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Kazinol-P from *Broussonetia kazinoki* enhances skeletal muscle differentiation via p38MAPK and MyoD



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ABSTRACT

The activation of MyoD family transcription factors is critical for myogenic differentiation, which is fundamental to the regeneration of skeletal muscle after injury. Kazinol-P (KP) from *Broussonetia kazinoki* (*B. kazinoki*), a natural compound, has been reported to possess an anti-oxidant function. In a screen of natural compounds for agonists of the MyoD activity, we identified KP and examined its effect on myoblast differentiation. Consistently, KP enhanced the myotube formation, accompanied with upregulation of myogenic markers such as MHC, Myogenin and Troponin-T. KP treatment in C2C12 myoblasts led to strong activation of a key promyogenic kinase p38MAPK in a dose, and time-dependent manner. Furthermore, KP treatment enhanced the MyoD-mediated trans-differentiation of 10T1/2 fibroblasts into myoblasts. Taken together, KP promotes myogenic differentiation through activation of p38MAPK and MyoD transcription activities. Thus KP may be a potential therapeutic candidate to prevent fibrosis and improve muscle regeneration and repair.

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1. Introduction

Muscle wasting is observed in various states with disease (cachexia), inactivity (atrophy) or aging (sarcopenia). Sarcopenia is defined as the age-related loss of muscle mass, associated with a decrease in strength, metabolic rate, and aerobic capacity [1]. In response to injury, skeletal muscle has a remarkable ability to regenerate. The activated quiescent satellite cells migrate to the site of injury where they proliferate, differentiate, and fuse to form myofibers [2,3]. Myogenic differentiation is a highly orchestrated multistep process and plays an important role in muscle regeneration, which is controlled by the family of myogenic bHLH factors (Myf5, MRF4, and MyoD) and two key intracellular signaling pathway including p38MAPK and AKT signaling pathways [4]. Especially, p38MAPK plays an essential role in the transition of myoblasts to differentiated myotubes through the activation of

MyoD family transcription factors by which regulates MyoD dimerization with E proteins [5].

Broussonetia kazinoki (paper mulberry) has long been used in the manufacture of paper in Korea, China, and Japan. According to Principles and Practice of Eastern Medicine (Donguibogam), this plant has been used for dermatologic disease such as burns and acne [6]. Even though the extract of *B. kazinoki* was reported to have anti-diabetic and antihyperglycemic effects [7], and to inhibit melanin synthesis [8], biological effects for myoblast differentiation have not been reported yet.

In this report, we have screened in house built phytochemical library for activation of MyoD-responsive reporter and myosin heavy chain (MHC) expression in C2C12 myoblasts to identify kazinol-P (KP) purified from *B. kazinoki* as a myogenic activator. The treatment with KP enhanced the level of myogenic marker proteins, the formation of larger multinucleated myotubes. It also activated p38MAPK, a key promyogenic kinase which activates MyoD in C2C12 myoblasts. Moreover, the MyoD mediated trans-differentiation of 10T1/2 fibroblasts into myoblasts mediated by MyoD was enhanced dramatically by KP treatment. These results indicate that KP has a promyogenic effect and a potential as a therapeutic candidate for improving muscle regeneration and inhibiting fibrosis occurring in age-related or pathological muscle diseases.

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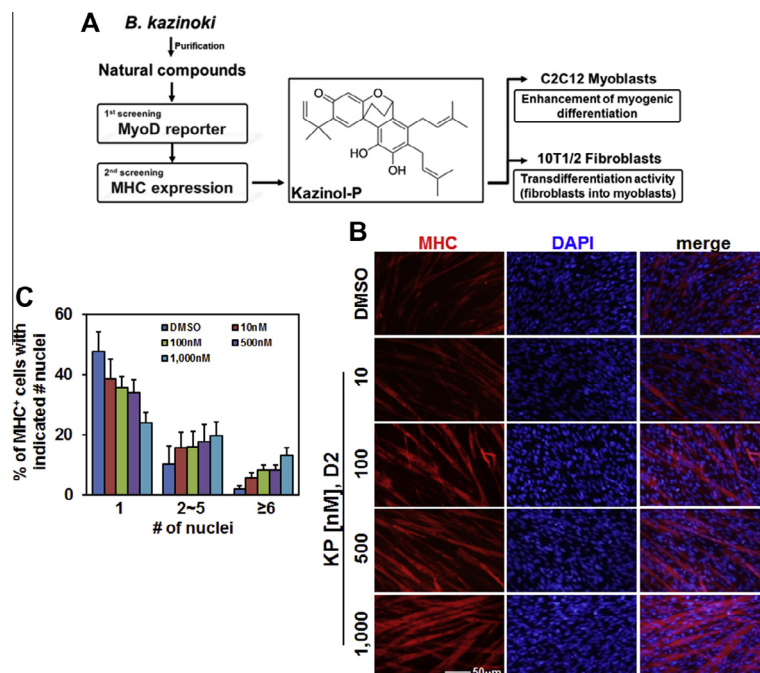


Fig. 1. Promotion of myotube formation in C2C12 myoblasts. (A) The experimental scheme and chemical structure of KP isolated from *B. kazinoki*. (B) C2C12 cells were treated with KP (10, 100, 500 and 1000 nM) and differentiated in DM for 48 h, fixed, and immunostained with an antibody to MHC (Red) followed by DAPI (blue) stain. Scale bars, 50 μm. (C) Quantification of myotube formation in this cell lines shown in B. Values represent the means of triplicate determinations \pm 1 SD. The experiment was repeated three times with similar results. Asterisks indicate significant difference from the control at * P < 0.05 and ** P < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and method

2.1. Materials and general procedures

Fetal bovine serum (FBS) and Dulbecco modified Eagle's medium (DMEM) were purchased from Thermo Scientific (Waltham, MA). Horse serum (HS) was obtained from WelGene (Daegu, Korea). Complete protease inhibitor cocktail was purchased from Roche Diagnostics (Indianapolis, IN). Lipofectamin 2000 was obtained from Invitrogen (Carlsbad, CA). SB203580 was obtained from CalBiochem (La Jolla, CA). Antibody against phospho38MAPK was purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Myogenin and MyoD were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against Cdo was purchased from Zymed Laboratories Inc. (South San Francisco, CA). MHC (MF-20) was obtained from the Developmental Studies Hybridoma Bank (Iowa, IA). Primary antibodies against pan-Cadherin, Troponin-T and p38MAPK and all other chemicals used were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Column chromatography was carried out over Silica gel (40–60 μm, Merck), LiChroprep RP-C18 (40–60 μm, Merck). Fractions obtained from column chromatography were monitored by thin layer chromatography (TLC) (RP-18 F254S and silica gel 60 F254, Merck). NMR spectra were obtained on a VARIAN UNITY INOVA 400 spectrometer (Palo Alto, CA). Mass spectra were determined on a JEOL JMS-AX505WA mass spectrometer.

2.2. Extraction and isolation of kazinol-P from the root of *B. kazinoki*

The root of *B. kazinoki* was purchased from Kyungdong Oriental Market, Seoul, Korea, in February 2007 and authenticated by Prof. K.S. Yang at Sookmyung Women's University. A voucher specimen (No. SPH 07002) was deposited in the herbarium of Sookmyung Women's University.

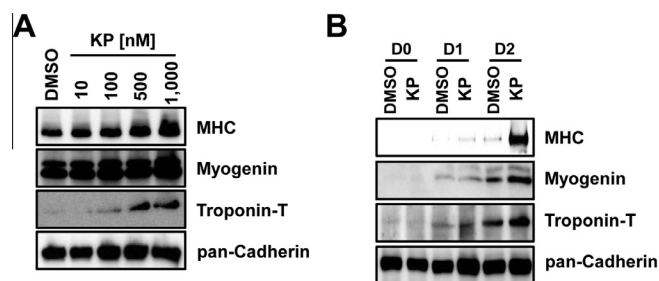


Fig. 2. Enhancement of the expression of muscle specific marker proteins. (A) C2C12 cells were treated with KP (10, 100, 500 and 1000 nM) in DM for 48 h. Lysates were Western blotted with antibodies to MHC, Myogenin, Troponin-T and pan-Cadherin as a loading control. (B) C2C12 cells were treated with KP (1000 nM) for D0, D1 and D2. Lysates were Western blotted with antibodies to MHC, Myogenin, Troponin-T and pan-Cadherin as a loading control.

Air-dried *B. kazinoki* root bark (0.6 kg) was extracted for 24 h at room temperature in 2 l of ethanol. The resultant extract (51 g) was dissolved in water and successively partitioned with *n*-hexane, ethyl acetate, chloroform and butanol. The ethyl acetate soluble fraction (31 g) was subjected to silica gel column chromatography and eluted with an *n*-hexane:acetone gradient system (20:1 \rightarrow 1:10) to yield 11 fractions. Fraction 7 (7.2 g) was subjected to silica gel column chromatography eluting with a chloroform:methanol gradient system (100:1 \rightarrow 10:1). The resultant sub-fraction 7–3 (210 mg) was further purified on an RP-C18 column via a gradient elution of methanol (30% \rightarrow 100%), thus yielding kazinol-P (18 mg).

The purity of the kazinol-P was evaluated by HPLC using a Waters 1525 system (Milford, MA, USA). A reverse phase column (ODS-2, 150 \times 4.6 mm i.d., GL Sciences Inc., Japan) was eluted with 80% methanol, and the resultant eluates were monitored with UV

detector at 280 nm. The chemical structure was determined by spectroscopic analyses as kazinol-P that was previously reported from *B. kazinoki* [9].

2.3. Cell culture and differentiation

C2C12 and 10T1/2 cells were cultured as described previously [10]. To induce differentiation of C2C12 myoblasts, cells at near confluency were changed from DMEM containing 15% FBS (growth medium, GM) to DMEM containing 2% HS (differentiation medium, DM) in a 5% CO₂ incubator at 37 °C and myotubes were observed at 48–72 h of differentiation. The efficiency of the myotube formation was quantified by a transient differentiation assay as previously described [11]. For inhibitor study of p38MAPK, C2C12 cells were treated with 1000 nM KP after pre-incubation with 100 nM SB203580 in fresh culture medium for 30 min. For the trans-differentiation study, 10T1/2 cells were cultured in DMEM supplemented with 10% FBS in 10 mm culture plates, and transfected with 10 µg of MyoD or control vector (pSuper). After incubation for 24–48 h, cells were treated with KP in 2% HS for 48 h.

2.4. Western blotting

Western blot analysis was performed as previously described [10]. The cells were lysed in an extraction buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄, complete protease inhibitor cocktail) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed. The primary antibodies used were anti-Cdo, anti-pan-Cadherin, anti-p38, anti-p-p38 (the phosphorylated, active form of p38MAPK), anti-MHC, anti-Myogenin, anti-Troponin-T and anti-MyoD. The signal was

detected using the Amersham ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL) and quantified with a FUJIFILM Luminescent Image Analyzer LAS-3000.

2.5. Immunocytochemistry and microscopy

Immunostaining for MHC expression was performed as described previously [10]. Briefly, C2C12 were seeded at a density of 3×10^4 cells/well. After 24 h, the cells were incubated in DM for 72 h to induce myotube formation. The differentiated cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in phosphate buffered saline, blocked, and stained with anti-MHC, followed by the treatment of an Alexa Fluor 568-conjugated secondary antibody (Molecular Probes). Images were captured and processed with a Nikon ECLIPSE TE-2000U microscope and NIS-Elements F software (Nikon) and Photoshop CS5 (Adobe).

2.6. Luciferase assay

5×10^4 C2C12 cells were plated 24 h in 24-well plates and transiently transfected with 4RTK reporter gene and MyoD plasmids using Lipofectamin 2000, according to the manufacturer's protocol. Cell culture, myoblast differentiation, and KP treatment were performed as described above. C2C12 cells were harvested and the luciferase assay was done by using the Luciferase kit (Promega, Madison, WI) according to manufacturer's protocol. Luciferase activities were measured on a Berthold luminometer, integrating light emission over 20 s. Transfection efficiencies were normalized by co-transfecting 50 ng of the β-galactosidase plasmid pCH110 (Amersham Pharmacia Biotech). All transfections were performed in duplicate at least three times.

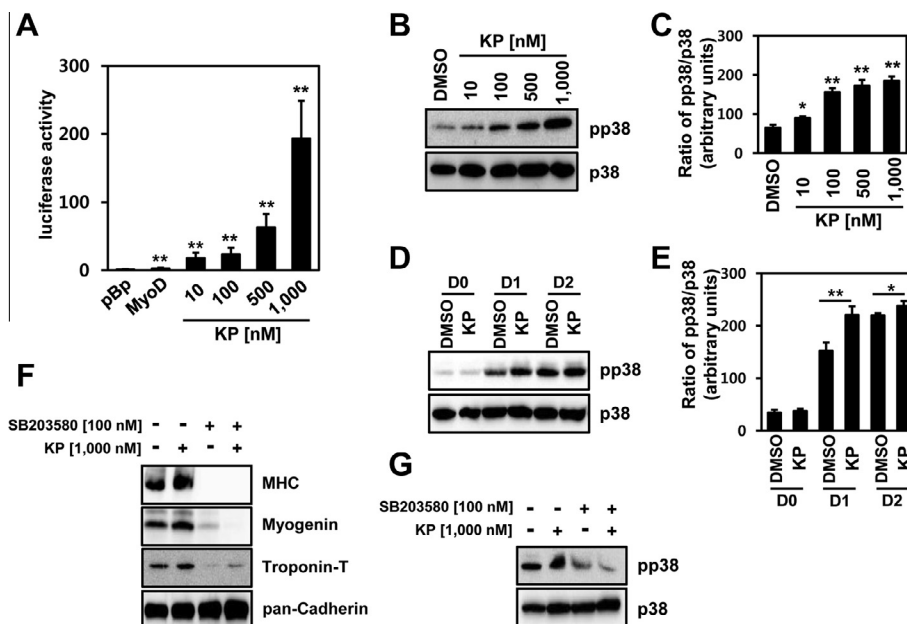


Fig. 3. Stimulation of the p38MAPK signaling pathway in myoblast differentiation. (A) C2C12 cells were transiently transfected with 4RTK-luciferase, LacZ and MyoD or pBp plasmid as a control vector. After 48 h in culture, the cells were lysed and luciferase and β-galactosidase activities were measured. Asterisks indicate significant difference from the control at * $P < 0.05$ and ** $P < 0.01$. (B) C2C12 cells were treated with the indicated amounts of KP followed by Western blotting with antibodies to p-p38MAPK (pp38) or p38MAPK (p38) as a loading control in DM for 48 h. (C) Quantification of the three replicate blots, which was shown in panel B. The intensity of the pp38 and p38 were quantified, and the values were obtained from the ratio of pp38/p38, respectively. Values represent the means of triplicate determinations ± 1 SD. Asterisks indicate significant difference from the control at * $P < 0.05$ and ** $P < 0.01$. (D) C2C12 cells were treated with KP (1000 nM) for D0, D1 and D2 and lysates were Western blotted with antibodies to pp38 or p38 as a loading control. (E) Quantification of the three replicate blots, which was shown in panel D. The intensity of the pp38 and p38 were quantified, and the values were obtained from the ratio of pp38/p38, respectively. Values represent the means of triplicate determinations ± 1 SD. Asterisks indicate significant difference from the control at * $P < 0.05$ and ** $P < 0.01$ (F and G). C2C12 cells were treated with 100 nM SB203580 for 30 min before treatment with 1000 nM KP, and then differentiated in DM for 48 h. Cell lysates were Western blotted with antibodies to MHC, Myogenin, Troponin-T and pan-Cadherin as a loading control, and antibodies to pp38 or p38 as a loading control.

2.7. Statistics

The experiments were done independently three times. The participants' *T*-test was used to access the significance of the difference between two mean values. * $P < 0.05$ and ** $P < 0.01$ were considered to be statistically significant.

3. Results and discussion

3.1. KP promotes myotube formation in C2C12 cells

To identify potential regulators of MyoD and myoblast differentiation, we have purified several kazinol compounds from *B. kazinoki*, and screened by two criteria: the activation of MyoD-responsive 4RTK reporter and the induction of MHC expression in C2C12 myoblasts (Fig. 1A). Among candidates, KP was selected from both screening experiments. We first confirmed whether KP has an agonistic effect on myoblast differentiation by inducing of the myotube formation. C2C12 myoblasts were induced to differentiate for 48 h in DM, fixed, and immunostained with anti-MHC antibodies, followed by DAPI stain to visualize nuclei. Photomicrographs of these cells showed that KP treatment formed larger myotubes with more nuclei per myotube, relative to the DMSO-treated C2C12 cells (Fig. 1B). MHC-positive cells were scored as mononucleate, containing two to five nuclei, or containing six or more nuclei. Treatment with KP significantly reduced the proportion of mononucleate cells with the substantially increased myotubes

containing six or more nuclei in a dose-dependent manner (Fig. 1C). These results suggest that the KP treatment enhances myoblast differentiation and myotube formation.

3.2. KP enhances the expression of muscle-specific proteins

Next we investigated the effect of KP on the expression of muscle specific proteins associated with myogenic differentiation including MHC, Myogenin and Troponin-T [4]. C2C12 cells were cultured in DM for 48 h in the presence of KP (10–1000 nM). The KP-treated C2C12 cells exhibited increased expression of MHC, Myogenin and Troponin-T in a dose-dependent manner, compared to the DMSO-treated control cells (Fig. 2A). When analyzing the expression level of these proteins during differentiation time course, C2C12 cells treated with a final concentration of 1000 nM KP exhibited dramatically enhanced expression of these proteins at 48 h, relative to those of DMSO-treated C2C12 cells (Fig. 2B). These data suggest that the KP treatment up-regulates the expression levels of muscle-specific proteins leading to enhanced myoblast differentiation.

3.3. KP up-regulates the activation of p38MAPK in myoblast differentiation

p38MAPK signaling pathway plays a critical role in skeletal muscle differentiation and the block of p38MAPK pathway inhibits induction of muscle-specific proteins and myogenic differentiation

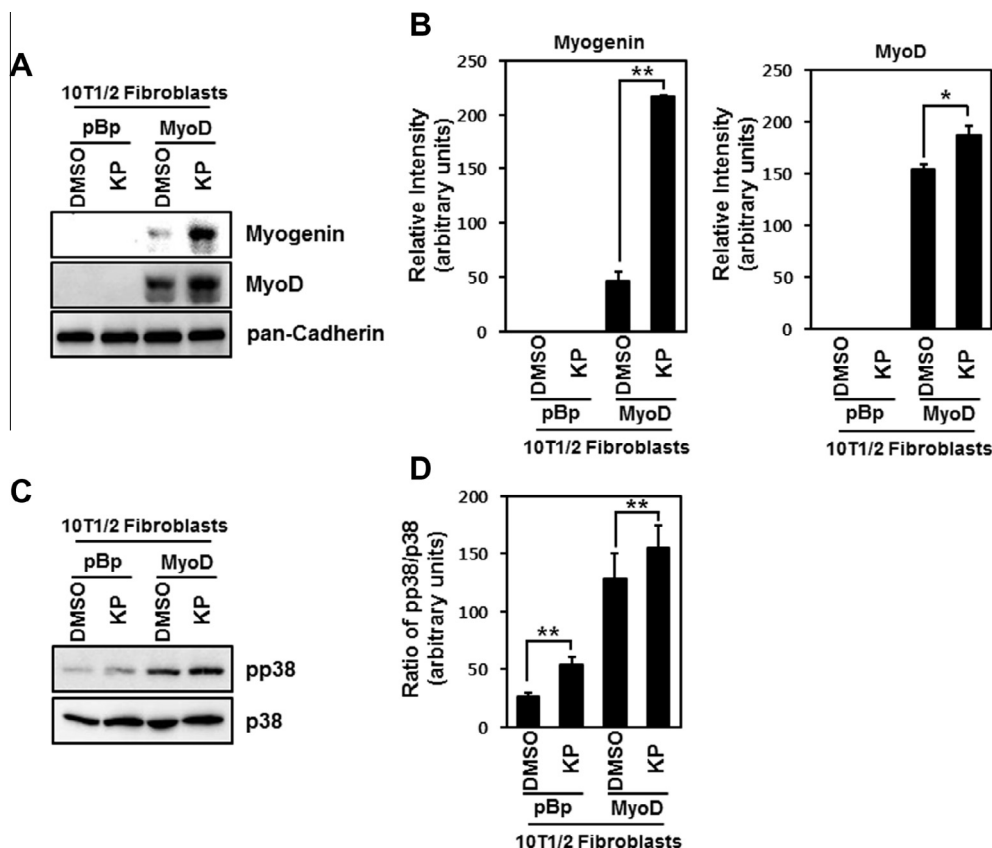


Fig. 4. MyoD-mediated trans-differentiation of 10T1/2 fibroblasts. 10T1/2 cells were transiently transfected with control (pBp) or MyoD expression vector (pBp-MyoD) and induced to differentiate with treatment of either 1000 nM KP or DMSO for 48 h. Cell lysates were Western blotted with antibodies to Myogenin, Troponin-T and pan-Cadherin (A), and pp38 and p38 (C). The experiment was repeated three times with similar results. (B) Quantification of the three independent experiments, one of which was shown in panel A. The intensity of myogenic-specific proteins was quantified, and the values were obtained from ratio of marker proteins to pan-Cadherin as a loading control. Values represent the means of triplicate determinations \pm 1 SD. The experiment was repeated three times with similar results. Significant difference from vehicle-treated group, * $P < 0.05$ and ** $P < 0.01$. (D) Quantification of the three blots replicate immunoblots, one of which was shown in panel C. The intensity of the pp38 and p38 was quantified, and the values were obtained from the ratio of pp38/p38, respectively. Values represent the means of triplicate determinations \pm 1 SD. Significant difference from vehicle-treated group, * $P < 0.05$ and ** $P < 0.01$.

[10,12–14]. Much evidence indicates that p38MAPK is an activator of MyoD [15]. Because KP enhanced the differentiation of C2C12 myoblasts at the early time point (D0 to D1), we then asked whether KP can activate a key promyogenic kinase p38MAPK which is critical to induce MyoD activation. To further confirm the effect of KP on MyoD-responsive reporter, C2C12 cells were transfected with 4RTK reporter, MyoD expression vector or control vector and treated with DMSO or KP for 48 h in DM, followed by the luciferase assay. In agreement with the screening data, KP enhanced the MyoD-dependent transcription activities in a dose-dependent manner (Fig. 3A). To explore whether KP activates p38MAPK signaling pathway during myogenic differentiation, we performed Western blot analysis. KP treatment significantly increased the level of an actively, phosphorylated form of p38MAPK (pp38) in a dose-dependent manner (Fig. 3B and C) and a differentiation day-dependent manner (Fig. 3D and E).

To assess the requirement of p38MAPK activation for the KP-mediated myogenic differentiation, C2C12 cells were treated with KP and a specific pharmacological p38MAPK inhibitor SB203580. C2C12 cells were treated with 100 nM SB203580 for 30 min before application of 1000 nM KP in DM for 48 h. As shown in Fig. 3F, the treatment with SB203580 inhibited the expression of muscle-specific proteins and the addition of KP did not recover the expression of these proteins. Moreover, consistent with Fig. 3F, KP treatment did not restore the phosphorylation of p38MAPK under the condition of pretreatment with SB203580 (Fig. 3G). These results indicate that KP requires activation of p38MAPK signaling to induce MyoD activation and myoblast differentiation.

3.4. KP has the potential for trans-differentiation of embryonic fibroblasts into myoblasts

The ectopic expression of MyoD in non-muscle cell types, such as 10T1/2 fibroblast cells can activate downstream myogenic regulators and induce trans-differentiation of such cells into myogenic cell types [16,17]. To further verify the effect of KP on MyoD activation, we assessed the effect of KP on the trans-differentiation efficiency of 10T1/2 fibroblasts into myoblasts in response to MyoD. 10T1/2 cells were transfected with the expression vector for MyoD or control pBp, treated with DMSO or KP and then induced to differentiate for 48 h, followed by Western blot analysis. As shown in Fig. 4A and B, no expression of muscle-specific proteins was observed in control pBp-transfected 10T1/2 cells treated. On the other hand, the KP treatment of MyoD-transfected 10T1/2 cells dramatically increased the expression levels of MyoD and Myogenin, compared to that of DMSO-treated MyoD-transfected 10T1/2 cells. Moreover, the trans-differentiation of 10T1/2 cells by MyoD transfection enhanced the level of pp38 and the treatment with KP in MyoD-transfected 10T1/2 cells further increased pp38 levels (Fig. 4C and D). Interestingly, KP increased the level of pp38 in pBp-treated 10T1/2 cells, which was further increased by MyoD-transfection. These data further support that KP enhances activation of MyoD most likely via activation of p38MAPK thereby not only promoting myoblast differentiation but also enhancing the MyoD-mediated trans-differentiation of fibroblasts into myoblasts.

Taken together, we identified a natural compound KP from *B. kazinoki* as a promyogenic agent which can significantly activate myogenic differentiation. The promyogenic effect of KP requires MyoD and p38MAPK activities and KP treatment enhances the

early differentiation events, such as expression of muscle-specific proteins like MHC, Myogenin and Troponin-T. In addition, the KP treatment can also increase the conversion efficiency of MyoD mediated-10T1/2 fibroblasts into myoblasts. This is the first finding to provide a new insight by which KP promotes myogenic differentiation. KP can be used as a potential tool to enhance myogenic differentiation of muscle stem cells or trans-differentiation capacities of non-muscle cells to treat various muscle diseases.

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