Original Paper

Expressions and Regulatory Effects of P38/ERK/JNK Mapks in the Adipogenic Trans-Differentiation of C2C12 Myoblasts

Renli Qi^{a,b} Hong Liu^a Qi Wang^{a,b} Jing Wang^a Feiyun Yang^{a,b,c} Dingbiao Long^c
Jinxu Huang^{a,b}

^aChongqing Academy of Animal Science, ^bKey Laboratory of Pig Industry Sciences, Ministry of Agriculture, ^cChongqing Key Laboratory of Pig Industry Sciences, Rongchang, China

Key Words

Trans-differentiation • Adipogenesis • MAPK • ERK1/2 • JNK • P38

Abstracts

Background/Aims: Myoblasts and muscle satellite cells have the potential to trans-differentiate into adipocytes or adipocyte-like cells. Previous studies suggest that mitogen-activated protein kinase (MAPK) is critical to adipogenic trans-differentiation of muscle cells. ERK1/2, P38 and JNK are three major MAPK family members; their activation and regulatory functions during adipogenic trans-differentiation of myoblasts are investigated. **Methods:** C2C12 myoblasts were cultured and induced for adipogenic trans-differentiation. Activation patterns of MAPKs were assayed using protein microarray and Western blot. Three specific MAPK blockers, U0126, SB20358 and SP600125, were used to block ERK1/2, P38 and JNK during trans-differentiation. Cellular adipogenesis was measured using staining and morphological observations of cells and expression changes in adipogenic genes. **Results:** Inhibitors reduced phosphorylation of corresponding MAPK and produced unique cellular effects. Suppressing P38 promoted adipogenic trans-differentiation and intensified adipolytic metabolism in differentiated cells. However, inhibition of ERK1/2 had the opposite effects on adipogenesis and no effect on adipolysis. Blocking JNK weakly blocked trans-differentiation but stimulated adipolysis and induced apoptosis. **Conclusion:** Three MAPKs participate in the regulation of myoblast adipogenic trans-differentiation by controlling adipogenic and adipolysis metabolism.

Introduction

Skeletal muscle cells and adipocytes are derived from common embryonic stem cells in vertebrates, so cell types can transform into each other under specific conditions. *In vitro* and *in vivo* studies suggest that during abnormal physiological processes or after exogenous

R. Qi and H. Liu contributed equally to this work.

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Dr. Dingbiao Long
and Dr. Jinxu Huang

Changlong road 51#, Rongchang, 402460 (China)
Tel. +86-023-46792097; Tel.+86-023-46792081
E-Mail longjuan880@163.com; 57816041@qq.com

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stimuli (drugs or cytokines), skeletal muscle precursor cells can lose myogenic ability and trans-differentiate into fat cells or adipocyte-like cells with fat production and storage capacity [1-5]. In contrast, studies also suggest that adipose-derived cells or adipose-derived stem cells (ADSCs) can differentiate into myocytes [6].

Adipogenic trans-differentiation occurs in muscle precursor and satellite cells. However, myotubes at the end stage of differentiation cannot transform into adipocytes [2]. Some studies have focused on molecular characterization and regulatory mechanisms underlying transformation between muscle and fat cells, and data show that trans-differentiation is complex involving reprogramming of many genes and cell fate changes [7, 8].

Results from our previous work [9] and that of others [3] suggest that a mitogen-activated protein kinase (MAPK) signaling pathway may contribute to adipogenic trans-differentiation of myoblasts. MAPK, a serine/threonine protein kinase, is found throughout various cells and consists of three major subtype groups: extracellular signal-regulated kinases (ERK); c-Jun amino-terminal kinases (JNK); and p38 MAPK (p38) [10, 11]. Once cells are stimulated with a drug or cytokines, MAPKs are activated and assist with differentiation, apoptosis, energy metabolism, or the stress response by regulating their downstream signaling cascades. Three major MAPK subtypes have unique effects on cells via these various signaling transduction pathways and some reports show that the three MAPK pathways participate in differentiation and adipogenesis of white adipocyte to promote and/or inhibit adipogenic and adipolytic metabolism in fat cells, depending on the signal [12-17]. Molecular mechanism of myoblast adipogenic trans-differentiation may be similar to that of adipocyte differentiation but there are no data to describe the regulatory function of MAPK in trans-differentiation until now.

Thus, we sought to assess the respective effects of ERK1/2, P38 and JNK on the adipogenic trans-differentiation of C2C12 myoblasts. Three MAPK inhibitors (U0126, SB20358 and SP600125) were used to reduce expressions and function of MAPKs during trans-differentiation. The blockers have good specificity and effectiveness that widely demonstrated by many previous studies [18, 19]. Our results show that ERK1/2 has a positive effect when P38 has negative effect on trans-differentiation of myoblasts. However, removing JNK has only a weak effect on adipogenesis and caused significant apoptosis. These results will help us understand the regulatory mechanism underlying transformation between muscle and fat cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), DMEM-F12 and fetal calf serum (FCS) were purchased from CIBCO (Grand Island, NY, USA). U0126, SB20358 and SP600125 were purchased from Beyotime inc (Nantong, China). Oil red O, insulin, dexamethasone, 3-isobutyl-1-methylxanthine, rosiglitazone, Acridine orange (AO) and Ethidium bromide (EB) were purchased from Sigma-Aldrich (St Louis, MO, USA). All antibodies and the PathScan Intracellular Signaling Array Kit were purchased from CST (MA, USA). RNA extraction kit, PrimeScript™ RT reagent Kit and SYBR Green II quantitation PCR Kit were purchased from TaKaRa (Dalian, China). The Caspase 3/7 activity assay kit was purchased from Promega (Los Angeles, CA, USA). BODIPY and DAPI were purchased from Invitrogen (Carlsbad, CA, USA).

Culture and cell treatment

C2C12 myoblast were seeded and cultured in DMEM supplemented with 10% FCS at 37 °C and 5% CO₂. To induce adipogenic trans-differentiation, cells were transferred from growth medium to DMEM-F12 medium supplemented with 1 mg/mL insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10 μM rosiglitazone when cells reached 90% confluence. After 2 days, cells were transferred to medium supplemented with only 1 mg/mL insulin and 10 μM rosiglitazone for another 2 days. Subsequently, medium was changed every 2 days until cells were well differentiated.

To determine effect of MAPKs on the adipogenic trans-differentiation of myoblast, U0126 (10uM), SB20358 (10uM) and SP600125 (4uM), three MAPK blockers were added to the culture medium respectively

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throughout the whole trans-differentiation process. Then the cells were harvested for detections and assays at different stage (0d, 2d, 4d, 8d) of trans-differentiation. In addition, to investigate the effect of MAPKs on lipolysis of trans-differentiated cell, the blockers only were used at the last stage (8th day) of trans-differentiation. All experiments were performed using three to five different cell clones.

Staining and morphologic observation

The adipogenesis and trans-differentiation degree were determined by Oil red O staining and microscopic examination. Briefly, cells were collected at different differentiation stages, washed three times with PBS (pH 7.2), and then fixed in 4% paraformaldehyde for 30 min. After washes, fixed cells were stained with a working solution of Oil Red o for 30 min at room temperature. Lastly, cells were washed with deionized water, and the stain was extracted with 100% avantin for colorimetric analysis at 510 nm (quantitative analysis of triglyceride).

In addition, intracellular lipid was stained by a boron-dipyrromethene (BODIPY) fluorescent dye and photographed by a inverted fluorescence microscope (DMI8, Leica, Germany).

Activation of P38/ERK/JNK MAPKs

A pathscan intracellular signaling array protein microarray was used to measure activation changes of ERK/P38/JNK during trans-differentiation according to following the manufacturer's protocol.

RNA extraction and qRT-PCR

mRNA of genes related to adipogenesis, adipolysis, myogenesis, and fatty acid oxidation were assayed with qRT-PCR. Total RNA was extracted from cells with RNAiso Reagent at an appointed time and 1.0 µg of each sample was reverse-transcribed to cDNA with a PrimeScript RT reagent Kit. PCR was performed using the Q6 qPCR system. Relative mRNA expression was calculated using the 2-ΔΔCt method. The 18S and GAPDH were used as reference genes with similar results and the standardized primers were purchased from the sangon biotech company (Shanghai, China). The specific primer sequences are shown in Table 1.

Western blot

Cells were collected at specified times and lysed in RIPA buffer. Protein expression was measured using Western blot with GAPDH as a loading control. All primary antibodies and horseradish peroxidase-conjugated secondary antibody were obtained from CST.

Apoptosis analysis

Cells were counted using an automatic cell counter (Countess, Life technology) 48 h after blocking. Caspase 3/7 activity was measured using a Caspase-Glo 3/7 Assay Kit according to the manufacturer's instructions. In addition, apoptotic cells were observed with AO/EB cell double staining in according to the method of Liu [20].

Statistical analysis

All statistical analyses for data were performed using SPSS 21.0 software (IBM, NY, USA). All data are means ± SD. One-way ANOVA was used to determine significance between groups (p < 0.05 was considered statistically significant).

Results

Activation Changes of P38/ERK/JNK during adipogenic trans-differentiation

With the adipogenic stimuli, trans-differentiated cells gradually rounded and many peri-nuclear small lipid droplets were seen. These droplets coalesced and enlarged over

Table 1. Sequences of qPCR primers

Gene	Sequence (5' → 3')
PPAR γ	F: AAGAGCTGACCAATGGTTG R: ACCCTGGATCCTTCACAAG
FASN	F: CGGCTCTCTTTCTTCTTGG R: TCCTTGCTGGCATCTGTAT
FABP4	F: GTCACCATCCGGTCAGAGA R: TGATGCTCTTCACCTTCCTGT
MyoD	F: GGCTCTCTGCTCCTTTGA R: GTAGGGAAGTGTGCGTGCTC
MyoG	F: AGGAAGTCTGTGTCGGTGGA R: AGGCGCTCAATGACTGTGAT
HSL	F: GATTCCGCATAGACCCAGAG R: CAGAGACGACAGCACTCAA
ATGL	F: ATGACCACCCTTTCCAACAT R: GATGCTACCCGCTGCTCTT
CPT1	F: GACTCCGCTCGCTCATTGC R: GGCAGATCTGTTTGAGGGCT
UCP2	F: TGTGGAAGCCTACAAGACCA R: CATAGGTCACCAGCTCAGCA

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Fig. 1. Adipogenic trans-differentiation of C2C12 myoblasts. (A) Lipid accumulation in trans-differentiated cells observed with Oil red o and BODIPY staining (100x). (B) Quantitative analysis of cellular triglycerides. (C) protein and (D) mRNA of regulators of adipogenesis. (E) mRNA of regulators of myogenesis. N=5, Lowercase letters indicate significant differences ($p < 0.05$).

time (Fig. 1A and 1B). Western blot and qRT-PCR confirmed that three key adipogenic regulators (peroxisome proliferator-activated receptor gamma—PPAR γ ; fatty acid synthetase—FASN; and fatty acid binding protein 4—FABP4) undergo significant mRNA and protein changes during trans-differentiation (Fig. 1C and 1D). This suggests lipid metabolism during transformation. Additionally, two factors in myogenesis—myogenic differentiation antigen (MyoD) and myogenin (MyoG)—had decreased expression during trans-differentiation (Fig. 1E) and this was consistent with phenotypic cell changes. Importantly, P38, ERK1/2 and JNK had different activation patterns during trans-differentiation (Fig. 2). P38 and JNK both had greater activation in early and late stages of trans-differentiation (Fig. 2B). However, ERK1/2 activation peaked in the early stage of trans-differentiation and then was reduced.

Suppressing P38/ERK/JNK differentially affected adipogenic trans-differentiation

To explore regulatory effects of MAPKs on adipogenesis and trans-differentiation, U0126, SB20358 and SP600125 were applied to cells during trans-differentiation. Western blot showed that phosphorylation of P38, ERK, and JNK were reduced with inhibitors (Fig. 3). MAPK

Fig. 2. Changes in activation of P38/ERK/JNK MAPKs during adipogenic trans-differentiation. (A) Activation of P38/ERK/JNK MAPKs measured with protein microarray. (B) Quantitative analysis of protein activation. N=3, lowercase letters indicate significant differences ($p < 0.05$).

Fig. 3. Inhibitors reduced phosphorylation of P38/ERK/JNK MAPKs. U0126(10uM), SB20358(10uM) and SP600125(4uM) significantly decreased phosphorylation of ERK P38 and JNK respectively.

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Fig. 4. Inhibiting MAPKs affected adipogenic trans-differentiation of myoblasts. (A) Adipogenesis observed with Oil red O and BODIPY staining (100x). (B) Quantitative analysis of cellular triglycerides. (C) Quantitative analysis of fluorescent intensity of BODIPY. (D) mRNA and (E) protein of adipogenic regulator. N=5, lower-case letters indicate significant differences ($p < 0.05$).

Fig. 5. Blocking MAPKs differently affected adipolytic metabolism in trans-differentiated cells. (A) and (B) mRNA of HSL and ATGL, two key regulators of lipolysis. (C) Protein of HSL and ATGL. (D) Concentrations of free fatty acids in cell culture media. (E) Concentration of Cholesterol in cell culture media. (F) and (G) mRNA of CPT1 and UCP2. N=5, lower-case letters indicate significant differences ($p < 0.05$).

changes caused different effects on adipogenesis (Fig. 4). SB20358 treatment (P38 inhibitor) strengthened adipogenic metabolism, increased lipid production, and up-regulated mRNA and protein of the adipogenic regulators to different extents. Thus, P38 is a negative regulator for trans-differentiation. However, U0126 treatment (ERK1/2 inhibitor) significantly inhibited trans-differentiation and down-regulated FASN mRNA and protein. So, activation of ERK1/2 was essential for trans-differentiation. Though JNK had increased activation at later stages of trans-differentiation, SP600125 had a weak negative effect on adipogenesis and expressions of adipogenic genes. Therefore, JNK may not be critical for adipogenic trans-differentiation.

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Suppressing P38/ERK/JNK affected adipolysis of trans-differentiated cells

Additionally, effects of P38/ERK/JNK on adipolytic metabolism of differentiated cells were evaluated by adding inhibitor at later stages of differentiation (8th day). SB20358 and SP600125 increased expression of adipose triglyceride (ATGL) and hormone sensitive lipase (HSL), two rate-limiting enzymes of adipolytic metabolism (Fig. 5A, 5B and 5C). Up-regulated ATGL and HSL accelerated intracellular lipid breakdown and promoted release of free fatty acids and cholesterol (Fig. 5D and 5E). In addition, mRNA of carnitine palmitoyl transferase I (CPT1) and uncoupling protein 2 (UCP2) increased after inhibitor treatment (Fig. 5F and 5G). CPT1 and UCP2, located in mitochondria, control fatty acid uncoupling of oxidative phosphorylation and mitochondria-derived reactive oxygen species [21, 22]. Thus, suppressing P38 and JNK activation can promote adipolysis and increase the ratio of oxidative phosphorylation of fatty acids in the mitochondria. Unlike SB20358 and SP600125, U0126 did not have distinct effects on adipolytic metabolism but moderately increased expression of CPT1.

Fig. 6. Blocking MAPKs affected apoptosis in trans-differentiated cells. (A) Cell numbers with different inhibitor treatments. (B) Caspase 3/7 activity. (C) BAX and APAF1 protein. (D) Apoptosis viewed with acridine orange/ethidium bromide double staining (100 x). N=5, lower-case letters indicate significant differences ($p < 0.05$).

Suppressing JNK caused apoptosis in trans-differentiated cells

We noted that cells were reduced after SP600125 treatment at later stages of trans-differentiation, and studies suggest that MAPKs participate in apoptosis [23, 24]. Data for all treatment groups show that (Fig. 6) suppressing JNK with SP600125 decreased cells relative to controls after 48 h (Fig. 6A and 6D). Caspase3/7, a crucial apoptotic executor was increased after SP600125 treatment compared to controls (Fig. 6B). Apoptosis protease-activating factor 1 (APAF1) and Bcl-2-associated X protein (Bax) protein were also increased with SP600125 treatment (Fig. 6C). SB20358 treatment caused mild apoptosis (Fig. 6B and 6D) but U0126 treatment had no apparent effect.

Discussion

Cell trans-differentiation is the transformation of one type of cell to another [25]. In 1895, Wolff reported that pigment epithelium of salamanders can differentiate into lens cells during lens regeneration [26]. Subsequently, studies reveal numerous reports of cell trans-differentiation in humans and animals. For instance, striated muscle cells are differentiated into neurons [27]; neural stem cells are trans-differentiated from bone marrow stem cells [28]; and skeletal muscle cells are differentiated into osteocytes [29, 30] or adipocytes. A recent report suggests that during wound healing in the mouse, regeneration of hair follicles is accompanied by the transformation of many myofibroblasts to create “physiologically mature” white adipocytes [31]. Since abnormal transformation between muscle and adipose tissue may impair growth and development of tissues and disturb organ function, under-

standing myoblast-to-adipocyte trans-differentiation is essential.

Past studies have focused on molecular characteristics and regulatory mechanisms of transformations between muscle cell and adipocytes. For instance, it has been found that thiazolidinediones and fatty acids reduced expression of myogenic genes in C2C12 myoblasts and inhibited muscle formation as well as induced adipocyte characteristics [32]. These observations indicate that PPAR gamma activators, such as LCFA or thiazolidinediones, convert the differentiation pathway of myoblasts into that of adipoblasts. Similar results were obtained with mouse skeletal muscle satellite cells but drug and fatty acid stimulations did not have a significant effect on multinucleated myotubes during the terminal differentiation phase [2].

MKK3 kinase is reported to have a role in trans-differentiation of muscle cells and rosiglitazone induced MKK3-inactivated C2C12 myoblast trans-differentiated adipocytes [3]. MKK3 is a protein kinase upstream of the MPAK signaling pathway and it may activate P38 MAPK in specific cells [33]. Thus, P38 may be a negative regulator during trans-differentiation and MAPK may participate in the regulation of transformation between muscle and adipose tissue. We found that microRNA expression differs during trans-differentiation and pathway enrichment analysis suggested targets of differentially expressed were mainly enriched in the MAPK signaling pathway, suggesting its importance in trans-differentiation [9].

MAPK signal pathways exist in most type cells and transmit extracellular stimuli to cause biologic reactions. The magnitude, duration, and location of MAPK signaling is strictly controlled to produce the correct biological response [34]. In white adipocytes, different MAPKs exert complex and various regulatory functions on proliferation, differentiation, and metabolism to promote and/or inhibit lipid production and accumulation via crosstalk with signal transduction networks and control of downstream transcription factors. However, MAPKs contributions are not agreed upon. For instance, P38 is thought to promote adipogenic differentiation of 3T3-L1[14] and human preadipocytes [15] and inhibit adipogenic differentiation of embryo fibroblasts [35]. Also, reports suggest both positive and negative regulation of ERK and JNK during adipocyte differentiation [36, 37].

We assessed roles of P38, ERK and JNK and noted different activation patterns and functions during adipogenic trans-differentiation of C2C12 myoblasts (Fig 7). P38 was the most expressed and activated of the MAPKs; suppressing P38 activation with an inhibitor significantly promoted adipogenic trans-differentiation of C2C12 cells. In contrast, ERK1/2 suppression inhibited adipogenesis, so ERK1/2 may be a positive regulator during trans-differentiation. JNK weakly inhibited adipogenesis but suppressing JNK stimulated adipolysis in late trans-differentiating cells and caused more apoptosis. The multidimensional functions of MAPKs reveal their complex and diverse molecular mechanisms during trans-differentiation. Our data help to clarify different effects of MAPKs during transformation of muscle cells and adipocytes.

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Fig. 7. Three MAPKs uniquely regulate adipogenic trans-differentiation of myoblasts.

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Disclosure Statement

The authors declare that they have no competing interests.

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