DE GRUYTER

Biologia **72**/6: 694—701, 2017
Section Cellular and Molecular Biology
DOI: 10.1515/biolog-2017-0078

Blebbistatin induces chondrogenesis of single mesenchymal cells via PI3K/PDK1/mTOR/p70S6K pathway

Hyoin Kim[1], Dong Hyun Kim[1], Bohyeon Jeong[1], Ju-Hee Kim[1], Sun-Ryung Lee[2] & Jong Kyung Sonn[1]*

[1] *Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu, South Korea; e-mail: sonnjk@knu.ac.kr*

[2] *Department of Biology, College of Natural Sciences, Jeju National University, Jeju-si, South Korea*

Abstract: Rearrangement of the actin cytoskeleton plays an inductive role in chondrogenic differentiation. Our previous study showed that blebbistatin, an inhibitor of myosin II, removes actin stress fibres and induces chondrogenesis of mesenchymal cells in monolayer cultures. In the present study, we investigated signalling pathways implicated in the induction of chondrogenesis by dissolving actin stress fibres after blebbistatin treatment. Blebbistatin increased the activity of phosphoinositide 3-kinase (PI3K). Inhibition of PI3K with LY294002 blocked blebbistatin-induced chondrogenesis without affecting blebbistatin-induced reorganization of actin filaments. Blebbistatin also upregulated the phosphorylation of phosphoinositide-dependent protein kinase 1 (PDK1), and inhibition of PDK1 with GSK2334470 suppressed blebbistatin-induced chondrogenesis, indicating that removal of actin stress fibres by blebbistatin induced chondrogenesis by activating PI3K/PDK1. Although inhibition of Akt activity by Akt inhibitor IV blocked blebbistatin-induced chondrogenesis, phosphorylation of Akt was not affected by blebbistatin. Blebbistatin increased the phosphorylation of mammalian target of rapamycin (mTOR) at Ser2448 and p70 ribosomal protein S6 kinase (p70S6K). Inhibition of mTOR with rapamycin almost completely abolished the phosphorylation of p70S6K. Inhibition of mTOR complex 1 (mTORC1) and complex 2 (mTORC2) with pp242 diminished phosphorylation of Akt at Ser473, whereas inhibition of mTORC1 with rapamycin did not. However, blebbistatin did not affect the phosphorylation of mTOR at Ser2481. Taken together, the present results suggest that blebbistatin induces chondrogenesis by activating the PI3K/PDK1/mTOR/p70S6K pathway. Our data also indicate that Akt activity is essential for chondrogenesis but is regulated by mTORC2, which is independent of blebbistatin treatment.

Key words: mesenchymal cells; chicken; chondrogenesis; cytoskeleton; blebbistatin; signalling molecules.

Abbreviations: BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FBS, foetal bovine serum; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PKD1, phosphoinositide-dependent protein kinase 1; p70S6K, p70 ribosomal protein S6 kinase.

Introduction

Cell shape and the cytoskeleton are closely associated with chondrogenesis. Embryonic limb mesenchymal cells undergo changes in their shape and cytoskeleton during chondrogenesis. Undifferentiated mesenchymal cells with a stellate morphology become round after differentiating into chondrocytes (Wezeman 1998). Mature articular cartilage contains actin microfilaments as a predominantly cortical structure (Langelier et al. 2000). Chondrogenesis of mesenchymal cells can be induced when they become rounded *in vitro*. Single limb mesenchyme cells cultured on type I collagen gels differentiate into cartilage in a round configuration (Solursh et al. 1982). Dedifferentiated chondrocytes cultured on chitosan membranes exhibit cortical distribution of the actin cytoskeleton and restored chondrocyte characteristics (Park et al. 2008). Similarly, disruption of the actin cytoskeleton is another important trigger of chondrogenesis. Cytochalasin D disrupts actin stress fibres, thus inducing the chondrogenesis of chick limb bud mesenchymal cells (Zanetti & Solursh 1984; Lim et al. 2000). Recent studies have shown that blebbistatin, a cell-permeable inhibitor of muscle and non-muscle myosin II (Straight et al. 2003), removes actin stress fibres and induces chondrogenesis (Kim et al. 2012).

Phosphoinositide 3-kinase (PI3K) plays a central role in many cellular processes, including proliferation, survival, adhesion, development, motility, and transformation (Krasilnikov 2000; Katso et al. 2001). The PI3K protein family includes 3 classes of enzymes (I–III) that generate 3' phosphoinositide lipids. Upon activation, class I PI3Ks convert phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] to phosphatidylinositol (3,4,5)-triphosphate [$PI(3,4,5)P_3$] that functions as a docking site for Akt and 3-phosphoinositide-

* Corresponding author

dependent protein kinase-1 (PDK1) and allows the latter to phosphorylate Akt at Thr308 (Cantley 2002). The PI3K/Akt signalling pathway has been shown to be involved in chondrogenesis, and PI3K activity is required for chondrogenesis (Bang et al. 2000; Hidaka et al. 2001; Lee et al. 2013). Insulin-like growth factor 1 induces chondrogenesis by activating PI3K and Akt (Oh & Chun 2003).

To elucidate the mechanisms through which reorganization of actin filaments induces chondrogenesis, we determined whether the PI3K signalling pathway is associated with blebbistatin-induced chondrogenesis.

Material and methods

Antibodies and reagents

Anti-PI3K p85, anti-GAPDH, anti-Akt, anti-phospho-Akt (Ser473), anti-PDK1, anti-phospho-PDK1 (Ser241), anti-phospho-p70S6K (Thr389), and anti-phosphorylated-molecular target of rapamycin (anti-phospho-mTOR; Ser2448), and anti-phospho-mTOR (Ser2481) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-type II collagen, anti-phospho-PI3K p85 (Tyr508), anti-p70S6K, and anti-phospho-Akt (Thr308) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Blebbistatin and LY294002 were purchased from Enzo Life Sciences (Plymouth, PA, USA). Cytochalasin D and GSK2334470 were from Tocris Bioscience (Ellisville, MO, USA). Rapamycin was purchased from LC Laboratories (Woburn, MA, USA).

Cell culture

Chick embryo wing bud mesenchymal cells were cultured as described previously (Kim et al. 2012). Briefly, wing buds were dissected from Hamburger-Hamilton stage 23/24 chick embryos and were collected in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution. The wing buds were then treated with 0.1% collagenase/trypsin for 10 min in a humidified incubator at 37℃ and 5% CO_2. F12 supplemented with 10% foetal bovine serum (FBS) was added to stop the reaction, and a cell suspension was obtained by pipetting the medium up and down several times. The cells were collected by centrifugation at 400 × *g* for 10 min and resuspended in F12 containing 10% FBS. Single cells were obtained by filtering the cell suspension through 8 layers of lens paper. Cells were counted using a hemocytometer. Cell cultures at low density where cells remain undifferentiated were performed by spreading the 3×10^6 cells over the entire 60 mm culture dishes and the cells were incubated for 1 h at 37℃. Cells were then treated with blebbistatin, LY294002, cytochalasin D, GSK2334470 or rapamycin dissolved in dimethyl sulfoxide (DMSO) for 1 or 2 days. Controls received DMSO vehicle at a concentration equal to that of agent-treated cells. For Western blot analysis with anti-PI3K p85 and anti-phospho-PI3K p85 (Tyr508) antibodies, which do not react well with samples from chicken, the distal subridge region of the limbs of E11.5 mouse embryos were dissected and mesenchymal cell cultures were prepared as described above.

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Next, the cells were rinsed with phosphate-buffered saline and blocked using 1% bovine serum albumin (BSA) to prevent nonspecific binding. The cells were then incubated with goat anti-type II collagen antibody in 1% BSA for 1 h at room temperature. After washing with phosphate-buffered saline, the cells were double stained with Alexa 555-conjugated anti-goat secondary antibody and Alexa 488-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) to detect type II collagen and F-actin, respectively, for 30 min at room temperature. Cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). The stained cells were visualized using a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) epifluorescence microscope.

Western blot

Cultures were extracted in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, and 1% NP-40) containing 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations of the cell lysates were measured spectrophotometrically using a BCA Assay Kit (Thermo Scientific Inc., Rockford, IL, USA). Next, 30 μg of the protein extract was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis and the resolved proteins were electrotransferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked using 4% non-fat dry milk in TBS with 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4℃ with primary antibodies. The membrane was then incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Membrane-bound proteins were detected using SuperSignal West Femto (Thermo Scientific) according to the manufacturer's protocol.

PI3K assay

PI3K activity was determined using a P13-Kinase Activity ELISA kit according to the manufacturer's protocol (Echelon Biosciences Inc., Salt Lake City, UT, USA). Briefly, the cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, and 1% NP-40). The cell lysate was centrifuged, and the supernatant was incubated with anti-p85 PI3K antibody at 4℃ for 1 h, with gentle rotation. Protein A agarose beads (60 μL of 50% slurry) were added, and the mixture was incubated at 4℃ overnight. Immunoprecipitated enzyme was mixed with phosphatidylinositol 4,5-bisphosphate and incubated at room temperature for 3 h. The kinase reaction was stopped by adding 100 mM EDTA, and the mixture was then transferred to a PI3K ELISA plate. Detector was added to the mixture, and the mixture was incubated for 1 h. The plate was then washed and incubated with the secondary detector for 30 min. The plate was washed again and incubated with 3,3',5,5'-tetramethylbenzidine solution for 20 min, and then the reaction was stopped by adding H_2SO_4. The plate was read at 450 nm using a plate reader. Protein concentrations in the cell lysate were measured as described above, and PI3K activities were corrected for protein content.

Results

Blebbistatin induces chondrogenesis by activating PI3K

Chick embryo wing bud mesenchymal cells were grown in the absence or presence of blebbistatin. Untreated cells exhibited numerous well-organized actin stress fibres and were not positive for type II collagen by immunostaining, whereas cells treated with blebbistatin

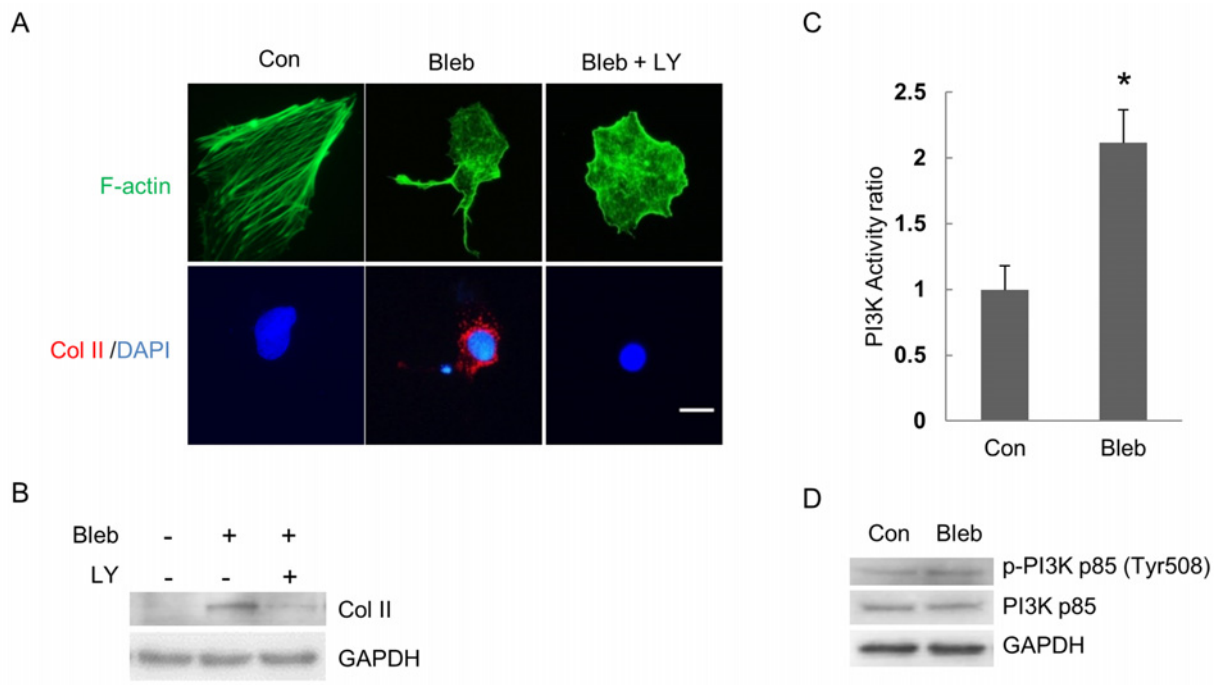

Fig. 1. Blebbistatin induces chondrogenesis by activating phosphoinositide 3-kinase (PI3K). Chick wing bud mesenchymal cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without 10 μM LY294002 (LY) for 2 days. (A) Cells were stained for F-actin (phalloidin, green), type II collagen (Col II, red), and nuclei were stained with DAPI (blue). Scale bar: 10 μm. (B) Cell lysates were analyzed for type II collagen by immunoblotting. (C) The cells were lysed, and the p85 subunit of PI3K was immunoprecipitated from cell lysates using an anti-PI3K antibody, followed by competitive ELISA, as described in Materials and methods. Data are expressed as means ± SEM of 3 independent experiments. $^*P < 0.005$ versus control group. (D) Mouse embryo limb bud mesenchymal cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without 10 μM LY294002 (LY) for 2 days. Cell lysates were analyzed for phospho-PI3K p85 (Tyr508) and PI3K p85. GAPDH served as a loading control for immunoblots. The images are representative of 3 or more independent experiments.

showed disappearance of stress fibres and were positively stained for type II collagen (Fig. 1A). Chondrogenic induction of blebbistatin was confirmed by Western blot using anti-type II collagen antibody (Fig. 1B).

To examine whether blebbistatin-induced chondrogenesis required PI3K activity, the mesenchymal cells were incubated with blebbistatin and LY294002, and chondrogenesis was examined using immunofluorescence and Western blot with anti-type II collagen antibody. As shown in Figure 1A,B, inhibition of PI3K with LY294002 blocked blebbistatin-induced expression of type II collagen. LY294002 did not block the disappearance of stress fibres induced by blebbistatin, indicating that PI3K signalling is involved in the chondrogenic process following dissolution of actin stress fibres by blebbistatin. To determine whether PI3K activity is regulated by blebbistatin treatment, PI3K activity was measured. The assay used immunoprecipitated enzyme to measure the generation of $PI(3,4,5)P_3$, a product of $PI(4,5)P_2$ conversion by PI3K. Blebbistatin increased PI3K activity by 2.01 fold (Fig. 1C). To confirm the above result, we tested whether blebbistatin affects the phosphorylation of PI3K subunit p85. Figure 1D shows that blebbistatin increased the phosphorylation of PI3K subunit p85 at Tyr508.

Blebbistatin activates PDK1 but not Akt

PDK1 functions downstream from PI3K in receptor tyrosine kinase signal transduction pathways (Vanhaesebroeck & Alessi 2000). To investigate the downstream components of the PI3K pathway, we determined whether PDK1 is involved in the PI3K pathway. Blebbistatin increased the phosphorylation of PDK1 (Fig. 2A). Inhibition of PDK1 activity with GSK2337740, a highly specific ATP-competitive PDK1 inhibitor (Najafov et al. 2011), blocked blebbistatin-induced chondrogenesis (Fig. 2B,C). LY294002 inhibited the phosphorylation of PDK1 that was increased by blebbistatin (Fig. 2A), indicating that blebbistatin activated the PI3K/PDK1 pathway.

Targets of PDK1 include many protein kinases belonging to the AGC family, including Akt and p70 ribosomal protein S6 kinase (p70S6K) (Mora et al. 2004). Therefore, we examined whether Akt is downstream of the PI3K/PDK1 pathway in blebbistatin-induced chondrogenesis. Blebbistatin treatment results in little change or even decreased level of Akt phosphorylation (Fig. 2D). However, inhibition of Akt with Akt inhibitor IV almost completely blocked blebbistatin-induced expression of type II collagen (Fig. 2E,F). These results indicate that Akt is not involved in the PI3K/PDK1 signalling pathway activated by blebbistatin, but the activity of Akt is essential for chondrogenesis.

mTOR/p70S6K1 is implicated in the PI3K/PDK1 pathway activated by blebbistatin

The kinase mTOR is central in the PI3K signalling pathway (Hay & Sonenberg 2004). Therefore, we deter-

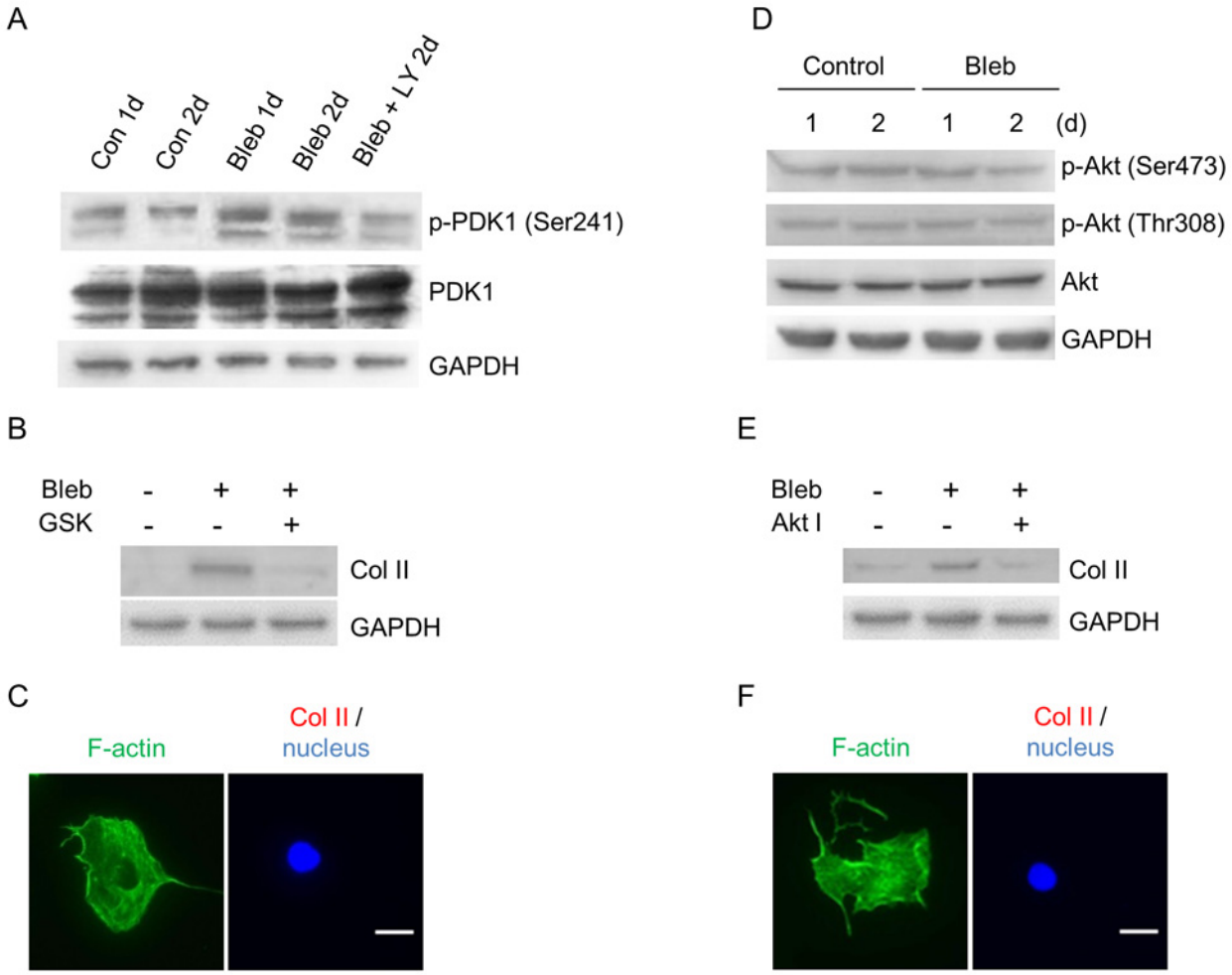

Fig. 2. Blebbistatin activates PDK1 but not Akt. (A) Mesenchymal cells were treated with 100 μM blebbistatin (Bleb) or 10 μM LY294002 (LY) for 1 or 2 days, and cell lysates were analyzed for phospho-PDK1 (Ser241) and PDK1 by immunoblotting. GAPDH served as a loading control. (B) Cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without 10 μM GSK2334470 (GSK) for 2 days and cell lysates were analyzed for type II collagen by immunoblotting. (C) Cells were incubated in the presence of blebbistatin and GSK2334470 for 2 days and stained for F-actin (phalloidin, green), type II collagen (Coll II, red). The cell nuclei were stained with DAPI (blue). Scale bar: 10 μm. (D) Mesenchymal cells were incubated in the presence or absence of blebbistatin for 1 or 2 days. Cell lysates were analyzed for phospho-Akt (Ser473), phospho-Akt (Thr308) and Akt by immunoblotting. (E) Cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without 0.5 μM Akt inhibitor IV (Akt I) for 2 days and cell lysates were analyzed for type II collagen by immunoblotting. (F) Cells were incubated in the presence of blebbistatin and Akt inhibitor IV for 2 days and stained for F-actin (phalloidin, green), type II collagen (Coll II, red). The cell nuclei were stained with DAPI (blue). Scale bar: 10 μm. GAPDH served as a loading control for immunoblots. The images are representative of 3 or more independent experiments.

mined whether mTOR is downstream of PI3K/PDK1. As shown in Figure 3A, blebbistatin increased the phosphorylation of mTOR at Ser2448. LY294002 and GSK2337740 inhibited the phosphorylation of mTOR that was increased by blebbistatin (Fig. 3B). In addition, rapamycin decreased phosphorylation of mTOR and blocked chondrogenesis induced by blebbistatin (Fig. 3C). p70S6K is a downstream effector of the mTOR pathway (Berven & Crouch 2000). Phosphorylation of p70S6K was increased by blebbistatin treatment (Fig. 3A), and decreased by LY294002, GSK2337740 (Fig. 3B), and rapamycin (Fig. 3C). These results suggest that blebbistatin induces chondrogenesis of single-cell cultures of mesenchymal cells by activating the PI3K/PDK1/mTOR/p70S6K pathway.

Akt is regulated by mTORC2 that is independent of blebbistatin treatment

In the previous section, we showed that Akt activity is essential for chondrogenesis, but is not regulated by blebbistatin treatment. Therefore, we sought to investigate how Akt is regulated. Phosphorylation of Akt at Ser473 in the carboxyl-terminal hydrophobic motif is induced by mTOR complex 2 (mTORC2) (Sarbassov et al. 2005). Whereas rapamycin inhibits mTORC1, pp242 inhibits both mTORC1 and mTORC2 (Apsel et al. 2008). Blebbistatin did not affect the phosphorylation of mTOR at Ser2481 (Fig. 4A). Although rapamycin did not affect the phosphorylation of Akt at Ser473, pp242 inhibited this phosphorylation. pp242 did not affect the phosphorylation of Akt at Thr308 (Fig. 4B), indicating that pp242 inhibits mTORC2. These results

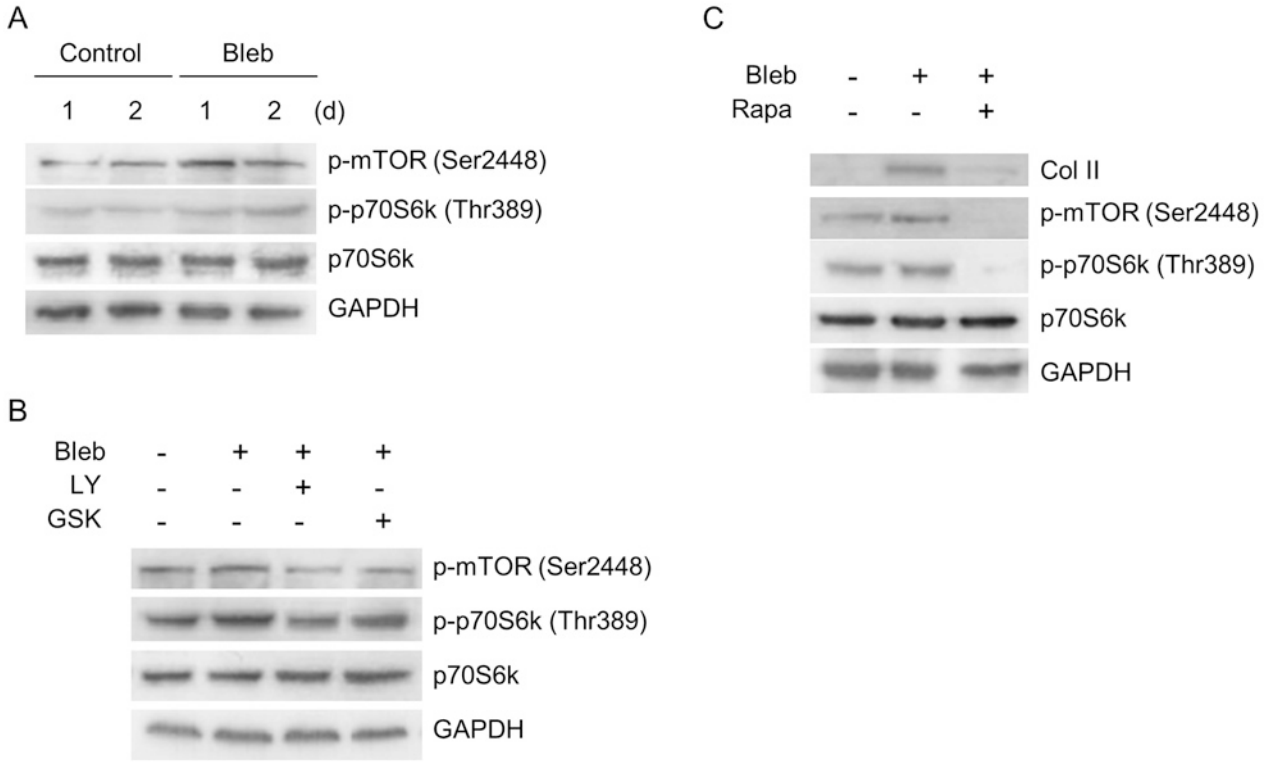

Fig. 3. Blebbistatin activates mTOR/p70S6K through the PI3K/PDK1 pathway. (A) Mesenchymal cells were incubated in the presence or absence of blebbistatin for 1 or 2 days. Cell lysates were analyzed for phospho-mTOR (Ser2448), phospho-p70S6K (Thr389), and p70S6K by immunoblotting. (B) Cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without LY294002 or GSK2334470 for 2 days. Cell lysates were analyzed for phospho-mTOR (Ser2448), phospho-p70S6K (Thr389), and p70S6K by immunoblotting. (C) Cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without 20 nM rapamycin (Rapa) for 2 days. Cell lysates were analyzed for type II collagen, phospho-mTOR (Ser2448), phospho-p70S6K (Thr389), and p70S6K by immunoblotting. GAPDH served as a loading control. The images are representative of 3 or more independent experiments.

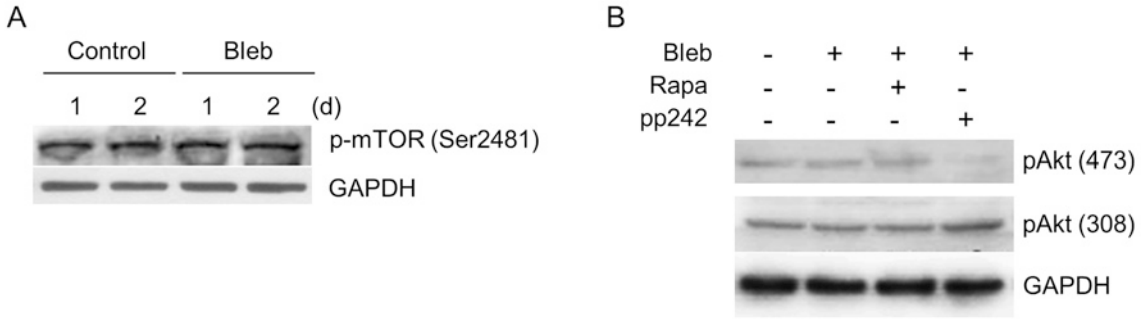

Fig. 4. Akt is regulated by mTORC2 independently of blebbistatin. (A) Mesenchymal cells were incubated in the presence or absence of blebbistatin for 1 or 2 days. Cell lysates were analyzed for phospho-mTOR (Ser2448). (B) Cells were incubated in the presence or absence of 100 μM blebbistatin (Bleb) with or without 20 nM rapamycin (Rapa) or pp242 for 2 days. Cell lysates were analyzed for phospho-Akt (Ser473) and phospho-Akt (Thr308). GAPDH served as a loading control. The images are representative of 3 or more independent experiments.

suggest that Akt is regulated by mTORC2 independently of blebbistatin treatment.

Discussion

Regulation of the actin cytoskeleton is one of the diverse functions of PI3K. PI3K is required for actin filament remodelling induced by growth factors (Rodriguez-Viciana et al. 1994; Wennström et al. 1994) and insulin (Tsakiridis et al. 1999). PI3K also induces actin filament remodelling through Akt and p70S6K1 (Qian et al. 2004). Conversely, mechanical properties of the microenvironment alter the activation of the PI3K pathway. For example, cyclic strain deforms cells and activates PI3K (Hoshino et al. 2007; Gayer et al. 2009). In the present study, blebbistatin removed actin stress fibres and increased PI3K activity and phosphorylation (Fig. 1). Inhibition of PI3K with LY294002 blocked blebbistatin-induced chondrogenesis, but did not influence blebbistatin-induced reorga-

nization of actin filaments. These results indicate that blebbistatin-induced changes in the actin cytoskeleton activated PI3K, leading to chondrogenic differentiation.

Several proteins function as downstream targets of PI(3,4,5)P_3 produced by PI3K, including PDK1 (Hirsch et al. 2007). In our study, blebbistatin increased PDK1 phosphorylation that was suppressed by LY294002 (Fig. 2). In addition, inhibition of PDK1 with GSK2334470 blocked blebbistatin-induced chondrogenesis, indicating that PDK1 is downstream of PI3K during blebbistatin-induced chondrogenesis. To our knowledge, this is the first report demonstrating that PDK1 is downstream of PI3K during chondrogenesis.

Activated PDK1 phosphorylates and activates the AGC family of protein kinases including Akt and p70S6K (Mora et al. 2004). Akt contains 2 regulatory phosphorylation sites: Thr308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domain. Akt is phosphorylated at Thr308 by PDK1 and becomes partially activated. Complete activation of Akt occurs when it is phosphorylated at both Thr308 and Ser473 (Song et al. 2005). Interestingly, our results show that phosphorylation of Akt at Thr308 and Ser473 was not significantly affected by blebbistatin, although inhibition of Akt blocked blebbistatin-induced chondrogenesis. The PI3K/Akt pathway has been shown to be involved in insulin-induced chondrogenesis (Hidaka et al. 2001; Fujita et al. 2004) and enhancement of chondrogenesis by hypoxia (Lee et al. 2013). Our present data suggest that Akt is not downstream of the PI3K/PDK1 pathway activated by blebbistatin and is presumably regulated by other signalling pathways.

Studies have shown that mTOR is implicated in chondrogenic differentiation. Rapamycin inhibits chondrogenic differentiation (Oh et al. 2001; Phornphuktul et al. 2008) and skeletal growth (Phornphuktul et al. 2009). Furthermore, mechanical stress, e.g., cyclic loading of embryonic chicken growth plate chondrocytes, induces sustained activation of mTOR (Guan et al. 2014). Consistent with these data, our present results show that removal of actin stress fibres by blebbistatin increased the phosphorylation of mTOR on Ser2448 and that inhibition of mTOR with rapamycin blocked blebbistatin-induced chondrogenesis (Fig. 3A). Further, phosphorylation of mTOR on Ser2448 was inhibited by LY294002 (Fig. 3B). mTOR interacts with several proteins to form two distinct complexes, namely mTORC1 and mTORC2. In mTORC1, mTOR is phosphorylated predominantly on Ser2448, whereas in mTORC2, mTOR is phosphorylated predominantly on Ser2481 (Copp et al. 2009). Thus, the current data suggest that PI3K activated by blebbistatin increased the activity of mTORC1.

mTOR has been implicated in the phosphorylation of p70S6K (Berven & Crouch 2000). mTORC1 is a major downstream effector of Akt (Slomovtiz & Coleman 2012). The signalling leading to activation of mTOR/p70S6K by insulin has been extensively studied and involves the canonical association of PI3K, PDK1, and Akt (Avruch et al. 2006; Corradetti & Guan 2006; Bertrand et al. 2008). In the present study, blebbistatin increased the phosphorylation of p70S6K (Fig. 3A), and inhibition of PI3K or PDK1 decreased the phosphorylation of p70S6K (Fig. 3B). Based on these results, we can assume that PI3K/PDK1/mTOR/p70S6K plays a role in blebbistatin-induced chondrogenesis. However, as our results show that Akt was not activated by blebbistatin treatment, it seems reasonable to rule Akt out of the above pathway. It has been shown that mTORC1 activity is controlled by a PI3K/Akt-independent pathway mediated by PDK1 (Finlay et al. 2012). In addition, although leucine-dependent activation of mTOR/p70S6K is prevented by PI3K inhibitors, it does not involve PKB/Akt activation (Peyrollier et al. 2000; Greiwe et al. 2001; Krause et al. 2002), but requires PDK1 (Sanchez Canedo et al. 2010). Our present data show that inhibition of PDK1 decreased the phosphorylation of mTOR (Fig. 3B). This result confirms that PI3K/PDK1 regulates the mTOR/p70S6K pathway.

As mentioned above, Akt was not regulated by blebbistatin. It has been documented that Akt is phosphorylated at Ser473 by mTORC2 (Sarbassov et al. 2005). However, the phosphorylation of mTOR on Ser2481 was not affected by blebbistatin in our study (Fig. 5A), indicating that mTORC2 is not involved in blebbistatin-induced chondrogenesis. Although the mTORC1 inhibitor rapamycin did not affect the phosphorylation of Akt on Ser473 and Thr308, the mTORC1 and mTORC2 dual inhibitor pp242 blocked the phosphorylation of Akt on Ser473, but not on Thr308 (Fig. 4B). These results indicate that Akt is regulated by mTORC2 independently of blebbistatin.

Taken together, our results suggest that reorganization of the actin cytoskeleton by blebbistatin induces chondrogenesis of single mesenchymal cells via the PI3K/PDK1/mTOR/p70S6K pathway. Our findings also show that mTORC2 regulates Akt independently of blebbistatin and Akt activity is essential for chondrogenesis.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2009749).

References

Apsel B., Blair J.A., Gonzalez B., Nazif T.M., Feldman M.E., Aizenstein B., Hoffman R., Williams R.L., Shokat K.M. & Knight Z.A. 2008. Targeted polypharmacology: discovery of dual inhibitors of tyrosine and phosphoinositide kinases. Nat. Chem. Biol. **4:** 691–699.

Avruch J., Hara K., Lin Y., Liu M., Long X., Ortiz-Vega S. & Yonezawa K. 2006. Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase. Oncogene **25:** 6361–6372.

Bang O.S., Kim E.J., Chung J.G., Lee S.R., Park T.K. & Kang S.S. 2000. Association of focal adhesion kinase with fibronectin and paxillin is required for precartilage condensation of chick mesenchymal cells. Biochem. Biophys. Res. Commun. **278:** 522–529.

Bertrand L., Horman S., Beauloye C. & Vanoverschelde J.L. 2008. Insulin signalling in the heart. Cardiovasc. Res. **79:** 238–248.

Berven L.A. & Crouch M.F. 2000. Cellular function of p70S6K: a role in regulating cell motility. Immunol. Cell Biol. **78:** 447–451.

Cantley L.C. 2002. The phosphoinositide 3-kinase pathway. Science **296:** 1655–1657.

Copp J., Manning G. & Hunter T. 2009. TORC-specific phosphorylation of mammalian target of rapamycin (mTOR): phospho-Ser2481 is a marker for intact mTOR signaling complex 2. Cancer Res. **69:** 1821–1827.

Corradetti M.N. & Guan K.L. 2006. Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? Oncogene **25:** 6347–6360.

Finlay D.K., Rosenzweig E., Sinclair L.V., Feijoo-Carnero C., Hukelmann J.L., Rolf J., Panteleyev A.A., Okkenhaug K. & Cantrell D.A. 2012. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. J. Exp. Med. **209:** 2441–2453.

Fujita T., Fukuyama R., Enomoto H. & Komori T. 2004. Dexamethasone inhibits insulin-induced chondrogenesis of ATDC5 cells by preventing PI3K-Akt signaling and DNA binding of Runx2. J. Cell. Biochem. **93:** 374–383.

Gayer C.P., Chaturvedi L.S., Wang S., Craig D.H., Flanigan T. & Basson M.D. 2009. Strain-induced proliferation requires thse phosphatidylinositol 3-kinase/AKT/glycogen synthase kinase pathway. J. Biol. Chem. **284:** 2001–2011.

Greiwe J.S., Kwon G., McDaniel M.L. & Semenkovich C.F. 2001. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. **281:** E466–E471.

Guan Y., Yang X., Yang W., Charbonneau C. & Chen Q. 2014. Mechanical activation of mammalian target of rapamycin pathway is required for cartilage development. FASEB J. **28:** 4470–4481.

Hay N. & Sonenberg N. 2004. Upstream and downstream of mTOR. Genes Dev. **18:** 1926–1945.

Hidaka K., Kanematsu T., Takeuchi H., Nakata M., Kikkawa U. & Hirata M. 2001. Involvement of the phosphoinositide 3-kinase/protein kinase B signaling pathway in insulin/IGF-I-induced chondrogenesis of the mouse embryonal carcinoma-derived cell line ATDC5. Int. J. Biochem. Cell Biol. **33:** 1094–1103.

Hirsch E., Costa C. & Ciraolo E. 2007. Phosphoinositide 3-kinases as a common platform for multi-hormone signaling. J. Endocrinol. **194:** 243–256.

Hoshino Y., Nishimura K. & Sumpio B.E. 2007. Phosphatase PTEN is inactivated in bovine aortic endothelial cells exposed to cyclic strain. J. Cell. Biochem. **100:** 515–526.

Katso R., Okkenhaug K., Ahmadi K., White S., Timms J. & Waterfield M.D. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu. Rev. Cell Dev. Biol. **17:** 615–675.

Kim M.J., Kim S., Kim Y., Jin E.J. & Sonn J.K. 2012. Inhibition of RhoA but not ROCK induces chondrogenesis of chick limb mesenchymal cells. Biochem. Biophys. Res. Commun. **418:** 500–505.

Krasilnikov M.A. 2000. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. Biochemistry (Moscow) **65:** 59–67.

Krause U., Bertrand L., Maisin L., Rosa M. & Hue L. 2002. Signalling pathways and combinatory effects of insulin and amino acids in isolated rat hepatocytes. Eur. J. Biochem. **269:** 3742–3750.

Langelier E., Suetterlin R., Hoemann C.D., Aebi U. & Buschmann M.D. 2000. The chondrocyte cytoskeleton in mature articular cartilage: structure and distribution of actin, tubulin, and vimentin filaments. J. Histochem. Cytochem. **48:** 1307–1320.

Lee H.H., Chang C.C., Shieh M.J., Wang J.P., Chen Y.T., Young T.H. & Hung S.C. 2013. Hypoxia enhances chondrogenesis and prevents terminal differentiation through PI3K/Akt/FoxO dependent anti-apoptotic effect. Sci. Rep. **3:** 2683.

Lim Y.B., Kang S.S., Park T.K., Lee Y.S., Chun J.S. & Sonn J.K. 2000. Disruption of actin cytoskeleton induces chondrogenesis of mesenchymal cells by activating protein kinase C-alpha signaling. Biochem. Biophys. Res. Commun. **273:** 609–613.

Mora A., Komander D., van Aalten D.M. & Alessi D.R. 2004. PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. **15:** 161–170.

Najafov A., Sommer E.M., Axten J.M., Deyoung M.P. & Alessi D.R. 2011. Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. Biochem. J. **433:** 357–369.

Oh C.D. & Chun J.S. 2003. Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1. J. Biol. Chem. **278:** 36563–36571.

Oh C.D., Kim S.J., Ju J.W., Song W.K., Kim J.H., Yoo Y.J. & Chun J.S. 2001. Immunosuppressant rapamycin inhibits protein kinase C alpha and p38 mitogen-activated protein kinase leading to the inhibition of chondrogenesis. Eur. J. Pharmacol. **427:** 175–185.

Park E.H., Kang S.S., Lee Y.S., Kim S.J., Jin E.J., Tak E.N. & Sonn J.K. 2008. Integrity of the cortical actin ring is required for activation of the PI3K/Akt and p38 MAPK signaling pathways in redifferentiation of chondrocytes on chitosan. Cell Biol. Int. **32:** 1272–1278.

Peyrollier K., Hajduch E., Blair A.S., Hyde R. & Hundal H.S. 2000. L-leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of system A amino acid transport. Biochem. J. **350:** 361–368.

Phornphutkul C., Lee M., Voigt C., Wu K.Y., Ehrlich M.G., Gruppuso P.A. & Chen Q. 2009. The effect of rapamycin on bone growth in rabbits. J. Orthop. Res. **27:** 1157–1161.

Phornphutkul C., Wu K.Y., Auyeung V., Chen Q. & Gruppuso P.A. 2008. mTOR signaling contributes to chondrocyte differentiation. Dev. Dyn. **237:** 702–712.

Qian Y., Corum L., Meng Q., Blenis J., Zheng J.Z., Shi X., Flynn D.C. & Jiang B.H. 2004. PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration. Am. J. Physiol. **286:** C153–C163.

Rodriguez-Viciana P., Warne P.H., Dhand R., Vanhaesebroeck B., Gout I., Fry M.J., Waterfield M.D. & Downward J. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature **370:** 527–532.

Sanez Canedo C., Demeulder B., Ginion A., Bayascas J.R., Balligand J.L., Alessi D.R., Vanoverschelde J.L., Beauloye C., Hue L. & Bertrand L. 2010. Activation of the cardiac mTOR/p70(S6K) pathway by leucine requires PDK1 and correlates with PRAS40 phosphorylation. Am. J. Physiol. Endocrinol. Metab. **298:** E761–E769.

Sarbassov D.D., Ali S.M. & Sabatini D.M. 2005. Growing roles for the mTOR pathway. Curr. Opin. Cell Biol. **17:** 596–603.

Slomovitz B.M. & Coleman R.L. 2012. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin. Cancer Res. **18:** 5856–5864.

Solursh M., Linsenmayer T.F. & Jensen K.L. 1982. Chondrogenesis from single limb mesenchyme cells. Dev. Biol. **94:** 259–264.

Song G., Ouyang G. & Bao S. 2005. The activation of Akt/PKB signaling pathway and cell survival. J. Cell. Mol. Med.**9:** 59–71.

Straight A.F., Cheung A., Limouze J., Chen I., Westwood N.J., Sellers J.R. & Mitchison T.J. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science **299:** 1743–1747.

Tsakiridis T., Tong P., Matthews B., Tsiani E., Bilan P.J., Klip A. & Downey G.P. 1999. Role of the actin cytoskeleton in insulin action. Microsc. Res. Tech. **47:** 79–92.

Vanhaesebroeck B. & Alessi D.R. 2000. The PI3K-PDK1 connection: more than just a road to PKB. Biochem. J. **346:** 561–576.

Wennström S., Hawkins P., Cooke F., Hara K., Yonezawa K., Kasuga M., Jackson T., Claesson-Welsh L. & Stephens L. 1994. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. Curr. Biol. **4:** 385–393.

Wezeman F.H. 1998. Morphological foundations of precartilage development in mesenchyme. Microsc. Res. Tech. **43:** 91–101.

Zanetti N.C. & Solursh M. 1984. Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. J. Cell Biol. **99:** 115–123.

Received May 12, 2017
Accepted June 9, 2017