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Developmental Biology 308 (2007) 474–484

DEVELOPMENTAL BIOLOGY

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MMP-2 functions as a negative regulator of chondrogenic cell condensation via down-regulation of the FAK-integrin β1 interaction

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Received for publication 30 November 2006; revised 31 May 2007; accepted 5 June 2007
Available online 12 June 2007

Abstract

Matrix metalloprotease-2 (MMP-2) has the capacity to degrade cartilage extracellular matrix molecules, the turnover of which is an essential event in chondrogenesis. Here, we investigated the functional role of MMP-2 in chondrogenesis of leg bud mesenchymal cells. Small interference RNA (siRNA)-mediated knockdown of *mmp-2* promoted precartilage condensation and chondrogenesis. Treatment with bafilomycin A1, an MMP-2 activator, or GM6001, an MMP inhibitor, at the pre-condensation stage resulted in the inhibition or promotion of chondrogenesis, respectively. By comparison, treatment at the post-condensation stage had little or no effect on chondrogenesis. These results indicate that MMP-2 is involved in the regulation of cell condensation. Inhibition of MMP-2 activity by *mmp-2* specific siRNA increased the protein level of fibronectin, and integrins α5 and β1. The interaction between focal adhesion kinase (FAK) and integrin β1 leading to tyrosine phosphorylation of FAK was also enhanced. Moreover, inactivation of p38MAPK down-regulated the level of MMP-2 mRNA and activity, and increased mesenchymal cell condensation in parallel with enhanced phosphorylation of FAK. Taken together, our data indicate that MMP-2 mediates the inhibitory signals of p38MAPK during mesenchymal cell condensation by functioning as a negative regulator of focal adhesion activity regulated by FAK via interactions with fibronectin through integrin β1.
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Keywords: Chondrogenesis; Condensation; FAK; Fibronectin; Integrin; MMP-2; p38MAPK

Introduction

Chondrogenesis is a prerequisite event for cartilage formation in the developing limb, involving mesenchymal cell recruitment/migration, condensation of progenitors, and chondrocyte differentiation and maturation (Goldring et al., 2006; Olsen et al., 2000). Precartilage condensation forming the cartilaginous template of the future skeleton is a critical step in the initiation of cartilage differentiation and is mediated by the cell adhesion molecule, N-cadherin, and extracellular matrix (ECM) molecules including fibronectin, proteoglycans, and collagens (DeLise et al., 2000; Johnson and Tabin, 1997; Knudson and Knudson, 2001; Knudson et al., 1999; Sandell and Adler, 1999). ECM molecules interact with cell adhesion molecules to activate intracellular signaling pathways involving focal adhesion kinase (FAK) and to initiate the transition of chondroprogenitor cells to fully committed chondrocytes (Bang et al., 2000; DeLise et al., 2000).

Modulation of cell–matrix interactions occurs through the action of unique proteolytic systems responsible for hydrolysis of a variety of ECM components. Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell–matrix composition through the turnover of ECMs and function as key regulators of cell–ECM interactions during development and differentiation (Mannello et al., 2005a; Mott and Werb, 2004;

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doi:10.1016/j.ydbio.2007.06.003

Somerville et al., 2003; Vum and Werb, 2000). MMPs are also involved in many pathological conditions including rheumatoid arthritis, cancer, and defective wound healing by regulating the integrity and composition of the ECM structure (Mannello et al., 2005b; Nelson and Tien, 2006; Sakai et al., 2001).

The MMPs are Ca^{2+}- and Zn^{2+}-dependent endoproteinases classified into three subgroups on the basis of substrate specificity: interstitial collagenases, stromelysins, and gelatinases (Visse and Nagase, 2003; Woessner and Nagase, 2000). MMP-2 is a gelatinase secreted as a 72-kDa precursor and is processed to an active 62- to 64-kDa form (Aimes and Quigle, 1995; Werb and Chin, 1998). MMP-2 has the capacity to degrade ECM molecules, including gelatins, fibronectin, laminin, aggrecan, and collagen I, IV, V, VII and X (McCawley and Matrisian, 2001; Nagase and Woessner, 1999; Visse and Nagase, 2003). MMP-2 is expressed at high levels in myoblasts, skeletal muscle satellite cells, Schwann cells of peripheral nerves, neural crest cells of avian embryos, and in differentiating adipocytes, suggesting a critical role for this enzyme in myogenesis, neurogenesis, and adipogenesis (Bouloumie et al., 2001; Duong and Erickson, 2004; El Fahime et al., 2000; Guerin and Holland, 1995; Krekoski et al., 2002). The detection of high levels of MMP-2 in osteoarthritic cartilage and synovial fluid suggests involvement of MMP-2 in the degradation of cartilage ECM (Imai et al., 1997; Mohtai et al., 1993; Tanaka et al., 2000). Consistent with this hypothesis, testican-1, an inhibitor of pro-MMP-2 activation, is expressed in joint and growth plate cartilage (Hausser et al., 2004). Together, these findings suggest that MMP-2 participates in the regulation of matrix turnover in cartilage and chondrogenic differentiation.

In the present study, we investigated the functional role of MMP-2 in chondrogenic differentiation using cultured chick leg bud mesenchymal cells. MMP-2 was found to function as a negative regulator of the integrin β1-mediated interaction between fibronectin and FAK at the focal adhesion site during cellular condensation. In summary, we propose that the p38MAPK signal transduction pathway downregulates tyrosine phosphorylation of FAK by upregulating the level of transcription and activation of MMP-2, thereby leading to inhibition of condensation of leg bud mesenchymal cells.

Materials and methods

Cell culture and treatment

Mesenchymal cells derived from the distal tips of leg buds from Hamburger–Hamilton (HH, Hamburger and Hamilton, 1951) stage 22/23 embryos of fertilized white Leghorn chicken eggs, were micromass-cultured as described previously (Jin et al., 2006). Cells at 2×10^7 cells/ml were plated onto 35 or 60 mm dishes in 3 or 19 drops of 15 μl each. The cells were incubated for 1 h at 37 °C under 5% CO_2 to allow attachment and were maintained in Ham's F-12 medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin (Gibco Invitrogen, Grand Island, NY). The cells were treated with 3 μM GM6001 (Chemicon, Temecula, CA), 10 nM bafilomycin A1 (Chemicon), 10 μM PD98059, 10 μM SB203580 (Calbiochem, San Diego, CA), 100 ng/ml insulin-like growth factor-1 (Gropep. Adelaide, Australia), 10 μg/ml herbimycin A, or 5 μg/ml cytochalasin D (Sigma, St. Louis, MO) unless the concentration is indicated.

Analysis of cell condensation and differentiation

Chondrogenic differentiation was measured by Alcian blue staining of sulfated cartilage glycosaminoglycans (GAG). To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures were collected at day 5 of incubation and stained with 0.5% Alcian blue 8GX, pH 1.0 (Hassel and Horigan, 1982; Lev and Spicer, 1964). Alcian blue bound to sulfated GAG was extracted with 6 M guanidine–HCl, and quantified by measuring the absorbance of the extracts at 600 nm. Binding of peanut agglutinin (PNA) was used as a specific marker for precartilage condensation (Maleski and Knudson, 1996). Briefly, cultures were rinsed twice with 0.02 M PBS, pH 7.2, fixed in methanol:acetone (1:1) for 1 min, air-dried, and then incubated with 100 μg/ml biotinylated PNA (Sigma) for 1 h. PNA binding was visualized using the VECTASTAIN ABC and DAB substrate solution kit (Vector laboratories Inc., Burlingame, CA).

Knockdown of mmp-2 mRNA

To generate constructs encoding small interfering RNA (siRNA) for *mmp-2*, oligonucleotides containing 60 nucleotides were synthesized as shown in Scheme 1, where "*mmp-2*: sense" and "*mmp-2*: antisense" represents 19 nucleotide sequences linked by a hair pin structure. The 19 nucleotide sequence for three different *mmp-2* specific antisense was siRNA-1; 5′-GTGCAATACCTGAATAAAT-3′ (nucleotide no. 273–292), siRNA-2; 5′-ACTGAGATTTAACCGAATA-3′ (nucleotide no. 590–609), si RNA-3; 5′-AGGCAGACATTATGATTAA-3′ (nucleotide no. 619–637), The oligonucleotides were annealed and then ligated into the pSUPER vector (Oligoengine, Seattle, WA) digested with *Bgl*II and *Xho*I. Positive clones from ampicillin resistant colonies were detected by the presence of an *Eco*RI–*Hin*dIII restriction fragment. Electroporation of mesenchymal cells with the siRNA-encoding vector was conducted using a square wave generator (BTX-830; Gentronics, San Diego, CA) with 20 ms, 200 square pulses.

5'-GATCCCC(*mmp-2*: sense)TTCAAGAGA(*mmp-2*: antisense)TTTTTA-3'
3'-GGG(*mmp-2*: sense)AAGTTCTCT(*mmp-2*: antisense)AAAAATTCGA-5'

Scheme 1.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted with Trizol (Gibco Invitrogen) and cDNA was synthesized from 1 μg RNA in 20 μl of master mix for reverse transcription containing 200 U/μl Superscript III (Invitrogen), 5 mM $MgCl_2$, PCR buffer, 1 mM dNTP, 1 U/μl RNase inhibitor, and 2.5 mM oligo dT in DEPC-treated distilled water. For semiquantitative PCR, synthesized cDNAs were subjected to 25 cycles of PCR amplification under the following conditions: 94 °C denaturing for 1 min, 55 °C annealing for 1 min and 72 °C extension for 1 min. PCR products were electrophoresed on a 2.0% agarose gel.

Quantitative real-time PCR reactions were performed using a Roche Light Cycler (Roche, Switzerland). Synthesis of double stranded DNA was monitored using SYBR Green I (Roche Laboratories). The PCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, 15 s at 60 °C and 17 s at 72 °C. To normalize the output, the expression of each gene of interest was divided by GAPDH gene expression, a commonly used housekeeping gene. Data are relative to the time point 12 h of control culture and are presented as a bar graph with standard deviations. Melting-curve analyses were done following each PCR to determine the output and detection quality (i.e., the formation of primer–dimers). Primers used for semiquantitative and quantitative real-time PCR are listed in Table 1.

Zymography

MMP-2 activity was determined using the gelatin zymography assay with 0.1% gelatin as a substrate in a 10% SDS-polyacrylamide gel. Mesenchymal cells were incubated in serum free media for 24 h and conditioned media (CM) was concentrated with 10% trichloroacetic acid precipitation. The precipitate was dissolved in 1X sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 10% Glycerol, 2% SDS, and 0.005% Bromophenol Blue) and separated by SDS-

Table 1

Primers	Sequence
Semiquantitative PCR primers	
mmp-2(F)	5′-ACTCCTGTACAAGTGCAG-3′
mmp-2(R)	5′-ACTCCTGTACAAGTGCAG-3′
gapdh (F)	5′-GATGGGTGTCAACCATGAGAAA-3′
gapdh (R)	5′-ATCAAAGGTG GAAGAATGGCTG-3′
Quantitative real time PCR primers	
mmp-2 (F)	5′-TCTGCAAGCACGACATTGTA-3′
mmp-2 (R)	5′-AACTCCATTCCAAGAATCCG-3′
gapdh (F)	5′-AGGCGAGATGGTGAAAGTCG-3′
gapdh (R)	5′-TCTGCCCATTTGATGTTGCT-3′

PAGE under non-reducing conditions. After electrophoresis, gels were washed three times with 2.5% Triton X-100 in water then incubated with 50 mM Tris buffer, pH 7.4, containing 0.2% Brij 35, 5 mM $CaCl_2$, and 1 mM NaCl, overnight at 37 °C in a closed container. Gels were then stained for 30 min with 0.25% Coomassie Blue R-250 in 10% acetic acid and 45% methanol, and destained for 30 min using an aqueous mix of 20% acetic acid, 20% methanol, and 17% ethanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Immunoprecipitation and immunoblot

Cells were lysed in buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, and protease inhibitors) for 30 min on ice. For Western blot analysis, proteins (30 μg) were separated by SDS-PAGE, and then probed with the following antibodies; paxillin (Signal Transduction Lab, Beverly, MA), FAK, pFAK, or Src (Santa Cruz Biotechnologies, Santa Cruz, CA), GAPDH, integrin α5, or integrin β1 (Chemicon), N-cadherin (BD Transduction Lab, Franklin Lakes, NJ), and fibronectin (Sigma). For Western blot analysis of MMPs, membrane fraction or CM was separated by SDS-PAGE, and then probed with the following antibodies; MT1-MMP, MT3-MMP, MMP-1, MMP-2, or MMP-13 (Chemicon). Membrane fractionation to analyze the level of MT1–MMP and MT3–MMP activity was performed. Briefly, micromass-cultured cells were sonicated and were centrifuged at 100,000×*g* for 1 h. The pellet was extracted with 20 mM Tris–HCl (pH 7.5) containing 1% SDS and 150 mM NaCl. After centrifugation at 15,000×*g* for 15 min, the supernatant was taken as the particulate membrane fraction.

The blots were developed with a peroxidase-conjugated secondary antibody, and reactive proteins were visualized by the ECL system (Amersham-Pharmacia, Buckinghamshire, UK). For immunoprecipitation, the cell lysates were incubated with anti-phospho tyrosine (Santa Cruz Biotech) or integrin β1 antibodies for 2 h at 4 °C, and then with a 50% slurry of protein A-Sepharose for the next 1 h. Immunocomplexes were eluted by boiling in sample buffer then were analyzed to SDS-PAGE followed by immunoblotting with antibody against FAK, c-Src or paxillin.

For analysis of MMP-2 activity MMP-2 protein was isolated from CM treated with or without SB203580 by using 0.5 μg of antibody against MMP-2. Immunocomplexes were precipitated by incubation with protein A-Sepharose for 1 h at 4 °C and analyzed by SDS-PAGE and Western blotting.

Results

Knockdown of MMP-2 stimulates precartilage condensation and chondrogenesis

To investigate the functional role of MMP-2 during chondrogenesis, we constructed MMP-2 specific siRNAs and transfected mesenchymal cells by electroporation. A vector encoding EGFP was used as a transfection control. The transfection efficiency was revealed by fluorescence microscopy to detect expression of the plasmid-encoded EGFP gene (Fig. 1A upper panel). Western blot analysis 48 h after transfection revealed that two siRNA vectors, siRNA-1 and siRNA-2 effectively knocked down the endogenous *mmp-2* mRNA level, but not ectopically expressed EGFP (Fig. 1A lower panel). Western blot analysis and gelatin zymography also revealed reduced levels of both the active and pro-forms of MMP-2 by two different MMP-2 siRNA, siRNA-1 and siRNA-2 but not by siRNA-3 (Fig. 1B).

Chondrogenic differentiation of mesenchymal cells is regulated at various stages including proliferation of chondroblast competent cells, precartilage condensation and formation of cartilage nodules (DeLise et al., 2000; Knudson and Knudson, 2001; Quarto et al., 1997). Therefore, we first examined the effect of siRNA-mediated *mmp-2* knockdown on the growth of mesenchymal cells by assessing cell numbers at days 1, 3, 5 after transfection. As shown in Fig. 1C, knockdown of *mmp-2* had little or no effect on the proliferation of chondroblast competent cells. We then examined the effect of siRNA on cellular condensation and chondrogenic differentiation. Mesenchymal cells were electroporated with the *mmp*-2 siRNA vector and cultured for 48 h. The effect of MMP-2 specific siRNA-1 and siRNA-2 on precartilage condensation and chondrogenesis was assessed by peanut agglutinin (PNA) binding for cellular condensation at day 3, and by Alcian blue staining for sulfated proteoglycans at day 5, respectively. Knockdown of endogenous MMP-2 in mesenchymal cells led to stimulation of precartilage condensation and chondrogenic differentiation (Fig. 1D). We also used Runx2 siRNA as another control for rule out of unspecific effect of siRNA. Runx2 siRNA did not show any effect on chondrogenic differentiation and condensation (data not shown). These results indicate that MMP-2 functions to negatively regulate cellular condensation and chondrogenesis of mesenchymal cells.

MMP-2 is involved in early regulation of chondrogenesis

To confirm the involvement of MMP-2 in the regulation of chondrogenesis, we tested the effect of an MMP-2 inhibitor or activator on chondrogenesis. Bafilomycin A1 is an inhibitor of H^+-ATPase. A previous report (Maquoi et al., 2003) showed that the activity of MT1–MMP on the cell surface is constitutively down-regulated through a vacuolar H^+-ATPase-dependent degradation process. Blockade of this degradation caused the accumulation of TIMP-free active MT1–MMP molecules on the cell surface and pro-MMP-2 activation was strongly enhanced as a consequence of this impaired degradation. GM6001 is a hydroxamic acid-based MMP inhibitor, known to inhibit broad spectrum of MMPs activity *in vitro* and *in vivo* (Hu et al., 2003; Galardy et al., 1994; Solorzano et al., 1997). It has recently been shown that GM6001 in the 1–10 μM range inhibits MMP-2 activity in various cell types (Macotela et al., 2006; Margulis et al., 2005; Shen et al., 2006). Mesenchymal cells were incubated with bafilomycin A1 (5–50 nM) or GM6001 (0.5–10 μM) for 24 h and MMP-2 activity was assessed using the gelatin zymography assay. MMP-2 activity was stimulated by 10 nM bafilomycin A1 and inhibited significantly by 3 μM GM6001 (Fig. 2A left panel) in

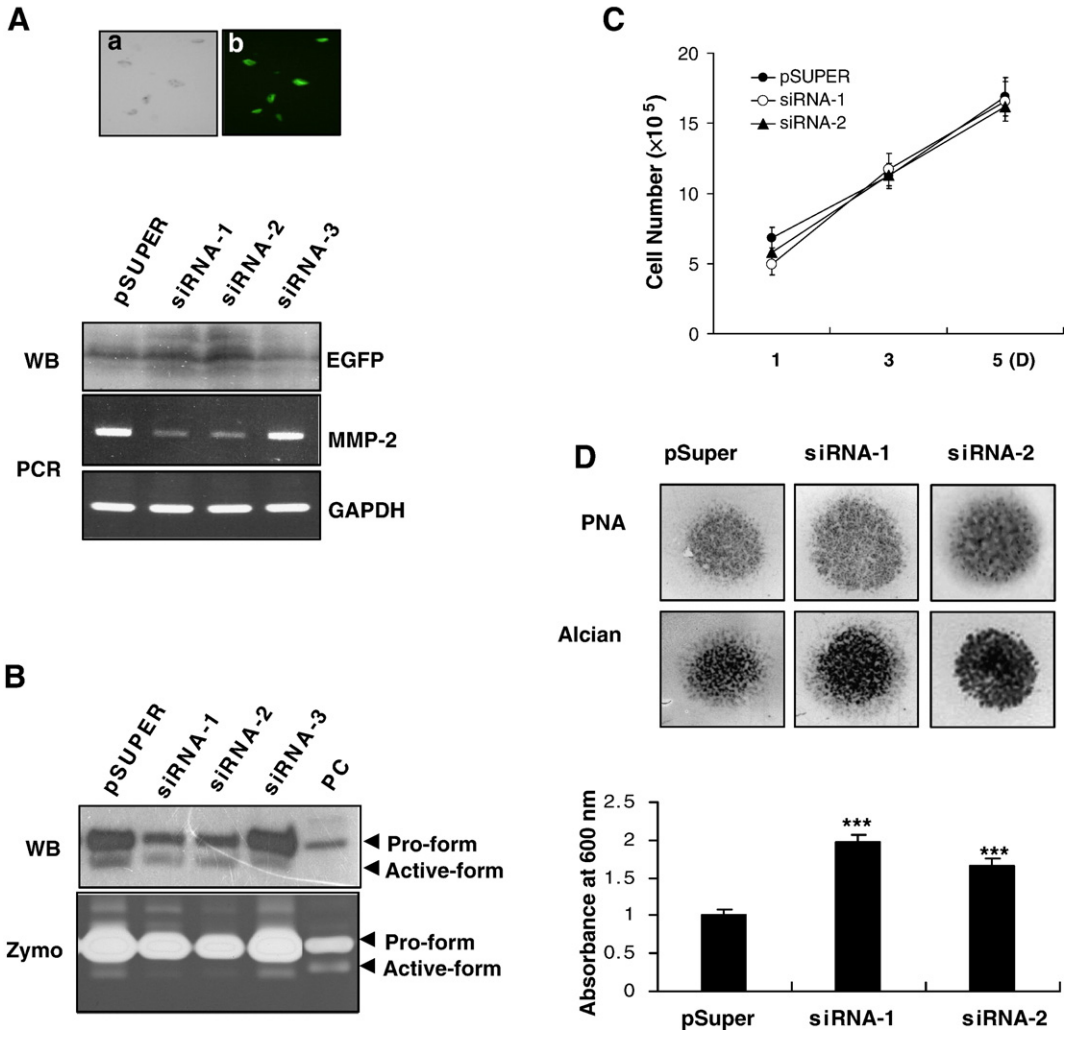

Fig. 1. siRNA-directed knockdown of *mmp-2* increases chondrogenesis. Leg bud mesenchymal cells were electroporated with either vector (pSUPER) as a mock or *mmp-2*-specific siRNA (siRNA-1, -2, or -3). (A) At 48 h, transfected cells were cytospin adhered to slides, fixed and observed under a microscope (bright field, a, fluorescent field, b; upper panel). The transfection efficiency was confirmed by Western blotting with anti-GFP antibody and RT-PCR using *mmp-2* specific primers at 48 h (lower panel). (B) Proteolytic activity of MMP-2 in conditioned media (CM) was analyzed by Western blotting using an anti-MMP-2 antibody (WB) and gelatin zymography (Zymo). PC indicates a positive control for MMP-2 activity. (C) The number of viable cells was determined at the indicated days. (D) Cultures were fixed at days 3 or 5 and stained with PNA (upper panel) or Alcian blue (lower panel), respectively. The data shown are representative of at least four independent experiments.

mesenchymal cells. To confirm the specificity of bafilomycin A1 or GM6001 for MMP-2 in this concentration, we tested the activity of other MMPs. Bafilomycin A1 slightly increased the activation of MT1–MMP governing the activation of proMMP-2 (Hernandez-Barrantes et al., 2000; Maquoi et al., 1998), whereas other MMPs such as MT3–MMP, MMP-3 and MMP-13 were not significantly regulated by bafilomycine A1 or GM6001 (Fig. 2A right panel).

Cellular condensation events are mediated by cell–matrix adhesion molecules including fibronectin, proteoglycans and collagens, which are cleaved by MMP-2 (DeLise et al., 2000; Nagase and Woessner, 1999; Visse and Nagase, 2003). To further investigate the functional role of MMP-2 during chondrogenesis, we examined the temporal action of MMP-2 on condensation and chondrogenic differentiation. Mesenchymal cells were treated with GM6001 (3 μM) or bafilomycin A1 (10 nM) under different exposure time periods. Exposure of chondroblast to GM6001 at early stage of chondrogenesis (early exposure, 0–48 h) gave a 2 fold increase in condensation spots and cartilage nodules, whereas the early exposure of chondroblasts to bafilomycin A1 inhibits cellular condensation and chondrogenesis (Figs. 2C and B). By comparison, exposure of chondroblasts to either GM6001 or bafilomycin A1 at late stage of chondrogenesis (late exposure, 48–72 and 72–96 h) showed a slight reduction in chondrogenic differentiation (Fig. 2B) or had little effect on condensation (Fig. 2C). These results suggest that MMP-2 is involved in regulation of chondrogenesis at the pre-condensation stage.

MMP-2 activity downregulates tyrosine phosphorylation of FAK

Precartilage condensation is regulated by cell adhesion molecules, the extracellular matrix and integrins (Bang et al.,

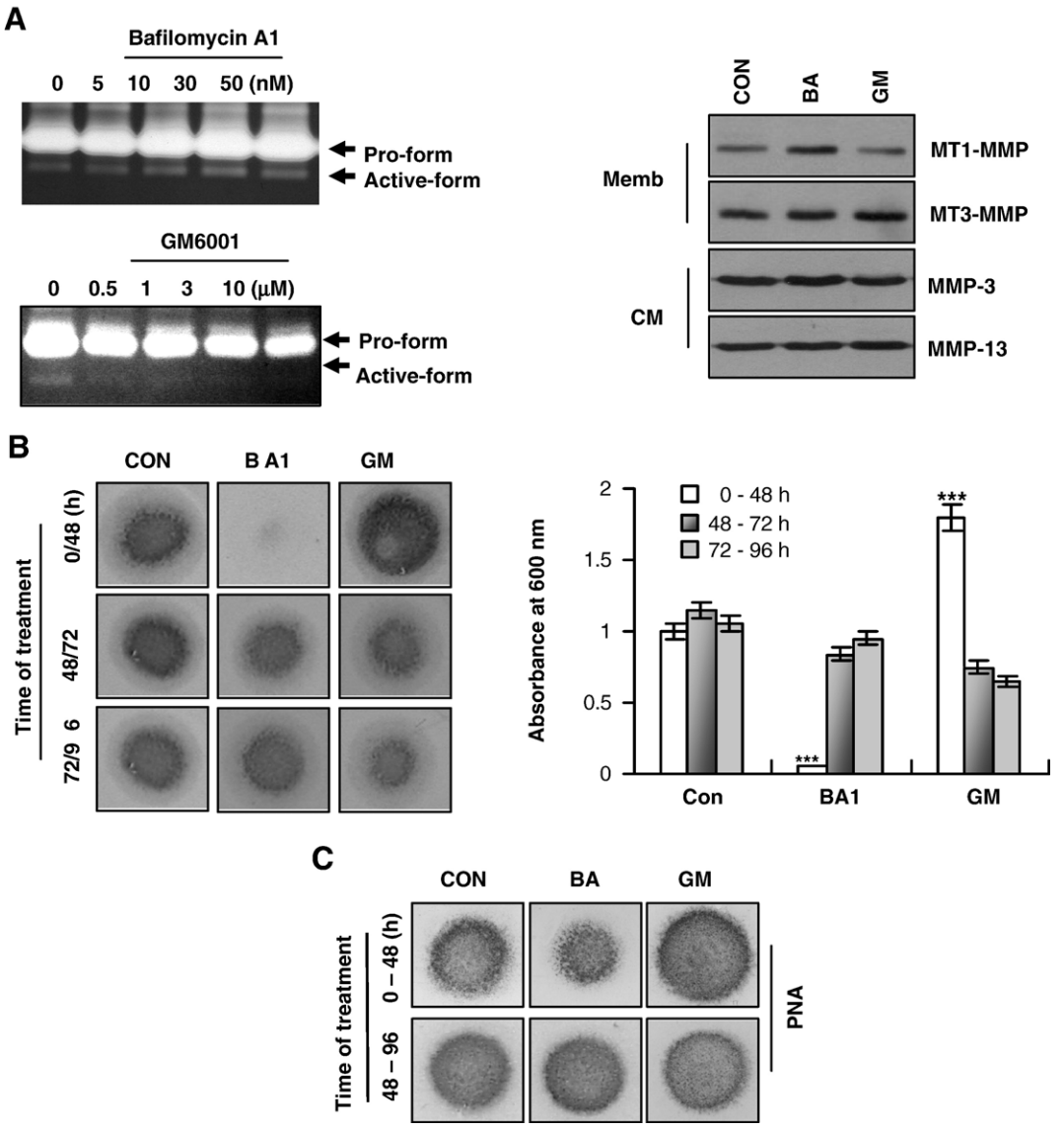

Fig. 2. MMP-2 is involved in the regulation of chondrogenesis at the pre-condensation stage. Mesenchymal competent cells were prepared as described in Materials and methods. (A) The cells were treated with various concentrations of bafilomycin A1 (BA) or GM6001 (GM), and cultured for 24 h under serum free conditions. MMP-2 activity was determined by gelatin zymography (left panel). Changes in the levels of MT1–MMP and MT-3 MMP on cell surface and the levels of MMP-3 and MMP-13 in conditioned media were analyzed by Western blotting using respective antibodies. Memb and CM indicate membrane fraction and conditioned media, respectively. (B and C) Cells treated with 10 nM of bafilomycin A1 or 3 μM of GM6001 for the early stage (0–48 h) or late stages (48–72 and 72–96 h) in the presence of 10% FBS were stained with Alcian blue at day 5 (B, left panel) or PNA (C) at day 3 of culture. Quantification of chondrogenesis was determined by measuring the absorbance of bound Alcian blue at 600 nm (B, right panel). The data shown are representative of at least four independent experiments. ***, statistically difference from control cells ($P<0.001$).

2000; Daniels and Solursh, 1991; Knudson and Knudson, 1993). To further investigate the regulation of cellular condensation by MMP-2, the expression levels of N-cadherin, fibronectin, and integrins α5 and β1 in mesenchymal cells transfected with the *mmp-2* siRNA vector (siRNA-2) or treated with bafilomycin A1 were assessed by Western blot analysis. The protein level of fibronectin, and the integrins α5 and β1 increased significantly in the *mmp-2*-knockdowned cells, and decreased significantly in the MMP-2 activated cells (Fig. 3A). By comparison, the protein level of N-cadherin remained unchanged following modulation of MMP-2 activity. These results suggest that MMP-2 is involved in the regulation of ECM and integrins, rather than N-cadherin, at the mesenchymal cell surface.

The cell-to-matrix interaction by fibronectin, and its receptor integrin β1, initiates the adhesion-mediated mesenchymal condensation process (Hentschel et al., 2004; White et al., 2003). FAK is localized to focal adhesions where it is activated by phosphorylation after integrin-mediated cell attachment (Chen et al., 2000; Giancotti and Ruoslahti, 1999; Mitra et al., 2005, 2006). For this reason we investigated whether modulation of protein levels of fibronectin and integrin β1 by MMP-2 affected FAK activation. Mesenchymal cells at 48 h after treatment of bafilomycin A1 or transfection with the *mmp-2* siRNA vector were subjected to SDS-PAGE and immunoblot analysis with the anti-phospho FAK antibody specific to Tyr (397), the phosphorylation of which is associated with kinase

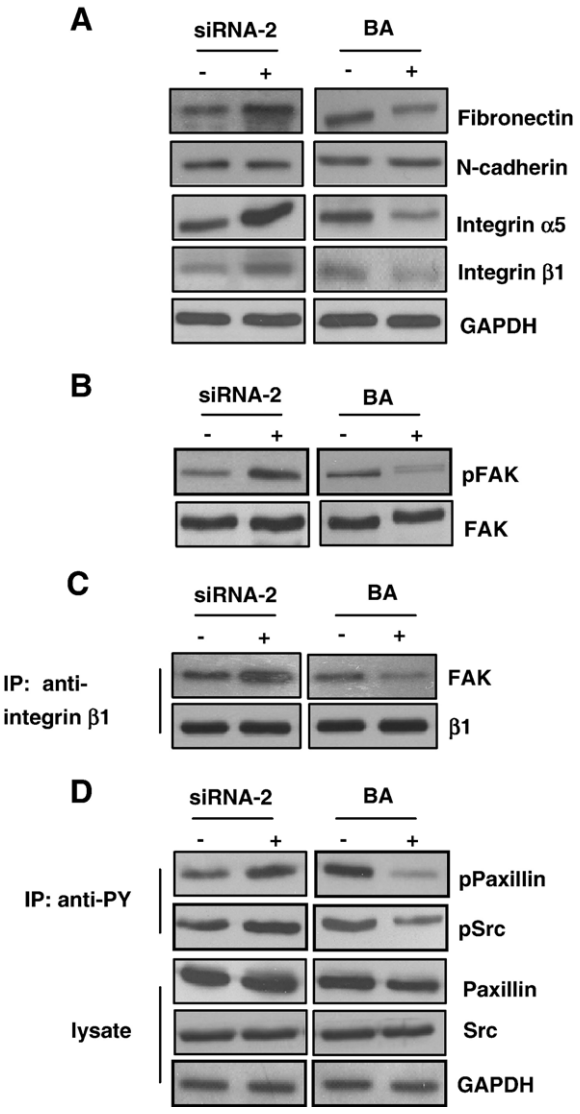

Fig. 3. MMP-2 induces down-regulation of ECM molecules at the protein level and their interaction with FAK. Mesenchymal cells were transfected with the MMP-2-specific siRNA (si MMP-2) construct or treated with bafilomycin A1 (BA) and harvested after 48 h. (A) Changes in the protein levels of FN, type II collagen, α5, β1 integrin and N-cadherin during chondrogenesis were analyzed by Western blotting using respective antibodies. (B) FAK, phosphorylated at tyrosine (397) site was detected using the anti-phosphotyrosine antibody. (C) Cell lysates were immunoprecipitated with the anti-integrin β1 antibody, and subjected to immunoblot analysis with the anti-FAK antibody. (D) Cell lysates were immunoprecipitated with anti-phosphotyrosine (PY) antibody. Immunoblot analysis of immunoprecipitates and cell lysates was performed with anti-paxillin or c-Src antibodies. GAPDH served as a loading control.

activation. The tyrosine phosphorylation of FAK increased after knockdown of *mmp-2*, but decreased after activation of MMP-2 by bafilomycin A1 (Fig. 3B). To determine if modulation of tyrosine phosphorylation of FAK is due to alterations in the association of FAK at the focal adhesion where fibronectin interacts with integrin β1, immunoprecipitation assays were performed on cells 48 h after transfection with the *mmp-2* siRNA vector or treatment of bafilomycin A1. Anti-integrin β1 antibody immunoprecipitates were probed with the anti-FAK antibody. The association of integrin β1 with FAK increased with siRNA-mediated knockdown of MMP-2, but decreased on bafilomycin A1 treatment (Fig. 3C). Adhesion of chondrocytes to the site of interaction between fibronectin and integrin β1 leads to tyrosine phosphorylation of cytoskeletal and signaling proteins localized at focal adhesions, including paxillin, c-Src and FAK (Gemba et al., 2002). The immunoprecipitation assay using the anti-phospho tyrosine antibody revealed that knockdown of MMP-2 increased the level of paxillin and c-Src tyrosine phosphorylation, whereas activation of MMP-2 by bafilomycin A1 reduced tyrosine phosphorylation of these proteins (Fig. 3D). These results indicate that the up-regulation of protein levels of fibronectin and integrin α5/β1 in the absence of MMP-2 leads to an increases in the tyrosine phosphorylation of focal adhesion molecules including FAK, c-Src, and paxillin, through stimulation of the interaction between FAK and fibronectin via integrin α5/β1. MMP-2-induced negative regulation of precartilage condensation appears to involve prevention of the focal adhesion complex assembly.

Integrin-mediated tyrosine phosphorylation of FAK is essential for precartilage condensation of mesenchymal cells (Bang et al., 2000; Takahashi et al., 2003). The role of tyrosine phosphorylation of FAK in the MMP-2-induced inhibition of cellular condensation was investigated by incubating mesenchymal cells with either GM6001, an MMP-2 inhibitor, or herbimycin A, a tyrosine kinase inhibitor, or with combination of GM6001 and herbimycin A. Tyrosine phosphorylation of FAK was significantly increased by GM6001 and significantly decreased by herbimycin A. Co-treatment of herbimycin A and GM6001 reversed the GM6001-stimulated phosphorylation of FAK without a change in the protein level (Fig. 4A). Precartilage condensation decreased significantly as a result of inhibition of tyrosine phosphorylation and, as expected, increased as a result of inhibition of MMP-2 activity. Herbimycin A also abolished GM6001-promoted cellular condensation (Fig. 4B). These results indicate that MMP-2 inhibits cellular condensation via the inhibition of tyrosine phosphorylation of FAK that mediates assembly of focal adhesion complex at the cell adhesion site.

The cytoskeleton is critical to the formation of the focal adhesion complexes regulated by the interaction between FAK and integrin β1 (Bang et al., 2000; Calandrella et al., 2005). We previously reported that cytochalasin D (CD), an inhibitor of actin polymerization, inhibits precartilage condensation via negative regulation of FAK phosphorylation (Bang et al., 2000). By comparison, insulin-like growth factor-1 (IGF-I), a growth and survival factor, triggers polymerization of F-actin, induces phosphorylation of FAK and paxillin, and enhances the interaction of integrin β1 with these focal adhesion proteins (Kim et al., 2004a; Sekimoto et al., 2005; Tai et al., 2003). To further investigate the involvement of MMP-2 in the regulation of the focal adhesion complex, mesenchymal cells were treated with CD or IGF-I in the presence or absence of MMP-2 inhibitor. CD treatment remarkably reduced phosphorylation of FAK, whereas IGF-1 treatment significantly increased phosphorylation of FAK (Fig. 4C, upper panel). Cotreatment of cells with IGF-1 and GM6001 synergistically increased tyrosine phosphorylation of FAK and precartilage condensation (Fig.

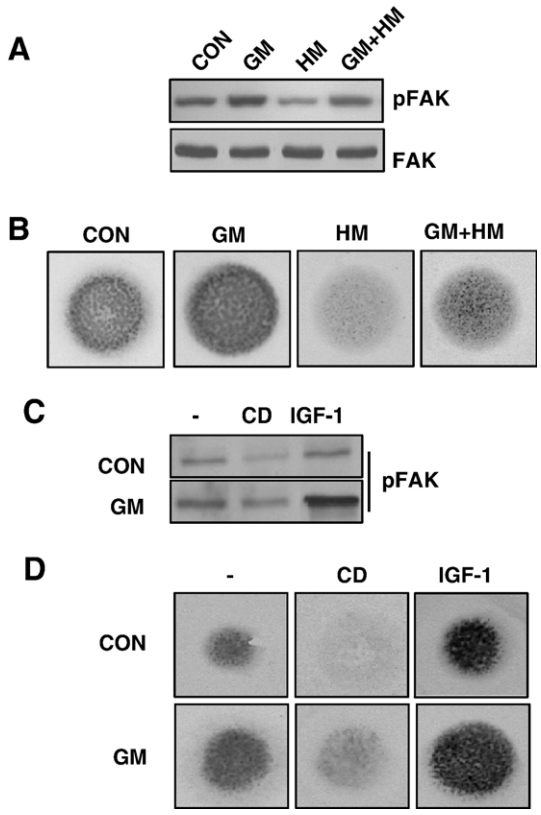

Fig. 4. The relationship between FAK phosphorylation and cell condensation. Chondrogenic competent cells were incubated with 3 μM GM6001 (GM), 10 μg/ml herbimycin A (HM), 5 μg/ml of cytochalasin D (CD) or 100 ng/ml IGF-1, for 48 h as indicated. (A and C) Immunoblot analysis of cell lysates was performed with anti-FAK or anti-phospho FAK antibodies. (B and D) The cells were stained with PNA at day 3 of culture. The data shown are representative of at least four independent experiments.

4C, lower panel). By comparison, the co-treatment of cells with CD and GM6001 inhibited the GM6001-enhanced tyrosine phosphorylation of FAK and precartilage condensation (Fig. 4D). These results indicate that MMP-2 is a negative regulator of tyrosine phosphorylation of focal adhesion components and acts by modulating the association between fibronectin and integrins α5 and β1 in response to extracellular signals during the cellular condensation of leg bud mesenchymal cells.

Involvement of p38MAPK in the down-regulation of MMP-2

Our previous reports have shown that ERK and p38MAPK regulate mesenchymal cell chondrogenesis by modulating the expression of cell adhesion molecules including N-cadherin, fibronectin and integrin α5/β1 (Choi et al., 1995; Oh et al., 2000). The pathway of MMP-2 and MT1-MMP activation involves MAPKs, including p38MAPK and ERK (Huang et al., 2005; Kim et al., 2004b; Munshi et al., 2004; Ruhul Amin et al., 2003). In the present study we investigated the involvement of MAPKs in the regulation of chondrogenesis by MMP-2. Mesenchymal cells were pretreated with PD98059, a specific inhibitor of MEK or SB203580, a specific inhibitor of p38MAPK and MMP-2 mRNA levels were analyzed by semiquantitative PCR and quantitative real time PCR. MMP-2 mRNA levels were decreased as a result of p38MAPK inhibition and not MEK–ERK inhibition (Fig. 5A). Gelatin zymography also demonstrated the decrease in MMP-2 activity as a result of p38MAPK inhibition and not MEK–ERK inhibition (Fig. 5B, upper panel). To clarify whether the decrease in MMP-2 activity by the inhibition of p38MAPK activity due to the modification at the post-translational level or the reduction of MMP-2 mRNA level, we analyzed MMP-2 activity after immunoprecipitation with MMP-2 antibody to get the same amount of MMP-2 protein. The level of MMP-2 activity was not changed by inhibition of p38MAPK pathway (Fig. 5B, lower panel). These results indicate that MMP-2 is regulated at the mRNA expression level but not at the activity level by the p38MAPK pathway during chondrogenesis.

The effect of SB203580 on mesenchymal cell condensation was also investigated. Cells were treated with SB203580 for the indicated time periods and precartilage condensation was analyzed by PNA staining. The early exposure of cells to SB203580 resulted in a significant increase in the number of PNA-bound condensation spots, whereas late exposure of cells to SB203580 showed little effect (Fig. 5C). These results suggest that in the process of cellular condensation MMP-2 mediates the inhibitory signals of the p38MAPK pathway. The effect of enhanced MMP-2 activity as a result of p38MAPK inhibition on the protein levels of fibronectin, and integrins α5 and β1, and on tyrosine phosphorylation of FAK was investigated. The inhibition of p38MAPK significantly increased the protein level of fibronectin, and integrins α5 and β1 at the pre-condensation stage (Fig. 5D). Consistent with these results, inhibition of p38MAPK promoted the phosphorylation of FAK, and cotreatment with bafilomycin A1 overcame the inhibition of FAK phosphorylation by bafilomycin A1 (Fig. 5E). Collectively, these results show that MMP-2 mediates the inhibitory signals of the p38MAPK pathway on the cellular condensation through the negative regulation of focal adhesion.

Discussion

Chondrogenesis is the earliest phase of skeletal development involving condensation of mesenchymal cells, the differentiation of mesenchymal cells into chondrocytes, and patterning of chondrifying tissues into skeletal structures. During condensation, communication between neighboring cells via adhesion and ECM molecules, such as type I collagen and fibronectin, provides important signals for subsequent chondrocytic differentiation. MMPs are candidate molecules for communicating these signals and mediating the progressive turnover of ECM molecules. As MMP-2 has wide substrate specificity for cartilage matrix constituents, we postulate that MMP-2 plays an important role in the regulation of chondrogenesis. In the present study, we demonstrate that MMP-2 inhibits cellular condensation by down-regulating the integrin β1-mediated interaction between fibronectin and FAK, and tyrosine phosphorylation of focal adhesion components, such as FAK, paxillin and c-Src at the focal adhesion site. The p38MAPK pathway induces the down-regulation of tyrosine phosphorylation of FAK, via the up-regulation of MMP-2, at the

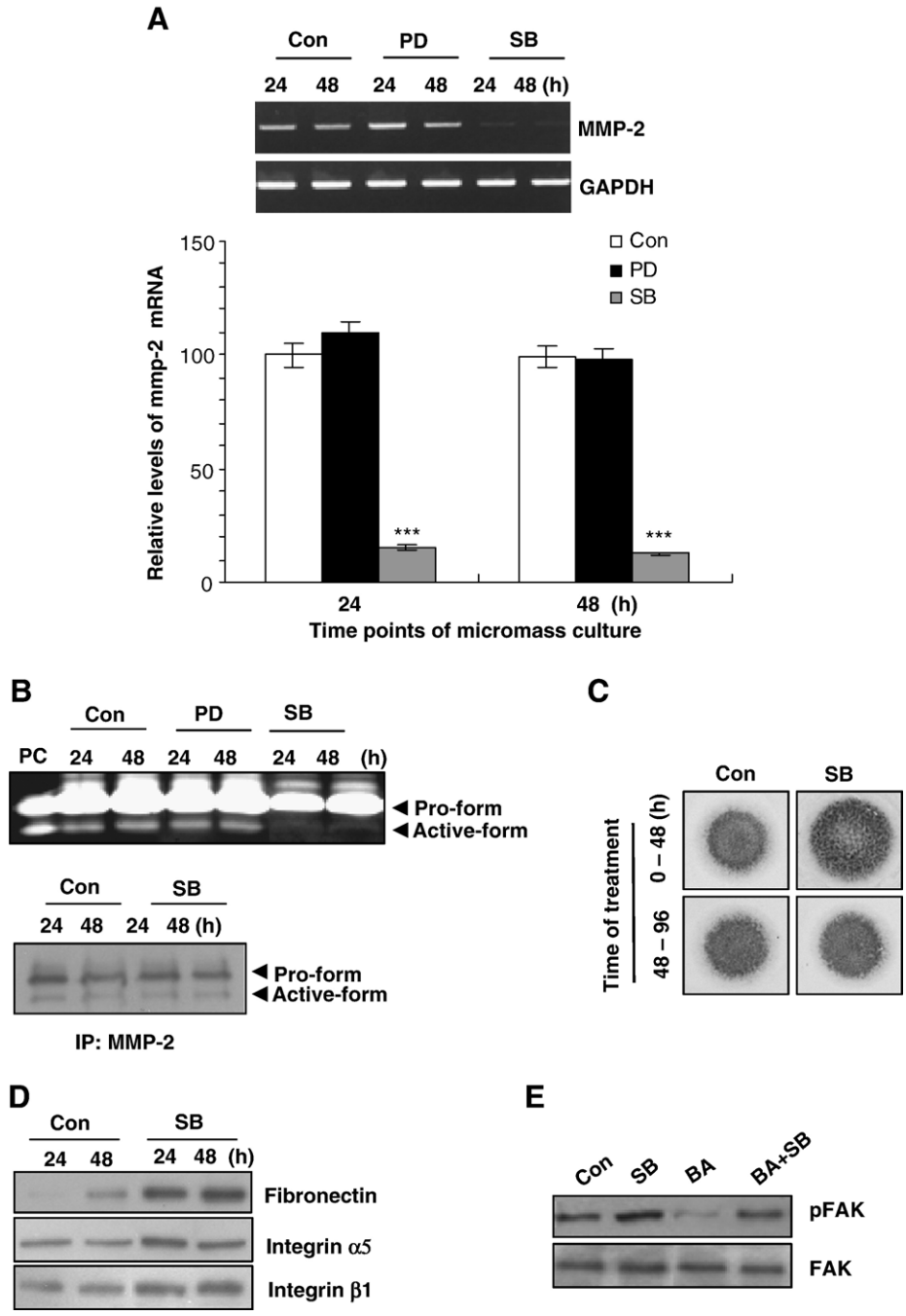

Fig. 5. MMP-2 mediates the inhibitory signals of p38MAPK. Micromass cultured leg bud mesenchymal cells were cultured in the absence or presence of 10 μM PD98059 (PD) or 10 μM SB203580 (SB). (A) RT-PCR (upper panel) and quantitative real time RT-PCR (qRT-PCR, lower panel) of cell extracts was performed to analyze the mRNA levels of *mmp-2*. Data of qRT-PCR represent the mean ± SD (***$P < 0.0001$) of three independent experiments each performed in triplicate. (B) Conditioned media from PD98059 or SB203580-treated cultures were collected at 24 and 48 h then analyzed by gelatin zymography (upper panel). MMP-2 activity treated with SB203580 was also analyzed after isolation of MMP-2 protein by using 0.5 μg of MMP-2 antibody (lower panel). (C) The cells were stained with PNA at day 3 of culture. (D and E) The cell lysates at the indicated time (D) or 48 h after culture (E) were subjected to immunoblot analysis with the respective antibody. The data shown are representative of at least four independent experiments.

transcription and protein activity levels, leading to inhibition of leg bud mesenchymal cell condensation.

Precartilage condensation is a multistep process mediated by cell adhesion and ECM molecules, leading to cell recruitment, cell-to-cell contacts, and concomitant changes in cell shape (DeLise et al., 2000; Knudson and Knudson, 2001; Sandell and Adler, 1999). In vivo, precartilage condensation is accompanied by direct cell–cell adhesion after the accumulation of cells at sites of transiently elevated fibronectin deposits (Hall and Miyake, 2000). These fibronectin deposits may be degraded by MMP-2 in the regulation of chondrogenesis. In the present study, MMP-2 induced the down-regulation of fibronectin and the fibronectin receptors, integrin α5 and β1, at the pre-condensation stage and inhibited the subsequent interaction of

these proteins with FAK. The MMP-2-induced down-regulation of the FAK-integrin β1 interaction leads to reduced tyrosine phosphorylation of focal adhesion components, such as FAK, c-Src, and paxillin. These results suggest that MMP-2 mediates the turnover of fibronectin, and its receptor, and is involved in the regulation of the FAK-integrin β1 interaction.

The modulation of MMP-2 activity by bafilomycin A1 or GM6001 revealed a different temporal effect on cellular condensation and chondrogenic differentiation. GM6001, an inhibitor of MMP-2 activity, increased cellular condensation, whereas bafilomycin A1, a stimulator of MMP-2 activity, decreased cellular condensation when leg bud mesenchymal cells were treated at the pre-condensation stage with GM6001 or bafilomycin A1. Treatment with GM6001 or bafilomycin A1 at the post-condensation stage had little or no effect on cellular condensation or chondrogenesis, indicating that modulation of fibronectin and its receptor by MMP-2 plays an important role in the regulation of cellular condensation and chondrocytic differentiation. As chondroblast cells differentiate into chondrocytes, ECM production switches from type I collagen, fibronectin, and its integrin α5/β1 receptor, to type II collagen and aggrecan (Cancedda et al., 1995; Silbermann and von der Mark, 1990). In chondrocytes, the force of tension promotes ECM adhesion of cells resulting in the inhibition of chondrogenesis and the concomitant up-regulation of integrins and FAK and the down-regulation of type II collagen (Takahashi et al., 2003). Therefore, cell attachment through ECM molecules is required for the cellular condensation process, but not for differentiation into chondrocytes. These findings suggest that MMP-2 has an inhibitory function in precartilage condensation rather than in chondrogenic differentiation.

Previous studies indicate that ECM molecules, including fibronectin and the integrins, are also involved in the regulating activities of MMP-2 mediated by the ERK pathway in several cancer cell lines (Hood and Cheresh, 2002; Leroy-Dudal et al., 2005; Mitra et al., 2003, 2006). Our data show that modulation of fibronectin and fibronectin receptor, integrin α5/β1 at the protein level and their association with FAK, resulting in activation of focal adhesion components for mesenchymal cell adhesion, is regulated by MMP-2 activity.

MAPK pathway components, including ERK1/2 and p38, are closely associated with the differentiation of chondrocytes (Nakamura et al., 1999; Oh et al., 2000: Yoon et al., 2000). During chondrogenesis of chick limb bud mesenchymal cells, p38MAPK promotes chondrogenesis by down-regulating the expression of adhesion molecules at post-precartilage condensation stages (Oh et al., 2000). In the present study the inactivation of p38MAPK by SB203580 lead to down-regulation of MMP-2 mRNA and protein, and up-regulation of adhesion molecules in mesenchymal cells at the pre-condensation stage. This increase in the level of adhesion molecules by inactivation of p38MAPK lead to significant stimulation of tyrosine phosphorylation of FAK and the subsequent promotion of cellular condensation. These results suggest that the inhibitory function of p38MAPK in cellular condensation is mediated by MMP-2, and that MMP-2 activity is controlled at both the temporal and spatial levels by various factors, including integrins and growth factors, during chondrogenesis in the developing limb. Taken together, our findings demonstrate that MMP-2 regulates the association of FAK and fibronectin via integrin β1, thereby leading to the tyrosine phosphorylation of focal adhesion components. In summary, MMP-2 functions as an important mediator of inhibitory signals from the p38MAPK pathway and functions to regulate cellular condensation of leg bud mesenchymal cells.

Acknowledgments

This research was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea Government (MOST) (R01-2006-000-10756-0) and (M1052701000005N270100000).

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