4P    | P2RX4         | Purinergic receptor P2X, ligand-gated ion channel 4                          | 1.56  | 2     |
| A0A088RT16| LNPEP         | Leucyl and cystyl aminopeptidase                                             | 1.54  | 1     |
| K9H4M9    | COLEC12       | Collectin subfamily member 12                                                | 1.54  | 1     |
| I3LS7     | VASN          | Vasorin                                                                     | 1.51  | 1     |
| K9H6H1    | SEMA3C        | Semaphorin-3C                                                                | 1.51  | 1     |
| F1S1H3    | ENTPD5        | Ectonucleoside triphosphate diphosphohydrolase 5                            | 1.51  | 2     |
| K9H6F2    | STOM_7         | Erythrocyte band 7 integral membrane protein isoform A                      | 1.50  | 1     |
| P2Z5M2    | S100A10       | Protein S100-A10                                                             | 1.48  | 1     |
| F1SJ65    | S1DT2         | SID1 transmembrane family, member 2                                          | 1.45  | 9     |
| I3LSW9    | TMBM1         | Protein ligand 3                                                            | 1.44  | 7     |
| A0A088RZM1| DERL1         | Derlin                                                                       | 1.43  | 5     |
| F1SA16    | GALNT1        | Polypeptide N-acetylglactosaminyltransferase                                 | 1.42  | 1     |
| I3LL68    | MXR2A         | Matrix remodeling associated 8                                               | 1.41  | 1     |
| P05027    | ATP1B1        | Sodium/potassium-transporting atpase subunit β-1                           | 1.41  | 1     |
| A0A088RZR8| ANPEP         | Alanyl (Membrane) aminopeptidase                                             | 1.39  | 1     |
| F1ZRZ0    | ITPR3         | Inositol 1,4,5-trisphosphate receptor 3                                      | 1.38  | 7     |
| K7GRK4    | SLC2A13       | Solute carrier family 2 member 13                                            | 1.37  | 4     |
| F1R7B8    | TTYH3         | Protein tewty homolog                                                        | 1.36  | 5     |
| F1SDT7    | ALDH3A2       | Aldehyde dehydrogenase 3 family member A2                                   | 1.36  | 1     |
| I3LT23    | B3GAT3        | Galactosylgalactosylxylosylprotein 3-β-glucuronosyltransferase              | 1.35  | 1     |
| Q95JH1    | ITGB3         | Integrin β 3                                                                | 1.35  | 2     |
| F1SMU0    | ERMP1         | Endoplasmic reticulum metallopeptidase 1                                    | 1.34  | 8     |
| I3LRE0    | BCAP29        | B-cell receptor associated protein 29                                       | 1.34  | 3     |
| A0A088RT32| TAP1          | Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)                  | 1.32  | 7     |
| F1SF40    | MAN1A1        | Alpha-1,2-Mannosidase                                                        | 1.32  | 2     |
| F1S2A1    | UGT1A6        | UDP-glucuronosyltransferase                                                   | 1.31  | 1     |
| F1S2C82   | MYOF          | Myoferlin isomer X4                                                         | 1.31  | 1     |
| K7GL5     | SLC4A11       | Solute carrier family 44 member 1                                           | 1.31  | 9     |
| I3LT19    | STX12         | Syntaxin 12                                                                  | 1.30  | 1     |
| F1SA10    | PTGFRN        | Prostaglandin F2 receptor inhibitor                                           | 1.30  | 1     |
| F1SA12    | TMEM205       | Transmembrane protein 205                                                   | 1.30  | 3     |
| F1RUC0    | LAMP2         | Lysosomal associated membrane protein 2                                     | 1.29  | 1     |
| F1RTA1    | ABC3C         | ATP-binding cassette subfamily C member 3                                   | 1.29  | 14    |
| B8XJS2    | ERLIN2        | ER lipid raft-associated 2 isoform 2                                        | 1.28  | 0     |
| M3TY5A    | KIAA0319L     | KIAA0319-like protein                                                        | 1.28  | 2     |
| Q9TWS3    | SLA-3         | Leukocyte antigen 3/2 (SLA-3/2)                                              | 1.27  | 1     |
| F1S268    | CREG1         | Cellular repressor of E1A stimulated genes 1                                | 1.27  | 1     |
| F1S846    | ILVBL         | ilvB acetylactate synthase like                                              | 1.26  | 1     |
| B1AP2     | SLA-2         | MHC class I antigen                                                          | 1.26  | 1     |
| I3LS60    | CARKD         | ATP-dependent (S)-NAD(P)H-hydrate dehydratase                                | 1.26  | 1     |
| A2RRQ8    | ITGAV         | Alpha v integrin subunit variant                                             | 1.25  | 1     |
| M3UZ26    | ATP11A        | Phospholipid-transporting atpase                                             | 1.25  | 7     |
| F1S4J4    | ABHD6         | Abhydrolase domain containing 6                                             | 1.25  | 1     |
| F1SPX9    | TMTC3         | Transmembrane and tetraticopeptide repeat containing 3                     | 1.24  | 9     |
| F1SK21    | SLC25A20      | Solute carrier family 25 member 20                                          | 1.24  | 5     |
| I3LB10    | GCCX          | Gamma-glutamyl carboxylase                                                   | 1.23  | 5     |
| F1RL36    | SLC25A16      | Solute carrier family 25 member 46                                          | 1.23  | 0     |
| M3VH66    | ANO10         | Anoctamin 10                                                                | 1.23  | 8     |

(continued on next page)
activity,” “protein complex binding,” 3 terms associated with transporter activity (“organic cation transmembrane transporter activity,” “ammonium transmembrane transporter activity,” and “substrate-specific transporter activity”), and 2 terms related to transducer activity (“signal transducer activity” and “molecular transducer activity”) (Fig. 3B). It was noteworthy that “transmembrane signaling receptor activity” was the most significantly enriched term (P = 0.003), implying the key role of MBPs in signal transduction.

Proteins assigned to “regulation of cytoskeleton organization” (P = 0.003), “regulation of cell adhesion” (P = 0.004), “organic cation transport” (P = 0.008), “syncytium formation by plasma membrane fusion” (P = 0.008), and “syncytium formation” (P = 0.008) were the top 5 most significantly enriched biologic process terms (Fig. 3C). MBPs involved in these terms were listed in Supplemental Table S2. Furthermore, 4 biologic process terms related to development were enriched, including “positive regulation of cell development,” “blood vessel development,” “epidermis development,” and “skin development.” Meanwhile, 2 terms were associated with the migration and proliferation of leukocyte, such as “regulation of leukocyte migration” and “regulation of leukocyte proliferation” (Fig. 3C). Moreover, 5 specific subclasses were also enriched, including “developmental process,” “response to stimulus,” “immune system process,” “biological regulation,” and “biological adhesion” (Fig. 3C).

### KEGG analysis of differentially expressed MBPs

We identified 9 KEGG pathways (P < 0.05) enriched with differentially expressed MBPs. Among them, 3 pathways were related to organismal systems, including “NOD-like receptor signaling pathway,” “Thyroid hormone signaling pathway,” and “Platelet activation” (Fig. 4A), and 2 pathways were related to environmental information processing, including “PI3K-AKT signaling pathway” and “Rap1 signaling pathway” (Fig. 4B). As displayed by the heat map, MBPs involved in these 5 pathways, such as ITGB3, ANTXR1, ITPR3, ITGAV, and EPHA2, were all up-regulated in myogenic precursors (Fig. 4A, B). In addition, “Apoptosis” was related to cellular processes (Fig. 4C).

### TABLE 2. (continued)

| Accession | Gene Name | Description | FC  | TMHMM*a |
|-----------|-----------|-------------|-----|----------|
| F1RQH9    | CD109     | CD109 molecule | 1.22 | 1        |
| F1SD42    | ADGRE5    | Adhesion GPCR E5 | 1.21 | 7        |
| A0A0B8S096| DAG1      | Dystroglycan 1 (Dystrophin-associated glycoprotein 1) | 1.20 | 1        |
| F1RY7     | ZDHHIC17  | Zinc finger DHHC-type containing 17 | 1.20 | 4        |

*aTMHMM, the number of predicted transmembrane helices.

### TABLE 3. Down-regulated membrane proteins in myogenic precursors compared with adipogenic precursors

| Accession | Gene Name | Description | FC  | TMHMM*a |
|-----------|-----------|-------------|-----|----------|
| F1SPN1    | FKBP11    | Peptidylprolyl isomerase | 0.83 | 1        |
| F1S6C7    | JAM3      | Junctional adhesion molecule 3 | 0.82 | 2        |
| K7GRK0    | SSR4      | Signal sequence receptor subunit 4 | 0.82 | 0        |
| F1SRK2    | ATG2      | Atlastin gtpase 2 | 0.82 | 2        |
| Q27HK4    | MTX1      | Metaxin-1 | 0.79 | 1        |
| K1XTE7     | FAD1      | Fatty acid desaturase 1 | 0.77 | 3        |
| F1RY7     | SRR1      | Signal sequence receptor subunit 1 | 0.75 | 1        |
| F1RM08    | SLC1A5    | Amino acid transporter (solute carrier family 1 member 5) | 0.74 | 9        |
| E7CX1     | VLDLR     | Very LDL receptor | 0.73 | 1        |
| I3LHM1    | GRIA2     | Glutamate receptor, ionotropic, AMPA2 (α2) | 0.71 | 3        |
| Q19K10    | RHCG      | Ammonium transporter Rh type C | 0.70 | 12       |
| I3LHY0    | LOC100622780 | Lamina-associated polypeptide 2 isoform ×1 | 0.70 | 1        |
| Q6GQH9    | SLC25A6   | ADP/ATP translocase 3 | 0.69 | 2        |
| I3LQM7    | SLC38A5   | Sodium-coupled neutral amino acid transporter 5 | 0.69 | 11       |
| A5D9P2    | SLC39A7   | Solute carrier family 39 (Zinc transporter), member 7 | 0.69 | 7        |
| F1SR8     | TMEM209   | Transmembrane protein 209 | 0.66 | 2        |
| F1S226    | VMP1      | Vacuole membrane protein 1 | 0.63 | 6        |
| F1RP4     | NUP210    | Nucleoporin 210 | 0.48 | 2        |

*aTMHMM, the number of predicted transmembrane helices.
this category, MBPs, such as ITPR3 and CTSD, were all upregulated in myogenic precursors. Also, in KEGG analysis, 3 enriched pathways were related to human diseases, including “Proteoglycans in cancer,” “Dilated cardiomyopathy (DCM),” and “Hypertrophic cardiomyopathy (HCM)” (Fig. 4D). Two up-regulated MBPs were involved in this category, including ITGB3 and ITGAV. It was worth noting that ANTXR1 and CTSD were respectively involved in 1 specific pathway, whereas ITGB3, ITGAV, ITPR3, and EPHA2 acted as hubs to connect at least 2 pathways (Fig. 5). In detail, ITGB3 linked 7 pathways, including “PI3K-AKT signaling pathway,” “Rap1 signaling pathway,” “Thyroid hormone signaling pathway,” and “Platelet activation” and 3 terms associated with human diseases. Following 5 pathways and 4 pathways connected, respectively, by ITGAV and ITPR3, EPHA2 acted as a hub to connect “PI3K-AKT signaling pathway” and “Rap1 signaling pathway.”

Role of MBPs in myogenic differentiation

Pax7+ cells are satellite cells that are required for skeletal muscle regeneration (32, 33), whereas Twist2+ cells are progenitors that contribute specifically to type IIb/x myofibers during muscle regeneration (34). As previously described (34), Twist2+ cells were sorted by flow cytometry and fluorescence-activated cell sorting analysis from the skeletal muscle of Twist2-CreERT2; R26-tdTO mice 10 d post-TMX treatment at 8 wk of age, whereas
Pax7+ cells were sorted from Pax7-CreERT2; R26-tdTO mice. Liu et al. (34) compared the transcriptome pattern of Twist2+ and Pax7+ cells during the myogenic differentiation (pre- and postmyogenic differentiation) in order to figure out the gene expression pattern of 2 kinds of cells and clarify their contributions to adult muscle development and regeneration.

Accordingly, we checked the mRNA expression pattern of these 85 MBPs during myogenic differentiation of Pax7+ and Twist2+ cells. As shown in Fig. 6A, a total of 22 MBPs whose transcripts were identified as differentially expressed genes, and among them, mRNA levels of 8 differentially expressed MBPs (IL6ST, SEMA3C, TMBIM1, DERL1, ATP1B1, GGCX, DAG1, and JAM3) were up-regulated during myogenic differentiation of Pax7+ and Twist2+ cells, whereas 14 MBPs were down-regulated, including VCAM1, EPHA2, ANTXR1, S100A10, GALNT1, ITPR3, TTYH3, ERMP1, PTGFRN, ITGAV, ITGB3, TMEM209, FADS1, and MTX1. Remarkably, the mRNA expression of EPHA2 was dramatically changed, which was decreased 12.61-fold and 25.47-fold during myogenic differentiation of Pax7+ and Twist2+ cells, respectively. In turn, we measured mRNA expression levels of these 22 MBPs of myogenic precursors.

**Figure 4.** KEGG analysis of differentially expressed membrane proteins (MBPs). KEGG pathways (P < 0.05) were classified into 4 groups: Organismal Systems (A), Environmental Information Processing (B), Cellular Processes (C), and Human Diseases (D). Specific KEGG pathways included in each group were listed below. Expression changes of differentially expressed MBPs involved in each group were exhibited by heat maps. Adi, adipogenic precursors; Myo, myogenic precursors.

**Figure 5.** Cluster of KEGG pathways and differentially expressed MBPs between adipogenic and myogenic precursors. KEGG pathways (P < 0.05) were based on differentially expressed MBPs. The green diamonds represent KEGG pathways, and red ellipses represent differentially expressed MBPs. Networks were visualized by Cytoscape (v.3.5.1).
derived from the skeletal muscle of neonatal pigs and found that mRNA expression level of IL6ST, TMBIM1, and ITGAV was significantly up-regulated during myogenic differentiation ($P < 0.05$, Fig. 6B), and conversely, levels of EPHA2, ATP1B1, ERMP1, and MTX1 were strongly down-regulated ($P < 0.01$, Fig. 6B). Importantly, the expression pattern of EPHA2, IL6ST, TMBIM1, ERMP1, and MTX1 during myogenic differentiation was similar in myogenic precursors derived from both mice and pigs. Meanwhile, the expression of ANTXR1, S100A10, ITPR3, GGCX, and ITGB3 tended to be altered during myogenic differentiation ($P < 0.1$, Fig. 6C), and the expression pattern of S100A10, ITPR3, and GGCX was similar during differentiation of myogenic precursors from mice and pigs. At the same time, 10 other MBPs were changeless during myogenic differentiation (Fig. 6D).

**The key role of EPHA2 in myogenic differentiation**

Notably, combining with RNA sequencing data previously obtained from mice, we found that EPHA2 mRNA expression was dramatically altered during myogenic differentiation. Furthermore, EPHA2 had the largest variation ($FC = 1.67$) between myogenic and adipogenic...
precursors among the 4 hub proteins (EPHA2, ITGB3, ITGAV, and ITPR3). Therefore, we focused on the role of EPHA2 in myogenic differentiation. After ALW-II-41-27 (EPHA2 inhibitor) treatment (0.5 μM) in GM for 12 h, the phosphorylation of EPHA2 was decreased ($P < 0.05$; Fig. 7A, B). AKT and ERK signaling are downstream targets of Eph receptors. As shown in Fig. 7A, B, the phosphorylation of AKT was significantly decreased by ALW-II-41-27 treatment ($P < 0.01$), whereas the phosphorylated ERK1/2 was stimulated ($P < 0.01$). After 4 d of myogenic induction, a deficiency of myotube formation was observed in cells treated with ALW-II-41-27 in GM (Fig. 7C), paralleled with a down-regulation of myosin ($P < 0.01$, Fig. 7D), although MYOD1 expression level was up-regulated ($P < 0.01$, Fig. 7D).

To further validate the role of EPHA2 in myogenic differentiation, EPHA2 knockdown was performed in myogenic precursors. The results showed that siEPHA2-2 exhibited the best knockdown efficiency (Supplemental Fig. S3). Therefore, siEPHA2-2 was used for further analysis. Remarkably, EPHA2 silencing significantly enhanced the phosphorylation of ERK1/2 ($P < 0.01$, Fig. 7E, F), whereas the phosphorylation of AKT was not changed (Fig. 7E, F), which differed from the effect of ALW-II-41-27. Upon 4 d of myogenic differentiation, myogenic precursors lacking EPHA2 also had obviously fewer multinucleated myotubes as revealed by myosin staining (Fig. 7G). Furthermore, the expression of MYOD1 and myosin was analyzed by Western blot. Indeed, expression of these 2 proteins was significantly blunted in EPHA2-silenced cells compared with cells that had been transfected with scrambled siRNA (Fig. 7H). Thus, EPHA2 was required for myogenic differentiation and may promote myogenic differentiation through ERK signaling.

**DISCUSSION**

Identification of mechanisms that modulate adipogenic and myogenic differentiation is a vital step toward the maintenance of skeletal muscle homeostasis. For pigs, cell surface marker of muscle-derived cells has not been established because of the limited knowledge of cell surface markers and available commercialized antibodies. Therefore, we had successfully established a preplate technique, a cell surface marker-independent method, to

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**Figure 7.** The role of EPHA2 in myogenic differentiation. Myogenic precursors were treated EPHA2 inhibitor ALW-II-41-27 or transfected with siEPHA2 in GM before myogenic differentiation. A, B) p-EPHA2 (Tyr588), p-AKT (Ser473), p-REK (Thr202/Tyr204) levels of myogenic precursors treated with DMSO control or the ALW-II-41-27 (0.5 μM) in GM for 12 h. E, F) p-AKT (Ser473) and p-REK (Thr202/Tyr204) levels of myogenic precursors treated with scrambled siRNA or siEPHA2 72 h in GM. GAPDH was used as a loading control. G, D, G, H) Representative immunofluorescence images (C, G) and MYOD1 and myosin levels analyzed by Western blot (D, H) on d 4 of myogenic differentiation for myogenic precursors treated with ALW-II-41-27 or transfected with siEPHA2. Myosin (red) and DAPI (blue) staining were shown. n = 3. Data are presented as means ±SEM. An unpaired Student’s t test was used. CON, control; ALW, ALW-II-41-27. Scale bars, 100 μm. *$P < 0.05$, **$P < 0.01$. 
isolate adipogenic and myogenic precursors from skeletal muscle of pigs (24, 25) by adapting the previous protocol (26) with some modifications. In this study, we proved again the purity of adipogenic and myogenic precursors by their distinct differentiation capacity upon adipogenic and myogenic induction (as shown in Fig. 1). In addition, we had previously evidenced that adipogenic precursors had significantly higher mRNA expression levels of PDGFRα (marker of adipogenic precursors) relative to myogenic precursors, whereas myogenic precursors had considerably higher mRNA expression levels of Desmin and Pax7 (markers of myogenic precursors) compared with adipogenic precursors (24). Based on the significantly divergent differentiation potential, we can reasonably deduce that the 2 subpopulation cells both have high purity.

Given the significant roles of MBPs in cell signal perception and cell isolation, we analyzed the distinct membrane protein profile in myogenic and adipogenic precursors using the iTRAQ-based membrane proteomic method. A total of 762 MBPs were successfully identified. Eighty five MBPs with values of P < 0.05 and FC ≥1.2 or ≤0.83 were defined as differentially expressed, including 67 up-regulated and 18 down-regulated in myogenic precursors.

Accumulating evidence demonstrates that cytoskeleton organization exerts an important regulatory effect in the balance between myogenic and adipogenic differentiation. Cytoskeleton organization was required for myoblast fusion (35), which was also confirmed in Nap1-induced myoblast fusion between mammals and *Drosophila* (36). Conversely, cytoskeleton played a negative role in the adipogenesis of human mesenchymal stem cells by regulating activities of p38 and ERK1/2 (37), although pathways regulating actin cytoskeleton were down-regulated accompanied with the inhibited adipogenic differentiation induced by TAGLN knockdown in human bone marrow–derived stromal stem cells (38). In this study, “regulation of cytoskeleton organization” was the most significantly enriched biologically processed term based on differentially expressed MBPs. Thus, it is conceivable that cytoskeleton organization exerts essential regulatory functions in myogenic and adipogenic differentiation and the consequent skeletal muscle health. Notably, biologic process terms like “syncytium formation by plasma membrane fusion” and “syncytium formation” were also enriched, and both CD109 and MYOF were included in them. A previous study uncovered that MYOF (Myoferlin), a membrane-anchored protein, was required for efficient myoblast fusion to myotubes (39). Myoblast fusion is a complex and tightly controlled process required for the formation of skeletal muscle fibers (40); however, the fusion of nonmuscle cells could not be induced by myomarker, a key MBP to control critical steps in myoblast fusion (31). Therefore, syncytium formation was a vital process to distinguish myogenesis and adipogenesis, although our understanding of the mechanisms regulating myoblast fusion remains limited, particularly in mammals (41).

By integrating GO enrichment analysis and KEGG analysis, another remarkable group enrichment was associated with environmental information processing, including biologic process term “regulation of cell adhesion” and 2 KEGG pathways (“PDK-AKT signaling pathway” and “Rap1 signaling pathway”). Knockdown of ITGB3, known as adhesion molecules, in C2C12 myoblasts impairs myoblast migration and myotube formation through the impeded Rac1 activity and myogenic gene expression, as well as the disruption of actin organization (42). Here, we presented evidence showing that ITGB3 and other 4 up-regulated MBPs were involved in “regulation of cell adhesion,” supporting the notion that cell adhesion played a positive role in myogenic differentiation. Furthermore, accumulating evidence has revealed the importance of AKT activation in myoblast differentiation and fusion (43, 44). As such, Rap1 protein undergoes important modifications during early and late myogenesis (45) and interferes with various MAPK-activating pathways in skeletal myogenic cells (46). Rap1 deficiency promoted adipogenesis of mouse embryonic fibroblasts *in vitro* and caused excessive accumulation of subcutaneous and intra-abdominal fat (47). It was noteworthy that pathways involved in environmental information processing were also enriched in our previous work as revealed by RNA sequencing analysis of adipogenic and myogenic precursors (24). In summary, adipogenic and myogenic precursors showed great differences in environmental information processing and, depending on which, we speculated that MBPs acted as receptors to transmit extracellular signals and initiate intracellular pathways to regulate myogenic or adipogenic differentiation.

As displayed by KEGG analysis, pathways involved in “organismal systems” also contributed to decipher the discrepancy between myogenic or adipogenic differentiation, mirroring the key signaling in regulating aspects of skeletal muscle homeostasis. For example, thyroid hormone (TH) plays an essential role in myogenesis, and T3 stimulated myoblast differentiation through the activation of TH receptor α and followed by Wnt/β signaling pathway (48). Although the role of integrin in TH-mediated cell migration has been described (49), the function of integrins identified in this study, such as ITGB3 and ITGAV, in TH-mediated myogenic differentiation was not clear. We supposed that ITGB3 and ITGAV might accept the stimulation of TH and transduce this signal to control myogenic differentiation *via* several intrinsic factors. The role of integrins in TH-regulated myogenic differentiation is of great interest and needs further study.

In our present study, we also characterized some key MBPs involved in cell differentiation. As highlighted by KEGG analysis, ITGB3, ITGAV, ITPR3, and EPHA2, which were all up-regulated in myogenic precursors, acted as hubs to connect at least 2 pathways. It has been reported that knockdown of ITGAV enhanced adipogenic differentiation of human adipose tissue stem cells (50), although the mechanism was not well documented and the role of ITGAV in myogenic differentiation still remained unclear. Cytosolic Ca²⁺ signals are fundamental for myogenic differentiation (24, 51), and the up-regulation of intracellular Ca²⁺-MLCK pathway was also observed in
myogenic precursors relative to adipogenic precursors derived from pig skeletal muscle in our previous study (24). It has been known that inositol 1,4,5-trisphosphate receptor (IP3R) is an intracellular calcium release channel located on the endoplasmic reticulum, and IP3R-mediated Ca2+ release is essential in the early step of myoblast differentiation (51). Being different from the role of IP3R1 in myoblast differentiation, IP3R3 appears to be the major player in Ca2+-dependent apoptosis (52). Given that apoptosis is often associated with cell differentiation (53), the relationship between IP3R3-Ca2+ pathway and myogenic differentiation merited further exploration. Besides ITGB3, ITGAV, and ITPR3, KEGG analysis also revealed that EPHA2 acted as a hub to connect PI3K-AKT and Rap1 signaling pathway. Intriguingly, the expression pattern of EPHA2 during myogenic differentiation of myogenic precursors in the present study was similar to that of Pax7+/Twist2+ cells isolated from mice (34). Ephrin-A/EPHA signal has been reported to facilitate IGF-I–induced myogenesis by suppressing the Ras-ERK1/2 cascade in myoblast cell lines (54). In concert, after the inhibition of EPHA2 with ALW-II-41-27 in GM or siRNA-mediated silencing of EPHA2, enhanced phosphorylation of ERK1/2 was observed in myogenic precursors, paralleled with the impaired myotube formation. It is conceivable that EPHA2 is required for myogenic differentiation and myotube formation through ERK signaling, although we cannot exclude other mechanisms that mediate EPHA2 function.

Over all, our study provided an insight into the cellular mechanism mediating divergent differentiation capacity of skeletal muscle–derived myogenic and adipogenic precursors through the view of MBP dynamics. The 2 cell subsets showed significant differences in cytoskeleton organization, syncytium formation, environmental information processing, and organimsal systems. Remarkably, the present study revealed that EPHA2 was required for myogenic differentiation and may promote myogenic differentiation through ERK signaling. This study cast a new light on understanding the role of MBPs in regulating cell differentiation and maintaining skeletal muscle homeostasis.

ACKNOWLEDGMENTS

This study was financially supported by the Major Project of National Natural Science Foundation of China (Grant No. 31790412), National Key R&D Program of China (Grant No. 2018YFD0500402), and National Natural Science Foundation of China (Grant No. 31672431). The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

J. Yin obtained financial support and oversaw this study; X. Zhang and J. Yin performed most of the experiments, designed the study, and wrote the manuscript; X. Zhang and K. Qiu isolated cells; X. Zhang, L. Wang, and D. Xu conducted quantitative RT-PCR and Western blotting analysis; and X. Zhang performed bioinformatics analysis and analyzed the data.

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