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Akt Signaling Regulates Actin Organization Via Modulation of MMP-2 Activity During Chondrogenesis of Chick Wing Limb Bud Mesenchymal Cells

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Abstract Endochondral ossification is initiated by the differentiation of mesenchymal precursor cells to chondrocytes. This process is characterized by a strong interdependence of cell shape and cytoskeletal organization accompanying the onset of chondrogenic gene expression, but the molecular mechanisms mediating these interactions are not known. In this study, we hypothesized that the activation of matrix metalloproteinase (MMP)-2 would be involved in the reorganization of the actin cytoskeleton and that this would require an Akt-dependent signaling pathway in chick wing bud mesenchymal cells. The pharmacological inhibition of Akt signaling resulted in decreased glycosaminoglycan synthesis and reduced the level of active MMP-2, leading to suppressed cortical actin organization which is characteristic of differentiated chondrocytes. In addition, the exposure of cells to bafilomycin A1 reversed these chondro-inhibitory effects induced by inhibition of Akt signaling. In conclusion, our data indicate that Akt signaling is involved in the activation of MMP-2 and that this Akt-induced activation of MMP-2 is responsible for reorganization of the actin cytoskeleton into a cortical pattern with parallel rounding of chondrogenic competent cells. J. Cell. Biochem. 102: 252–261, 2007.

Key words: Akt; MMP-2; actin cytoskeleton; chondrogenic differentiation

During development, most bones form through endochondral ossification, in which bones are first laid down as cartilage precursors [Karsenty and Wagner, 2002]. This process involves a precise series of events including aggregation and differentiation of mesenchymal cells, proliferation, hypertrophy and death of chondrocytes [DeLise et al., 2000]. It also involves a transition of multiple types of cells, with varying cellular morphologies. Chondrogenesis is characterized by drastic changes in cell shape from a fibroblastoid to round or polygonal morphologies [von der Mark and von der Mark, 1977]. This transition is accompanied by changes in gene expression and is reverted when chondrocytes dedifferentiate, for example in osteoarthritis and in monolayer cultures in vitro [Sandell and Aigner, 2001; Schnabel et al., 2002]. The molecular mechanisms responsible for these interactions are largely unknown, but reorganization of actin filaments is a critical regulatory factor for chondrogenesis [Daniels and Solursh, 1991; Kim et al., 2003]. Chondrocytes display mostly cortical organization of their actin filaments, whereas precursor cells or dedifferentiated chondrocytes are characterized by a more fibrillar organization [Idowu et al., 2000; Langelier et al., 2000].

Phosphatidylinositol 3-kinase (PI3K)-Akt signaling plays a prominent role in several processes considered the hallmark of cancer [Testa and Bellacosa, 2001]. Akt/protein kinase B (PKB), a serine/threonine protein kinase, plays a critical role in controlling the balance between cell survival and apoptosis [Franke

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et al., 1997]. Phosphorylation of Akt is promoted by phophatidylinositol-3,4,5-triphosphate, and phasphatidylinositol-3,4-bisphosphate generated by phosphoinositide 3-kinase. This facilitates transmembrane signaling by serving as membrane-localization elements to recruit target proteins to specific sites in response to various growth/survival factors [Hemmings, 1997; Klippel et al., 1997]. Although it has been reported that Akt promotes cancer cell invasion via increased motility and metalloproteinase production [Kim et al., 2001], the function of Akt in chick mesenchymal cells has not yet been clarified.

In the present study, we investigated the functional role of Akt at late stage of chondrogenic differentiation using cultured chick wing bud mesenchymal cells. Akt was found to be a positive regulator of matrix metalloproteinase (MMP)-2 induction in reorganization of the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture and Treatments

Micromass cultures of mesenchymal cells, derived from the distal tips of Leghorn chicken embryos at Hamburger–Hamilton (HH) stage 22/23 were established as described previously [Jin et al., 2006]. The cells were suspended at a density of 2×10^7 cells/ml in Ham's F-12 medium containing 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin (Gibco Invitrogen, Grand Island, NY). The cells were plated on 60 mm Corning culture dishes at 19 drops of 15 μl cell suspension per dish, and incubated for 1 h at 37°C under 5% CO_2 to allow attachment. The cells were maintained in 2 ml of culture medium for the indicated time periods in the absence or presence of the following reagents: 10 μM GM6001, 10 μM PD98059, Akt inhibitor IV (Chemicon, Temecula, CA), or 100 nM Bafilomycin A1 (Sigma Chemical Co., St. Louis, MO).

Analysis of Cell Differentiation

Chondrogenesis was measured by Alcian blue staining of sulfated cartilage glycosaminoglycans. Alcian-blue-bound sulfated glycosaminoglycans were extracted with 6 M guanidine–HCl and quantified by measuring the absorbance of the extracts at 600 nm.

Western-Blot Analysis

Proteins (30 μg) were separated by 10% polyacrylamide gel electrophoresis containing 0.1% SDS and transferred to nitrocellulose membranes (Schleicher and Schuell, Germany). The membranes were incubated for 1 h at room temperature in blocking buffer (20 mM Tris–HCl, 137 mM NaCl, pH 8.0, containing 0.1% [v/v] Tween and 3% [w/v] non-fat dry milk), and probed with antibodies against Type II collagen (Santa Cruz Biotech, Santa Cruz, CA) and HSP70 (Stressgene, San Diego, CA). The blots were developed with a peroxidase-conjugated secondary antibody and reactive proteins were visualized by the electrochemiluminescence (ECL) system (Pierce Biotechnology, Inc., Rockford, MN).

Gelatin Zymography

Chick leg bud mesenchymal cells were incubated in serum-free media for 24 h after drug treatment. Total protein content of the cells was determined by the bicinchoninic acid method (Pierce Biotechnology, Inc., Rockford, MN). Loading amounts were standardized to the middle protein concentration for all samples from that particular iteration of the experiment. The conditioned media was then concentrated by 10% (v/v) trichloroacetic acid (TCA) precipitation. The precipitate was dissolved in 1× non-reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 10% [v/v] glycerol, 2% [w/v] sodium dodecyl sulfate, and 0.005% [w/v] Bromophenol blue) and separated by electrophoresis on 7.5% SDS-PAGE co-polymerized with gelatin (0.1%, w/v) as the substrate. After electrophoresis was complete, the gel was incubated for 30 min at room temperature in 2.5% Triton X-100 solution, and incubated at 37°C for 12–18 h in 50 mM Tris–HCl buffer, pH 7.6, containing 5 mM $CaCl_2$, 0.2 M NaCl, and 0.02% (w/v) Brij 35. The gels were stained with 0.05% (v/v) Coomassie Brilliant Blue R-250, 50% (v/v) ethanol and 10% (v/v) acetic acid, and then destained with 25% (v/v) ethanol and 10% (v/v) acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie-blue-stained gelatin.

Actin Staining

Cells grown on coverslips were treated with various chemicals as indicated in the figure

legends, washed three times with phosphate-buffered saline (PBS), then fixed and permeabilized as described above. Each culture was then stained with Alexa488-phalloidin (Molecular Probes, Eugene, OR) prepared in PBS containing 1% (v/v) bovine serum albumin for 1 h at room temperature in a lightproof box. The cultures were then washed three times with water and mounted with Gel/Mount (Biomedia, Foster City, CA). The slides were examined using a confocal microscope (MRC 1024/ES, Bio-Rad Laboratory, CA). Cultures were imaged at scan speed 8 (1.76 μs per voxel, 1 s per section) in 1-μm-thick Z-sections for a total of 8–10 slices.

Statistical Analysis

Results of cell-adhesion experiments were pooled from five replicate samples derived from three independent experiments and expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey post hoc comparison of groups was used to test for significant effects. Differences were considered significant if $P < 0.05$.

RESULTS

Reorganization of the actin cytoskeleton to a cortical pattern with parallel rounding of cells and fewer stress fibers as visualized by phalloidin staining was observed after 4 days of culture (Fig. 1A). Treatment with cytochalasin D (CD), which causes disruption of the actin cytoskeleton [Lim et al., 2000], caused chondrogenic differentiation as assessed by Alcian blue staining of sulfated proteoglycan (Fig. 1B). These results show that microtubule polymerization is vital for chondrogenesis, in agreement with earlier studies that have shown reduced levels of glycosaminoglycan and proteoglycan production in colchicine-treated chondrocytes [Madsen et al., 1979; Takigawa et al., 1984].

An increase in the limb core cell density, which allows increased cell contact and the formation of gap junctions, is critical to chondrogenic development. With time in culture, the total cell number was significantly increased (Fig. 2A). We have previously demonstrated the negative role of Erk activation during chondrogenesis on the initiation of chondrogenic differentiation of chick limb bud mesenchymal cells [Oh et al., 2000; Yoon et al., 2000]. However, no data on temporal profiles of Akt expression and activation during chondrogenesis are available.

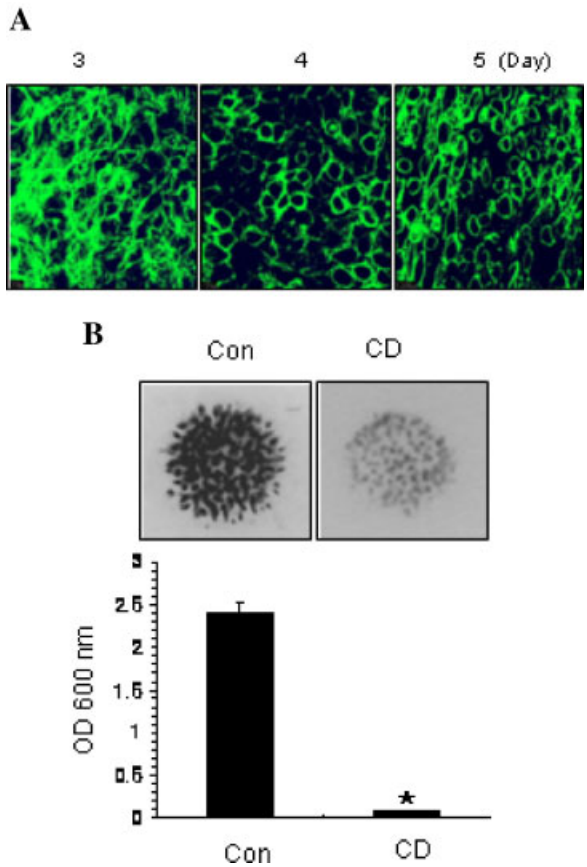

Fig. 1. Reorganization of cortical actin is essential for chondrogenic differentiation. Mesenchymal competent cells were prepared as described in the Materials and Methods. **A**: Cells were grown on coverslips and immunostained for F-actin with Alexa488-phalloidin at indicated days. **B**: Cells were treated cytochalasin D (CD) and stained with Alican blue at 5 days of culture. Asterisk (*) denotes statistically significant differences compared with control cells ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We examined the expression and phosphorylation levels of Akt during reorganization of the actin cytoskeleton to cortical pattern occurs—days 3–5. There were no marked changes in Akt protein levels. However, Akt phosphorylation increased as chondrogenesis progressed, as shown by the expression level of type II collagen, which is a chondrogenic differentiation marker (Fig. 2B). Activation of Akt has been shown to block the action of several pro-apoptotic proteins such as Bad [Hayakawa et al., 2000], and block cytochrome *c* release from the mitochondria through the regulation of Bcl-2 [Davies et al., 1999], so stress derived from Akt-induced increased cell density would be simply induced actin reorganization. To investigate this, chondroblasts cultured in a density-dependent

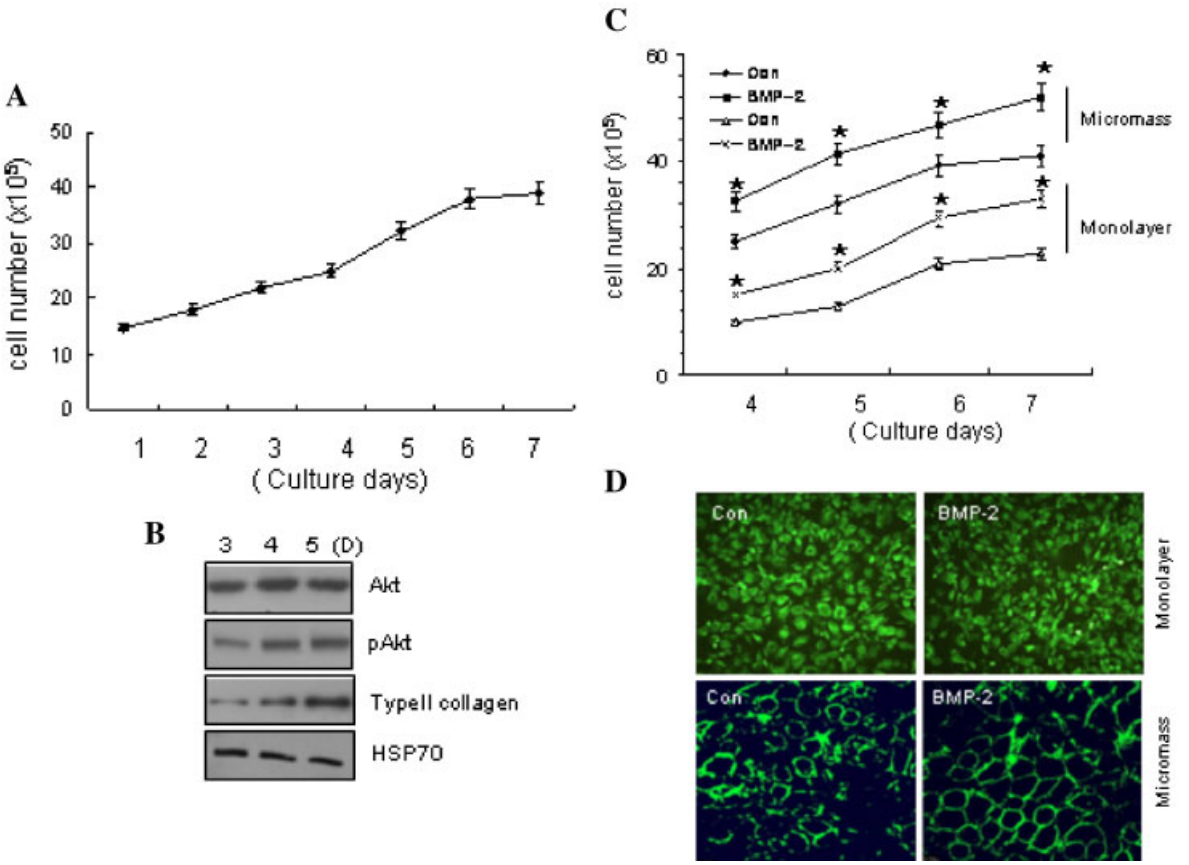

Fig. 2. Mesenchymal competent cells were prepared as described in Material and Methods. **A**: The number of viable cells in the control culture was determined at the indicated days. **B**: Changes in the protein levels of Akt, pAkt, and type II collagen during the late stage of chondrogenesis (i.e. 3, 4, 5 days of culture) were analyzed by Western blotting using respective antibodies. **C**: Monolayer- or micromass-cultured cells were treated with 100 ng/ml BMP-2 and the number of viable cells was determined at the indicated days. **D**: Cells were immunostained for F-actin with Alexa488-phalloidin at 4 days of culture. The data shown are representative of at least four independent experiments. Asterisk (*) denotes statistically significant differences compared with control cells ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

manner (monolayer vs. micromass) were treated with 100 ng/ml bone morphogenetic protein-2 (BMP-2), which suppresses apoptosis of cardiac myocytes and osteoblasts through activation of the SMAD pathway [Hay et al., 2001; Izumi et al., 2001], and actin organization was also analyzed. BMP-2 stimulated the proliferation of chick chondroblasts (Fig. 2C), but this increased cell density did not affect the organization and distribution of the actin cytoskeleton both in monolayer- and micromass-cultured chondroblasts (Fig. 2D), indicating that the stress from increased cell density may not cause actin reorganization.

We then asked whether inhibition of Akt and Erk singling would affect chondrogenesis by analyzing the effect of the Akt inhibitor IV (500 nM), on Alcian blue staining. Alcian blue stains for glycosaminoglycans is an established marker of chondrogenesis. When cells were exposed to Akt inhibitor IV on 3– 4 days of culture, the level of Alcian blue staining was reduced in micromass culture, indicating suppressed chondrogenic differentiation (Fig. 3A, upper panel). Inhibition of glycosaminoglycan production by the Akt inhibitor was confirmed quantitatively by dye extraction and measurement of absorbance (Fig. 3A, lower panel). We also investigated whether Akt signaling is associated with disruption of the actin cytoskeleton. Treatment with Akt inhibitor IV on 3–4 days of culture did not affect cell proliferation and viability (Fig. 3B,C). However, actin stress fibers were markedly intensified with Akt inhibitor IV treatment, pointing to the interaction of the actin cytoskeleton and Akt signaling is independent on cell density (Fig. 3D).

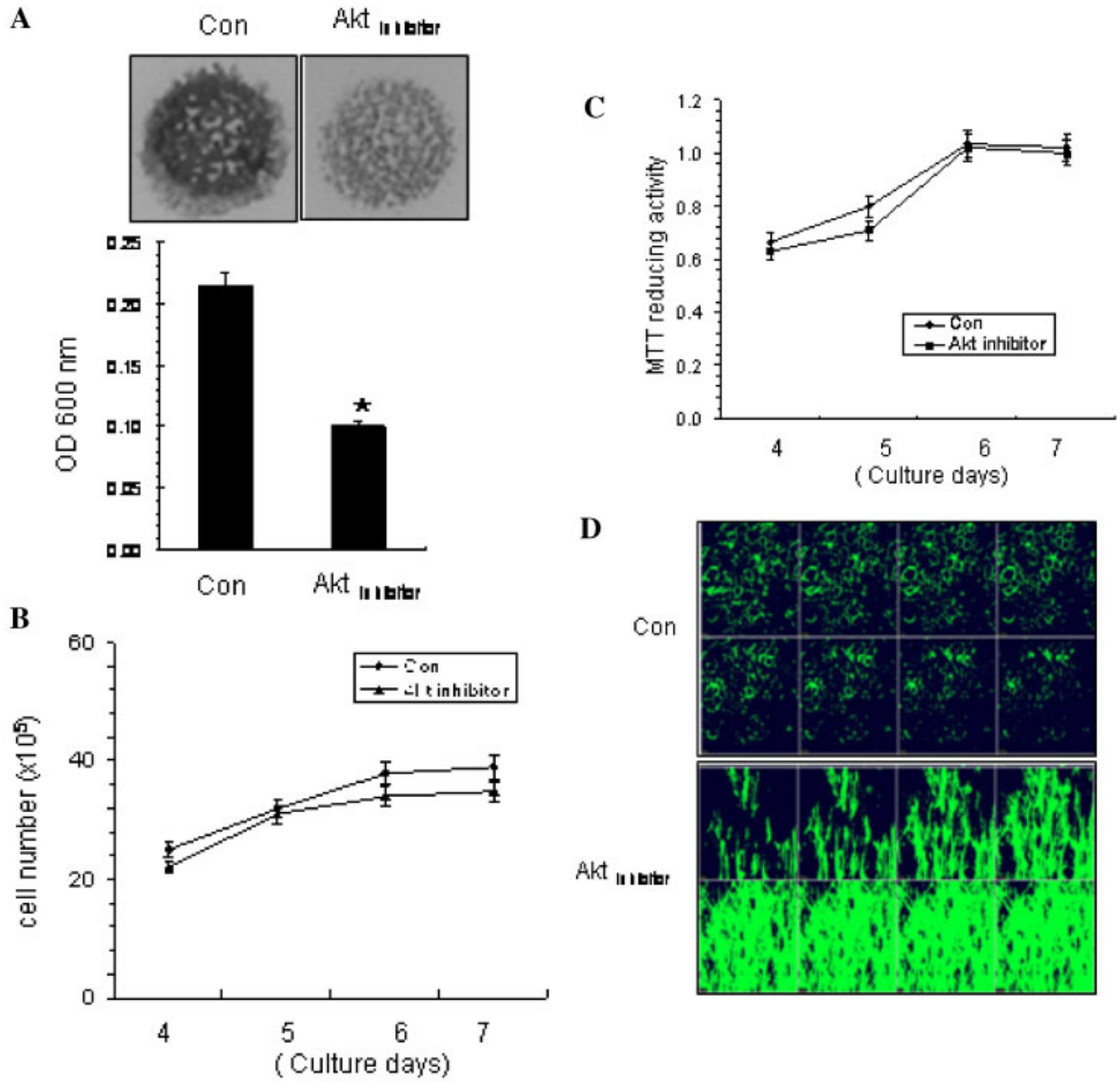

Fig. 3. Inhibition of Akt signaling suppresses chondrogenic differentiation. The mesenchymal cells were cultured at a density of 2×10^7 cells/ml with or without 500 nM Akt inhibitor IV ($Akt_{inhibitor}$) at 3 days of culture for 24 h. **A**: Cells were stained with Alcian blue at day 5 of culture (**upper panel**). Quantification of chondrogenesis was carried out by measuring the absorbance of bound Alcian blue at 600 nm (**lower panel**). **B**: The number of viable cells in the control culture was determined at the indicated days. **C**: Cell viability was determined using the MTT assay. **D**: Cells were immunostained for F-actin with Alexa488-phalloidin at 4 days of culture and imaged at scan speed 8 (1.76 μs per voxel, 1 s per section) in 1-μm-thick Z-sections for a total of 8 slices. The data shown are representative of at least four independent experiments. Asterisk (*) denotes statistically significant differences compared with control cells ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The regulation of MMPs is thought to be associated with cytoskeletal changes. There are few systematic studies on the expression patterns of the different MMPs and their inhibitors in cartilage and bone [Tamamura et al., 2005; Fichter et al., 2006; Soder et al., 2006]. MMP-9 (92 kDa type IV collagenase/gelatinase B) and MMP-2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase) are known to have an important roles in osteoclastic bone resorption [Parikka et al., 2005; Reinhardt et al., 2005] and cartilage remodeling [Lee et al., 2006; Milner et al., 2006]. To examine the time points and level of induction of gelatinases during the late period of micromass culture, conditioned media of chondroblasts taken at 3-, 4- and 5-day time points were analyzed by zymography. Conditioned media exhibited distinct bands on gelatin

gels, indicating the presence of MMPs with gelatinolytic and/or collagenolytic activity. The level of active MMP-2 was increased at 4 days of culture, but was then reduced by day 5 (Fig. 4A). Although activation of Erk functions as a negative signaling [Oh et al., 2000; Yoon et al., 2000], PD98059 did not affect the level of active MMP-2. This indicates that the regulation of MMP-2 activity is not related to Erk signaling. However, the increased level of active MMP-2 at 4 days of culture was significantly reduced when cells were exposed to 500 nM Akt inhibitor IV (Fig. 4B). Treatment with bafilomycin A1 did not affect the viability and proliferation of chondroblasts at 3–4 days of culture (Fig. 4C,D). We then investigated whether the increase in MMP-2 activation is accompanied by chondrocyte-specific changes in cellular morphology and actin organization (Fig. 4E). Cells were treated with 10 nM of bafilomycin A1 or 3 μM GM6001 in the absence or presence of Akt inhibitor IV. Bafilomycine A1 is an inhibitor of

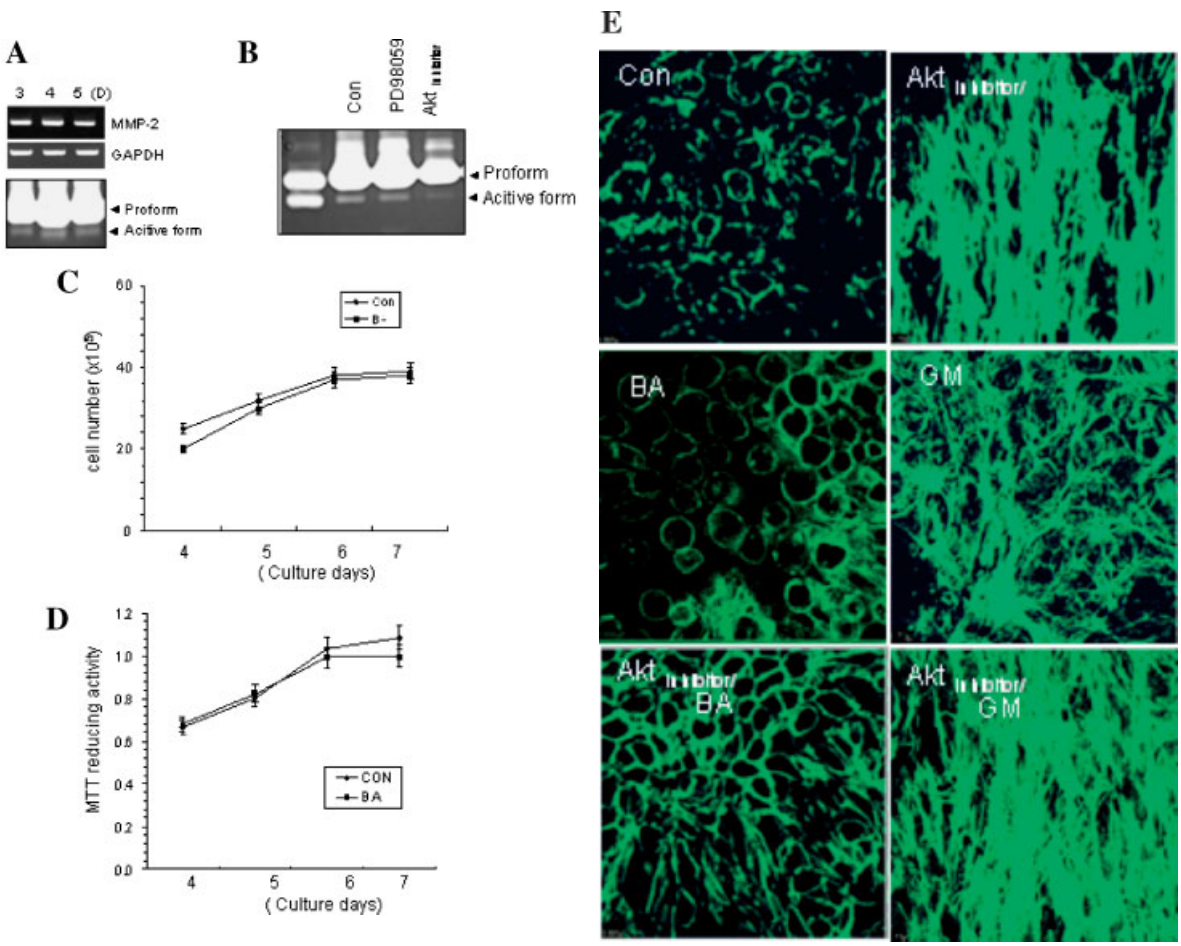

Fig. 4. Akt-induced MMP-2 activation is responsible for reorganization of the actin cytoskeleton. **A**: The mesenchymal cells were cultured at a density of 2×10^7 cells/ml and RT-PCR of cell extracts was performed to analyze the mRNA levels of *mmp-2* (**upper panel**) and the proteolytic activity of MMP-2 in conditioned media (CM) was analyzed by gelatin zymography (**lower panel**). **B**: The mesenchymal cells were cultured at a density of 2×10^7 cells/ml with or without 500 nM Akt inhibitor IV ($Akt_{inhibitor}$) at 3 days of culture for 24 h. Proteolytic activity of MMP-2 in conditioned media (CM) was analyzed. **C**: Cells were treated with bafilomycin A1 (BA) at 3 days of culture for 24 h and the number of viable cells in the control culture was determined at the indicated days. **D**: Cell viability was determined using the MTT assay. **E**: The mesenchymal cells grown on coverslips were cultured at a density of 2×10^7 cells/ml with or without 100 nM bafilomycin A1 (BA) and 10 μM GM6001 (GM) in the absence or presence of 500 nM Akt inhibitor IV ($Akt_{inhibitor}$) at 3 days of culture for 24 h. Cells were immunostained for F-actin with Alexa488-phalloidin at 4 days of culture. **F**: Cells were stained with Alcian blue at 5 days of culture (upper panel). Quantification of chondrogenesis was carried out by measuring the absorbance of bound Alcian blue at 600 nm (lower panel). The data shown are representative of at least four independent experiments. Asterisk (*) denotes statistically significant differences compared with control cells ($P < 0.01$).

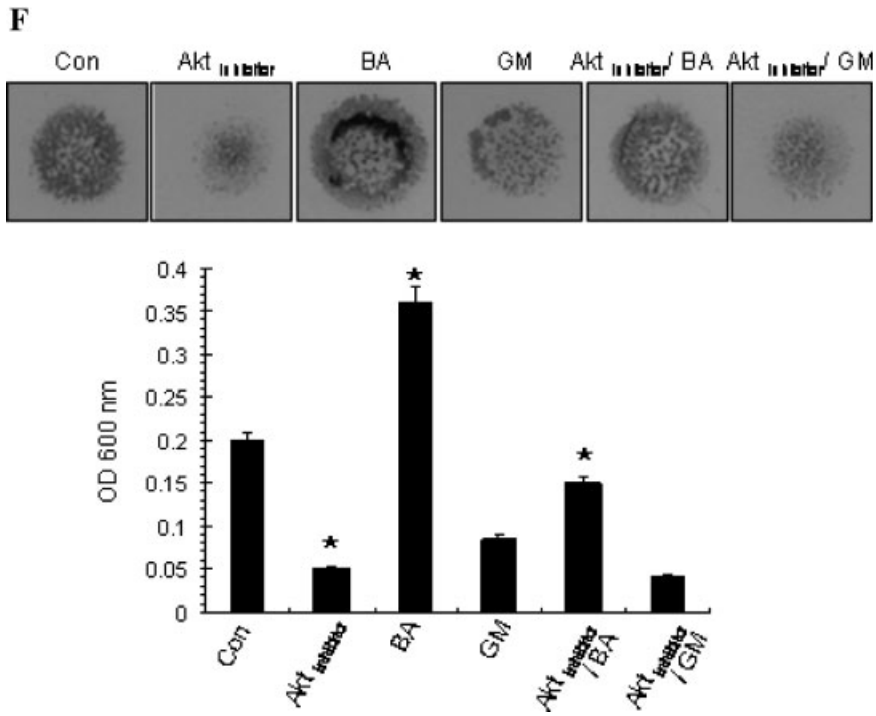

Fig. 4. (*Continued*)

H^+-ATPase. A previous report [Maquoi et al., 2003] showed that the activity of membrane type 1 MMP (MT1-MMP) on the cell surface is constitutively downregulated by a vacuolar H^+-ATPase-dependent degradation process. Blockade of this degradation led to the accumulation of tissue inhibitors of metalloproteases (TIMP)-free active MT1-MMP molecules on the cell surface and pro-MMP-2 activation was strongly increased owing to this impaired degradation. GM 6001 is a well-known inhibitor of broad spectrum of MMPs [Shen et al., 2006] and MMP-2 activity has been shown to be inhibited using GM6001 by 1–10 μM GM6001 in various cell types [Margulis et al., 2005]. Cultures treated with 100 μM bafilomycin A1 showed increased cell rounding and cells treated with 10 μM GM6001 displayed increased stress fibers compared with control cultures, which indicates that MMP-2 activation supports the establishment of a chondrocyte-specific cell shape and actin organization. In addition, co-treatment with bafilomycin A1 reduced the number of Akt-inhibition-induced stress fibers. The suppressed chondrogenic differentiation by Akt inhibitor IV was stimulated by co-treatment of bafilomycin A1 (Fig. 4E).

DISCUSSION

The molecular links between cytoskeletal organization and gene expression in chondrocytes are not well understood, despite the well-known relationship between cell shape and differentiation status in these cells and despite the recently discovered roles of actin-regulating pathways in the control of lineage commitment in undifferentiated mesenchymal cells. Chondrogenesis is characterized by drastic changes in cell shape from a fibroblastoid to a round or polygonal morphology [von der Mark and von der Mark, 1977]. In agreement with this, we also observed reorganization of the actin cytoskeleton to a cortical pattern with parallel rounding of cells. With increasing culture time, the total number of cells was significantly increased with a concomitant increased in the level of Akt phosphorylation. However, this increase in cell proliferation did not affect the actin organization, whereas the increase in Akt activation affected the actin organization during chondrogenic differentiation. This shows that the stress from increased cell density is not responsible for actin reorganization. In this study, we hypothesized that the Akt pathway that regulates actin polymerization controls cell

morphology during chondrogenic cell differentiation of chick limb bud mesenchymal cells. We showed that activation of Akt plays an important role in coordinating actin organization, cell shape and chondrogenic phenotype.

Akt (also called PKB), a serine/threonine kinase, is involved in promotion of cell survival, proliferation and metabolic responses [Downward, 1998; Lawlor and Alessi, 2001]. Akt activation is a multistep process involving coordinated actions of several catalytic and noncatalytic molecules such as Bad, cytochrome *c*, and Bcl-2 [Davies et al., 1999; Hayakawa et al., 2000]. Recently, it was shown that loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression in glioma cells is responsible for increased expression of MMP-2 or -9 [Koul et al., 2001; Kubiatowski et al., 2001; Park et al., 2002], which indicates that PTEN may mediate regulation of MMP expression. However, there is little evidence for the involvement of PTEN and Akt in MMP induction. Our data show that inhibition of the Akt pathway at a late period of micromass culture suppresses MMP-2 activation and glycosaminoglycan synthesis, a marker of chondrogenic differentiation and that this Akt inhibition also disrupted the organization and distribution of the actin cytoskeleton. Furthermore, activation of MMP-2 repressed the disruption of actin organization induced by inhibition of Akt. Our data provide insights into the emerging role of Akt in the regulation of actin remodeling via modulating MMP-2 activation.

In this study, several observations were coincided the idea that there is a connection among Akt, MMP-2 and the actin cytoskeleton. The maximum temporal levels of pAkt and active MMP-2 were coincided with the time when the actin cytoskeleton undergoes reorganization to a cortical pattern with parallel rounding of cells and the number of stress fibers is reduced. In addition, modulation of MMP-2 and Akt signaling affected the reorganization of the actin cytoskeleton and the destruction of the actin cytoskeleton induced by Akt inhibition was restored by activation of MMP-2. It is not clear how MMP-2 is functionally/structurally linked with cytoskeleton dynamics, but integrins could be a linker. There is a physical association of MMP-2 and β1-integrin in endothelial cells [Levkau et al., 2002] and β1-integrins induce the activation of MMP-2 [Mitra et al., 2004] via MT1-MMP, and forming a trimolecular complex promotes cell motility [Zigrino et al., 2001].

Taken together, our data indicate that Akt signaling is involved in activation of MMP-2 and that the activation of MMP-2 is responsible for reorganization of the actin cytoskeleton to a cortical pattern with parallel rounding of cells.

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