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Matrix metalloproteinase-13 influences ERK signalling in articular rabbit chondrocytes[1]

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Summary

Objective: Matrix metalloproteinase-13 (MMP-13) is an extracellular MMP that cleaves type II collagen, the major protein component of cartilage, with high specificity and has been implicated in the pathology of osteoarthritis. The present study aimed to characterize the binding and internalization kinetics of MMP-13 in normal rabbit chondrocytes and whether MMP-13 affected cell signalling.

Methods: Rabbit chondrocytes were used in [^{125}I]-MMP-13 binding assays to investigate the MMP-13 binding kinetics and Western analysis allowed for the assessment of intracellular signalling cascades.

Results: Rabbit chondrocytes were found to express the cartilage-specific genes aggrecan and type II collagen throughout their *in vitro* culture period. Appreciable specific cell-association of [^{125}I]-MMP-13 was detected after 10 min of exposure to the ligand and equilibrium was obtained after 2 h. Binding of [^{125}I]-MMP-13 to chondrocytes was specific and approached saturation at 75 nM. Internalization of MMP-13 was evident after 20 min, reached a maximum at 30 min and had returned to baseline by 90 min. Addition of receptor-associated protein (RAP) inhibited the internalization of MMP-13 indicating a likely role for low-density lipoprotein receptor-related protein-1 (LRP1) in this process. Interestingly the presence of MMP-13 induced phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2) protein showing that there is initiation of a signalling process in response to MMP-13 being bound and internalized by rabbit chondrocytes. However, this activation does not involve the MMP-13 internalization receptor LRP1.

Conclusion: These studies demonstrate and characterize the MMP-13 binding and internalization system in rabbit chondrocytes and indicate that MMP-13 may regulate the phenotype of the chondrocytes through this receptor system.

Key words: MMP-13, Cartilage, Matrix metalloproteinases, ERK, LRP-1.

Abbreviations: OA osteoarthritis, MMP matrix metalloproteinase, TIMP tissue inhibitor of metalloproteinase, LRP1 low-density lipoprotein receptor-related protein-1, ERK1/2 extracellular signal-regulated kinase, RAP receptor-associated protein.

Introduction

The maintenance of articular cartilage requires a balance between catabolism and synthesis of the extracellular cartilage matrix, dual functions performed by a single cell, the chondrocyte. Osteoarthritis (OA), the most common arthritis, is characterized radiologically as narrowing of the joint space due to loss of articular cartilage[1]. Evidence suggests that disruption of the normal balance of cartilage synthesis and degradation is a pathophysiological catalyst for this multifactorial disease[2]. Ultimately the erosion of type II collagen and aggrecan, the principal structural components of articular cartilage[3], compromise the cartilage matrix leading to irreversible cartilage destruction in OA[4,5].

Matrix metalloproteinases (MMPs) are enzymes involved in the remodelling of the extracellular matrix during development, wound healing and disease (reviewed in Ref. 6). This proteolytic family of enzymes is divided into four groups: collagenases, gelatinases, stromelysins and membrane metalloproteinases. MMP-13 (collagenase-3) acts at physiological pH to degrade fibrillar collagens (types I, II and III) and aggrecan[7]. Cleavage of collagen by collagenase MMPs occurs at an internal helical site to produce 1/4 and 3/4 fragments[8] and at an aminotelopeptide site, which is thought to initiate fibrillar depolymerization[9,10].

MMP-13 is secreted from cells as an inactive proenzyme and is activated via autocatalysis, by other MMPs and proteases (reviewed in Ref. 11). The activated MMP-13 then acts on the local extracellular structures prior to proteolytic inactivation, inhibition by tissue inhibitors of metalloproteinases (TIMPs)[12,13] or receptor-mediated endocytosis and degradation[14]. Our laboratory has previously described a specific endocytotic receptor for MMP-13 on osteoblastic[14] and fibroblastic cells[15]. The internalization mechanism

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on the osteoblast and fibroblast requires two different receptors. An unknown specific MMP-13 cell surface receptor is required for binding the MMP-13 protein to the cell surface and the low-density lipoprotein receptor-related protein-1 (LRP1) is involved in the internalization of MMP-13. Recently we have reported that normal human chondrocytes and synoviocytes and those from OA patients are also capable of internalizing MMP-13[16]. The human OA chondrocytes had reduced ability to bind MMP-13 and thus were consequently impaired in their ability to internalize and degrade the enzyme[16]. MMP-13 is overexpressed by chondrocytes in OA cartilage[17–19] and type II collagen is the preferred substrate for MMP-13[20]. The use of synthetic collagenase inhibitors has provided evidence that MMP-13 is the major collagenase involved in the chondrocyte mediated cleavage of type II collagen[21]. In addition the targeted expression of constitutively active MMP-13 generates an OA-like pathology in transgenic mice[22]. Despite the known involvement of MMP-13 in OA and these interesting and potentially clinically important observations of the MMP-13 internalization mechanism in OA chondrocytes a comprehensive assessment of the binding and internalization mechanism has not been performed on normal chondrocytes, due to the difficulty of obtaining them in abundance.

In addition to the actions that MMPs have on molecules of the extracellular matrix, the fact that they are able to bind to cell surface receptors suggests that these molecules may also be able to signal into the cell thus directing cellular function. The known component of the osteoblast MMP-13 internalization complex, LRP1, is a promiscuous receptor able to interact and bind many proteins. Most recently LRP1 has been shown to regulate intracellular signalling pathways and in doing so influence pathological disease[23].

Our laboratory has previously documented a process whereby osteoblastic cells remove MMP-13 from the surrounding milieu by binding the enzyme to a specific receptor. The enzyme is then internalized and degraded through the actions of the endocytotic receptor, the low-density LRP1. Furthermore we have also identified this process of MMP-13 internalization in human chondrocytes and found that it is significantly reduced in human chondrocytes from patients with OA. In these present investigations we have characterized the MMP-13 receptor-mediated internalization system in normal rabbit chondrocytes and also identified that the presence of the exogenous MMP-13 protein is able to lead to activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) intracellular signalling enzymes.

Materials and methods

MATERIALS

MMPs-2, -3, -8, and -9 were purchased from R&D Systems. Bovine collagen type II was obtained from Morwell MD Biosciences Inc. Antibodies for ERK1/2 and phospho-ERK1/2 were purchased from Santa Cruz (Santa Cruz, CA) and the AKT and phospho-AKT antibodies came from Cell Signalling (Beverley, MA). All other reagents unless otherwise stated were purchased from Sigma–Aldrich (St Louis, MO).

CELL CULTURE

Rabbit chondrocytes were harvested as previously described[24] from the humerus and femur of New Zealand white rabbits by shaving off the articular cartilage. The cartilage was incubated with agitation for 18–24 h in DMEM (Invitrogen) containing 2 mg/ml of collagenase A (Roche Diagnostic), 0.1 mg/ml hyaluronidase, and 0.15 mg/ml DNase at 37°C in 5% CO_2. Following an overnight incubation the cells were washed 2× in DMEM and seeded at 2×10^5 cells/cm^2. Rabbit chondrocytes were cultured and used in experiments for a maximum of three passages. Mouse embryo fibroblast (MEF1) cells were cultured and maintained in DMEM with 10% foetal bovine serum (FBS) as previously described[15].

PRODUCTION OF RECOMBINANT PROTEINS

Recombinant mouse MMP-13 [which has 87% amino acid sequence homology with human MMP-13[25] and 85% nucleotide homology with rabbit MMP-13] had previously been subcloned into the pET30a expression plasmid (Stratagene, La Jolla, CA) and transformed into BL21 *Escherichia coli*[16]. Expression of recombinant protein was induced with the addition of isopropyl β-D-thiogalactoside (IPTG; 0.4 mM, Boehringer Mannheim) to a culture in log-phase growth. After 4 h, bacteria were centrifuged, and the pellet stored overnight at −70°C. The bacterial pellet was then resuspended in 50 mM Tris–HCl, 200 mM NaCl, 5 mM $CaCl_2$, 1 mM PMSF, 1× protease inhibitors and 0.25 mg/ml lysozyme, incubated on ice for 30 min, freeze thawed 3× and centrifuged for 30 min at 9000*g* at 4°C. The pellet was resuspended in 20 mM Tris–HCl pH 7.6, 6 M guanidine HCl, 1 mM PMSF and 1× protease inhibitors using a Dounce homogenizer and solubilized by rocking at room temperature for 30 min. The solution was then centrifuged and the supernatant collected. All subsequent steps were performed at 4°C. Purification of the protein was performed using a HiTrap nickel column sequentially washed with water and then wash buffer (20 mM Tris–HCl pH 7.6 and 3 M guanidine HCl). The protein was then diluted 1:1 with 20 mM Tris–HCl pH 7.6 and passed through the column. The column was washed with elution buffer 1 (20 mM Tris–HCl pH 7.6, 3 M guanidine HCl and 20 mM imidazole) and eluted with elution buffer 2 (20 mM Tris–HCl pH 7.6, 3 M guanidine HCl and 500 mM imidazole) in 500 μl fractions. The protein-containing fractions were diluted 10-fold by drop wise addition with stirring into refolding buffer (glycerol 20%, 50 mM Tris–HCl pH 7.6, 200 mM NaCl, 50 μM $ZnSO_4$, 2.3 M Urea, 0.05% Brij, 2.5 mM oxidized glutathione, 2.5 mM reduced glutathione) and then allowed to incubate without stirring for 2 h at 4°C. The protein was then dialysed in 1 L of dialysis buffer (50 mM Tris–HCl pH 7.6, 200 mM NaCl, 2 M urea) for 1.5 h and then in 3 L overnight. Finally the protein was concentrated and immediately frozen at −70°C. Purified His-tagged MMP-13 is enzymatically active (as determined by gelatin zymography) and has a molecular mass of 62 kDa compared to 58 kDa native enzyme due to the presence of the 44 amino acid residue N-terminal purification tag. Purification of the GST-tagged receptor-associated protein (RAP) protein was performed as previously described[26].

RADIOIODINATION OF MMP-13

Purified recombinant mouse MMP-13 was labelled with [^{125}I] using the chloramine T method[27]. Briefly, 25 μg of purified enzyme, 1 mCi Na^{125}I (Amersham) and 4 μg of chloramine T were incubated for 10 min at room temperature in enzyme buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$). The reaction was quenched by the sequential addition of

10 μg of sodium metabisulfite, 7.5 μg sodium iodide and 220 μl elution buffer (enzyme buffer with 0.8 M NaCl). The final reaction mixture was then applied to an Econopac DG desalting column (Biorad) and eluted sequentially with 500 μl of elution buffer at 4°C. SDS-PAGE followed by autoradiographic analysis of the eluted fragments demonstrated that the [^{125}I]-MMP-13 co-migrated with the unlabelled purified MMP-13 protein. In addition >95% of the [^{125}I]-MMP-13 precipitated in 20% trichloracetic acid and the specific activity ranged between 2 and 27 μCi/μg.

RT-PCR

Total RNA was isolated from monolayer cultures of primary rabbit chondrocytes and cells cultured following one, two or three passages, using Tri-reagent (Sigma). RT-PCR was performed on 1 μg of total RNA using the Superscript One Step RT-PCR kit (Invitrogen). Oligonucleotides able to amplify rabbit MMP-13 (sense: 5′-CCT CCT GGA CCA AAT TAT GGA GG-3′, anti-sense: 5′-AAC AGC TCT GCA TCC ACC TGC CTG-3′), $T_m = 58$°C, amplified 395 bp fragment; collagen I (sense: 5′-CCC CAG CCA CAA AGA GTA TAC A-3′, antisense: 5′-AGG TCC AGG ACG ACC ATC TTC 3′), $T_m = 65$°C, amplified 643 bp fragment; collagen II (sense: 5′-ATG GAA TTC CTG GAG CCA AAG G-3′, antisense; 5′-AGG TCC AGG ACG ACC ATC TTC-3′), $T_m = 62$°C, amplified 529 bp fragment; aggrecan (sense: 5′-TGG CTG ATC TCA GGT A-3′, antisense: 5′-TGT GTA GCG TAT GGC GTC GTA-3′), $T_m = 60$°C, amplified 442 bp fragment, and β-actin (sense: 5′-GAC TAC CTC ATG AAG ATC CTC-3′, antisense: 5′-AGG AAG GAG GGC TGG AAC AG-3′), $T_m = 58$°C, amplified 242 bp fragment, were used to specifically amplify these genes. The products were then analysed on a 2% agarose gel.

BINDING ASSAYS

Binding assays were performed on confluent cells in 24 well (2.5 cm^2) plates. Cells were incubated with the indicated concentrations of [^{125}I]-MMP-13 at 4°C for the specified period of time. Non-specific binding and binding competition analysis were performed by adding 100-fold excess of unlabelled protein to triplicate wells. After binding the cells were rinsed 3× with cold DMEM, lysed with 1 M NaOH and assayed for radioactivity. Cell enumeration was performed with a haemocytometer on replicate wells.

INTERNALIZATION AND DEGRADATION ASSAYS

After binding 5 nM per well of [^{125}I]-MMP-13, as described above, the cells were rinsed 3× with cold DMEM. Warm (37°C) DMEM (250 μl) was added to the wells and the cells were placed at 37°C for the indicated time period to allow receptor-mediated endocytosis and intracellular processing of the ligand. Following incubation the cells were treated with 0.25% Pronase E for 10 min to remove cell surface proteins. The cells were then centrifuged to separate the cell surface fraction (supernatant) from the internalized fraction (pellet). In experiments measuring MMP-13 degradation, media overlying the cells were collected at the specified times following internalization. These supernatants were incubated with 20% TCA in ethanol (250 μl) and 2% BSA in Tris buffer (pH 7.5, 1 ml) for 2 h on a rocking platform to precipitate intact proteins. The mixture was then centrifuged and the radioactivity in the supernatant (representing the non-precipitable degraded protein) was measured.

IMMUNOBLOTTING EXPERIMENTS

Cells were seeded in 6-well culture plates and the following day serum starved for 24 h. The cells were then incubated with 50 nM MMP-13 for the indicated time, then washed in room temperature PBS and lysed in lysis buffer (20 mM Tris−HCl pH 8.0, 2 mM EDTA pH 8.0, 10% glycerol, 1% Triton-X100, 1× protease inhibitors (Roche Diagnostic), 1 mM sodium vanadate and 50 mM β-glycerophosphate). After lysis the cells were incubated on ice for 20 min prior to microfuging for 5 min to remove cellular debris. A protein assay was then performed on the cell lysate (BioRad) and 10 μg of protein was separated under reducing conditions on a 10% SDS-PAGE gel. The proteins were then transferred to PVDF and incubated in TBST (20 mM Tris−HCl pH 7.6, 137 mM NaCl and 0.1% Tween 20) with 5% non-fat milk and the indicated antibody overnight at 4°C. Following washing the filters were incubated in a 1:10,000 dilution of the appropriate horseradish peroxidase-conjugated IgG and the signal detected using an ECL plus kit (Amersham).

STATISTICAL ANALYSIS

Receptor kinetics were determined by non-linear regression using the GraphPad InPlot 4.0 program. Multi-group mean values were compared using a one-way analysis of variance (ANOVA) and values of $P < 0.05$ were considered significant.

Results

RABBIT CHONDROCYTES RETAIN THEIR CHONDROCYTIC PHENOTYPE IN SHORT TERM CELL CULTURE

The growth of primary chondrocytes in culture is challenging and like many cell types these cells undergo a process of dedifferentiation with sequential passaging. To ensure the phenotypic integrity of the chondrocytes used in this study the cells were assessed for the expression of the cartilage-specific genes aggrecan and type II collagen throughout the culture period (Fig. 1). The mRNA of the extracellular matrix protein aggrecan was expressed at consistently high levels in primary cells as well as cells passaged once, twice and three times, however, the expression of type II collagen, a specific marker of articular cartilage, decreased with subsequent passage of the chondrocytes and was only present at very low levels after the third passaging of the cells. Concurrently with the decline in expression of type II collagen there was an increase in the expression of type I collagen seen in the cells. The inappropriate synthesis of type I collagen by primary chondrocytes has been noted previously[28]. In addition the loss of type II collagen expression in cultured chondrocytes has also been reported[28] and is indicative that the cells are undergoing dedifferentiation. As a consequence of these data only cells passaged once or twice were used in the subsequent studies. Interestingly the rabbit chondrocytes expressed consistent levels of MMP-13 mRNA throughout the culture period.

BINDING OF MMP-13 TO RABBIT CHONDROCYTES WAS SATURABLE AND SPECIFIC AND SUGGESTIVE THAT THERE ARE SEVERAL RECEPTOR CLASSES PRESENT

To determine the amount of time required for the [^{125}I]-MMP-13 ligand to establish equilibrium, a time course of

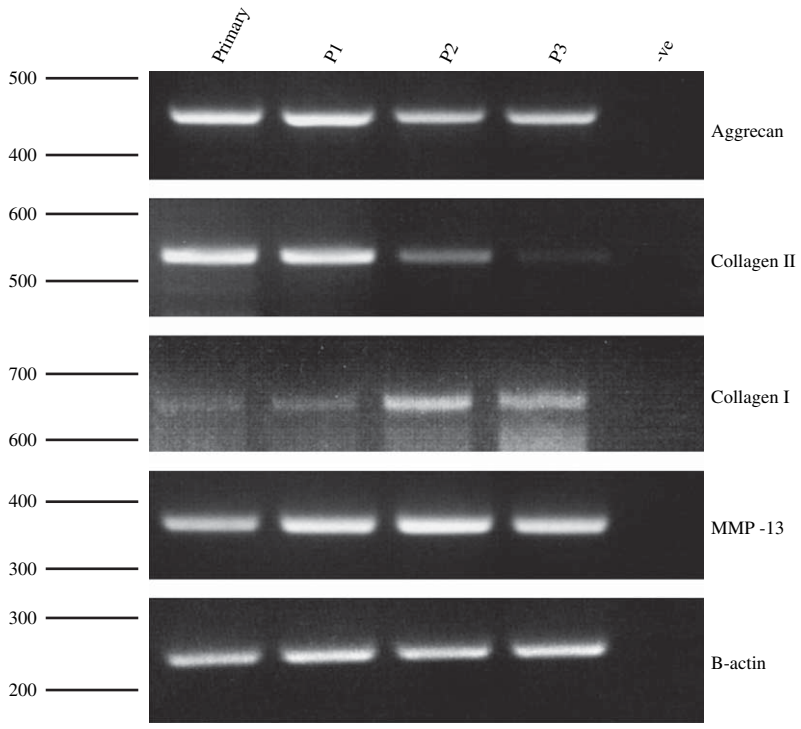

Fig. 1. Phenotyping cultured rabbit chondrocytes by RT-PCR. Total RNA was isolated from rabbit chondrocytes in primary culture or after passages 1, 2 or 3. RT-PCR was performed with 1 μg of total RNA and sense and anti-sense primers of the gene of interest. Thirty amplification cycles of standard PCR conditions were employed for all genes except collagen I where 40 cycles were used.

binding was performed. Appreciable specific cell-associated [^{125}I]-MMP-13 was detected after a 10-min exposure of rabbit chondrocytes to the ligand and equilibrium was approached after 2 h [Fig. 2(A)]. Thus in all subsequent binding experiments a 2-h incubation period was used.

Incubation of increasing concentrations of [^{125}I]-MMP-13 with rabbit chondrocytes showed that the binding of MMP-13 was specific and saturable [Fig. 2(B)] and suggested that there are several receptor classes present. Non-linear regression analysis by the GraphPad InPlot program indicated a high affinity binding site with a K_d of 12.7 nM and a B_{max} of 0.79 fmol/10^6 cells or ~5000 receptors/cell. The low affinity site was calculated to have a K_d of 58.6 nM and a B_{max} of 4.12 fmol/10^6 cells or ~25,000 receptors/cell. The binding of [^{125}I]-MMP-13 to rabbit chondrocytes was competitive as increasing concentrations of cold MMP-13 were able to compete off the hot ligand [Fig. 2(C)]. A log transformation of these data [Fig. 2(C), insert] shows that the competition curve decreases over more than one log phase, which further suggests that MMP-13 is binding to a multiple receptor system.

Receptor specificity for MMP-13 to the chondrocyte cell surface was demonstrated in competition experiments with a panel of extracellular matrix proteins. The rabbit chondrocytes were incubated with 5 nM of [^{125}I]-MMP-13 and 100-fold molar excess of the potential competitors. Binding of [^{125}I]-MMP-13 to rabbit chondrocytes was highly specific, none of the proteins tested were able to compete off [^{125}I]-MMP-13 except cold MMP-13 [Fig. 3(A)]. In addition we assessed the specificity of the receptor binding system to MMP-13 by performing binding competition experiments with a range of cold MMPs. Again the rabbit chondrocytes were incubated with 5 nM [^{125}I]-MMP-13 and a 100-fold excess of the indicated MMP. Collagenases 1 and 2 (MMP-1 and -8) were tested along with stromelysin (MMP-3) and gelatinase A (MMP-2). MMP-1 did partially compete for binding with [^{125}I]-MMP-13, however, none of the other MMPs were able to compete for binding as effectively as cold MMP-13 [Fig. 3(B)].

INTERNALIZATION AND DEGRADATION OF A SINGLE COHORT OF [^{125}I]-MMP-13 IN RABBIT CHONDROCYTES

By binding [^{125}I]-MMP-13 to the rabbit chondrocytes at 4°C, washing off excess ligand and allowing the cells to become metabolically active at 37°C, it is possible to track the localization of the ligand within the cells. As demonstrated in Fig. 6(A) the basal level of [^{125}I]-MMP-13 in the cells was not zero prior to internalization, which likely indicates the cells were internalizing MMP-13. Following 20 min at 37°C, there was a significant increase in the amount of radioactivity detected within the cells [Fig. 4(A)], however, there was no change in the amount of radioactivity released from the chondrocytes in the form of trichloroacetic acid-soluble degradation products at this early time [Fig. 4(B)]. The internalization of a single cohort of [^{125}I]-MMP-13 was complete by 60 min and the amount of cell-associated radioactivity had returned to the initial basal level [Fig. 4(A)]. In contrast the amount of degraded [^{125}I]-MMP-13 released from the cell as trichloroacetic acid-soluble degradation products increased significantly between 30 and 60 min [Fig. 4(B)].

RABBIT CHONDROCYTES EXPRESS LRP1 PROTEIN AND RAP INHIBITS THE INTERNALIZATION OF THE MMP-13 LIGAND

An immunoblot was performed to determine if the rabbit chondrocytes express the LRP1 protein. The LRP1 protein

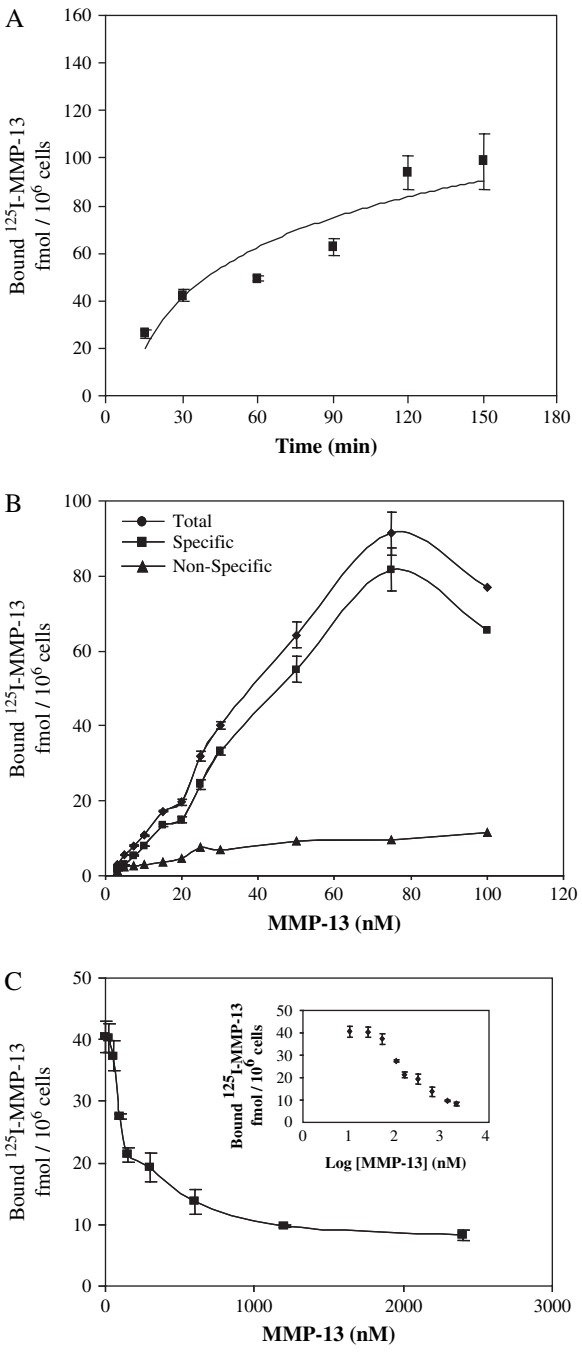

Fig. 2. Binding kinetics of [^{125}I]-MMP-13 to rabbit chondrocytes. (A) Confluent chondrocytes were incubated with 5 nM [^{125}I]-MMP-13 for up to 150 min at 4 °C. At the indicated time points the cells were assayed to quantify cell-associated [^{125}I]-MMP-13. Specific binding (total - non-specific) is displayed as means ± S.E.M. for triplicate wells of a representative experiment. (B) Increasing concentrations of [^{125}I]-MMP-13 were added to confluent chondrocytes. Non-specific binding was assessed by adding a 100-fold excess of MMP-13 to wells containing [^{125}I]-MMP-13. The cells were incubated at 4 °C for 2 h prior to lysis with 1 M NaOH. Total, specific binding (total - non-specific) and non-specific binding are displayed as means ± S.E.M. for triplicate wells. These data were the mean ± S.E.M. of two experiments. (C) Rabbit chondrocytes were incubated in the presence of 5 nM [^{125}I]-MMP-13 and increasing concentrations of cold MMP-13 for 2 h at 4 °C. After washing the cells were lysed with 1 M NaOH. Mean values ± S.E.M. are plotted for triplicate wells of a representative experiment. Inset shows a log transformation of the data.

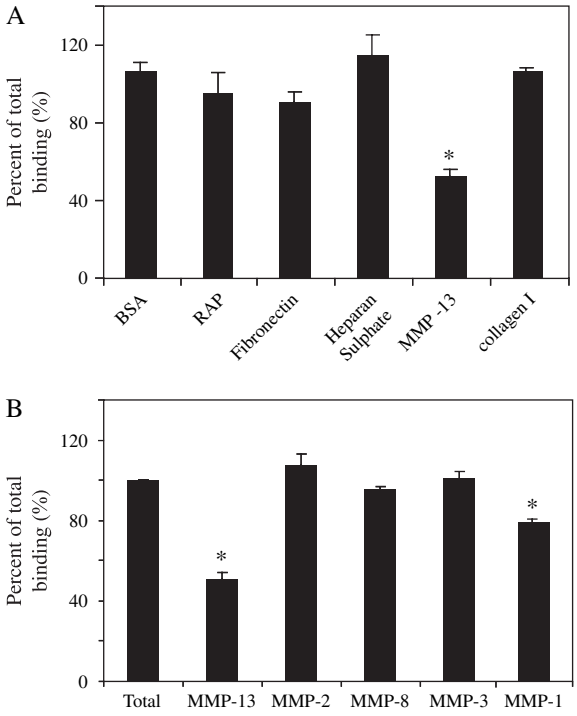

Fig. 3. Specificity of [^{125}I]-MMP-13 binding to rabbit chondrocytes. Rabbit chondrocytes were incubated with 5 nM [^{125}I]-MMP-13 for 2 h at 4 °C in the absence or presence of potential competitors at a concentration of 500 nM. Following washing the cells were lysed in 1 M NaOH and the amount of surface bound ligand determined. Mean values ± S.E.M. for three (A) and two (B) experiments are plotted. *Indicates $P < 0.05$ compared to radioactive ligand alone.

is composed of two subunits (515 and 85 kDa) and both subunits can be clearly seen in the rabbit chondrocytes and the rat osteoblastic UMR 106-01 cells [Fig. 5(A)]. In order to further characterize the possible involvement of LRP1 in the internalization of [^{125}I]-MMP-13 by rabbit chondrocytes, the RAP (LRP1 chaperone protein) was used in an internalization experiment. RAP competitively binds to LRP1 and thus prevents the binding of other ligands to this receptor. Cells not treated with RAP were able to internalize the MMP-13 protein as previously shown [Figs. 4(A) and 5(B)], however, in the presence of RAP the rabbit chondrocytes were significantly impaired in their ability to internalize [^{125}I]-MMP-13 [Fig. 5(B)]. Similar to previous observations in both osteoblasts and fibroblasts, there was no significant change in the amount of [^{125}I]-MMP-13 bound to the cell surface of the chondrocytes [Fig. 5(B), inset] in the presence of the RAP, however, RAP was able to inhibit the internalization of MMP-13 in the rabbit chondrocytes as we have also seen in osteoblasts and fibroblasts[15].

BINDING OF [^{125}I]-MMP-13 TO RABBIT CHONDROCYTES INDUCES PHOSPHORYLATION OF ERK

The fact that MMP-13 is able to bind to a cell surface receptor or binding protein raises the possibility that it is also able to initiate signalling into cells. The LRP1 component of the MMP-13 internalization system is known to be phosphorylated in response to activation of the PDGFR[23]. To assess the involvement of MMP-13 in the activation of the ERK1/2 pathway in chondrocytes, whole cell lysates

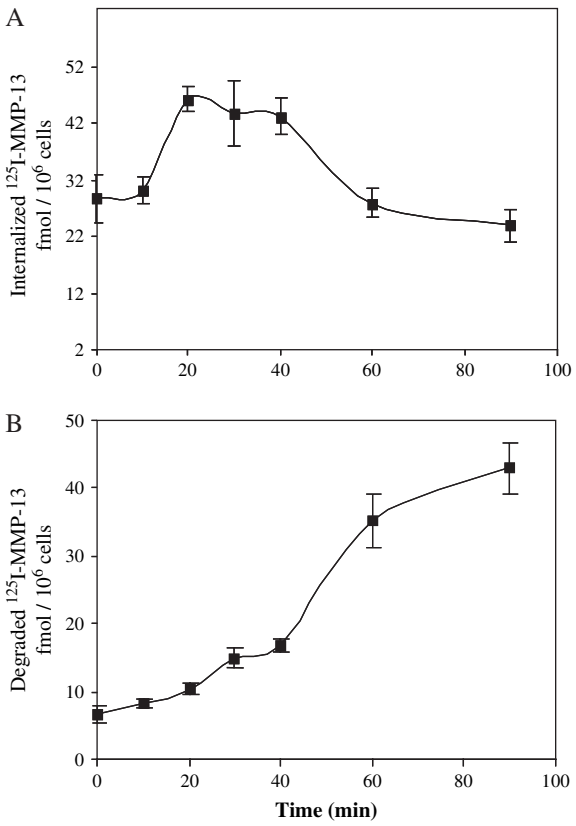

Fig. 4. Internalization and degradation of a single cohort of [^{125}I]-MMP-13 in rabbit chondrocytes at 37°C. Cells were incubated at 4°C for 2 h in the presence of 5 nM [^{125}I]-MMP-13. After washing the cells 3× with cold MEM, 250 μl of warm MEM was added to each well and the cells were moved to 37°C. At each time point, the cell media were collected to assess degraded [^{125}I]-MMP-13 (panel B) and the cells were then washed once with MEM. The cells were treated with 0.25% Pronase for 10 min at 4°C. The cell suspension was collected and centrifuged to separate the cell pellet shown in panel A (defining internalized [^{125}I]-MMP-13) from the supernatant (representing the degraded protein). Mean values ± S.E.M. are plotted of a representative experiment.

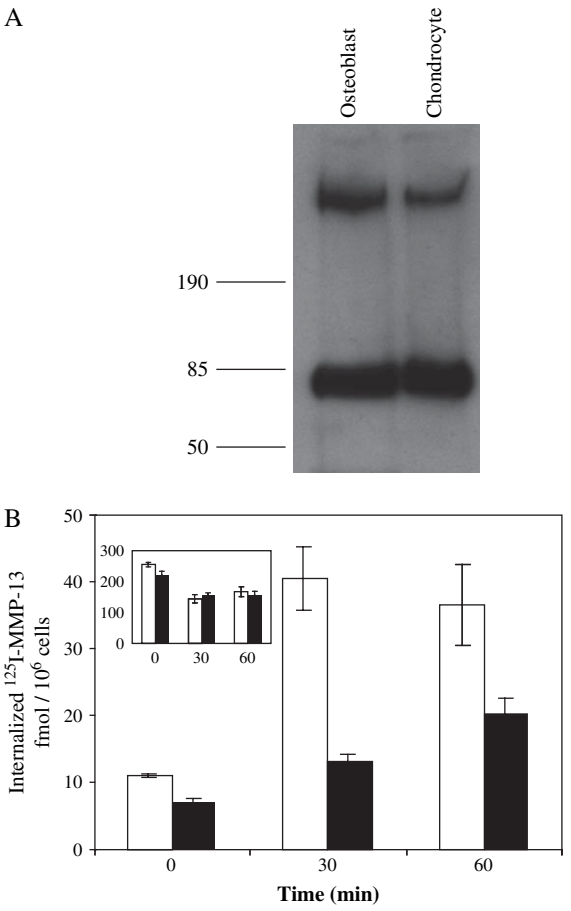

Fig. 5. Rabbit chondrocytes express LRP1 and RAP inhibits internalization of a single cohort of [^{125}I]-MMP-13. (A) Total protein (10 μg) from rabbit chondrocytes and rat UMR 106-01 osteoblastic cells were immunoblotted with anti-LRP1 antibodies. (B) Rabbit chondrocytes were incubated with 5 nM [^{125}I]-MMP-13 in either the absence (white bars) or the presence (black bars) of RAP. Following incubation the cells were assessed for internalization and surface bound (inset) radioactive ligand as described in the Materials and methods. Mean values ± S.E.M. of triplicate wells are plotted for a representative experiment.

from rabbit chondrocytes untreated or treated with MMP-13 were run on an SDS-PAGE gel and immunoblotted with either anti P-ERK or anti P-Akt antibodies. As a positive control, treatment of rabbit chondrocytes with 10 ng/ml of EGF induced strong phosphorylation of both ERK and Akt [Fig. 6(A,B)]. Treatment of rabbit chondrocytes with 50 nM MMP-13 induced phosphorylation of ERK which was detectable after a 10-min exposure to the enzyme and was very prominent after 30 and 60 min of MMP-13 treatment [Fig. 6(A)]. However, while EGF induced a strong phosphorylation of Akt, MMP-13 treatment had no effect on Akt activation at any of the time points assessed [Fig. 6(B)]. We have also reported that MMP-13 is able to bind to the surface and be internalized by other cell types including osteoblastic cells and fibroblasts. To investigate if MMP-13 also stimulates phosphorylation of ERK1/2 in these cells, MEF1 cells were treated with 50 nM MMP-13. Although MEF1 cells have a higher level of background ERK phosphorylation than that seen in rabbit chondrocytes, MMP-13 treatment stimulated phosphorylation of ERK1/2 in MEF1 cells in a similar time course to that observed in the rabbit chondrocytes. A detectable increase in ERK phosphorylation occurred after 20 min MMP-13 treatment and a greater response occurred after 60 min [Fig. 6(C)]. Interestingly, the observed ERK1/2 phosphorylation seemed to predominate in chondrocytes compared to fibroblast cells, possibly suggesting that the MMP-13 receptor system may produce different biological responses in these two cell types.

To assess the involvement of LRP1 or the unidentified specific MMP-13 receptor in the phosphorylation of ERK1/2, chondrocytes were treated with hMMP-1 or mMMP-13 in either the presence or absence of RAP. RAP inhibited internalization of MMP-13 without altering binding of mMMP-13 to the cell surface [Fig. 5(B)] or stimulation of phosphorylation of ERK1/2 [Fig. 6(D)], suggesting that the specific MMP-13 receptor is involved in both MMP-13 binding and ERK1/2 activation, however, the LRP1 is only required for MMP-13 internalization and is not involved in ERK1/2 phosphorylation.

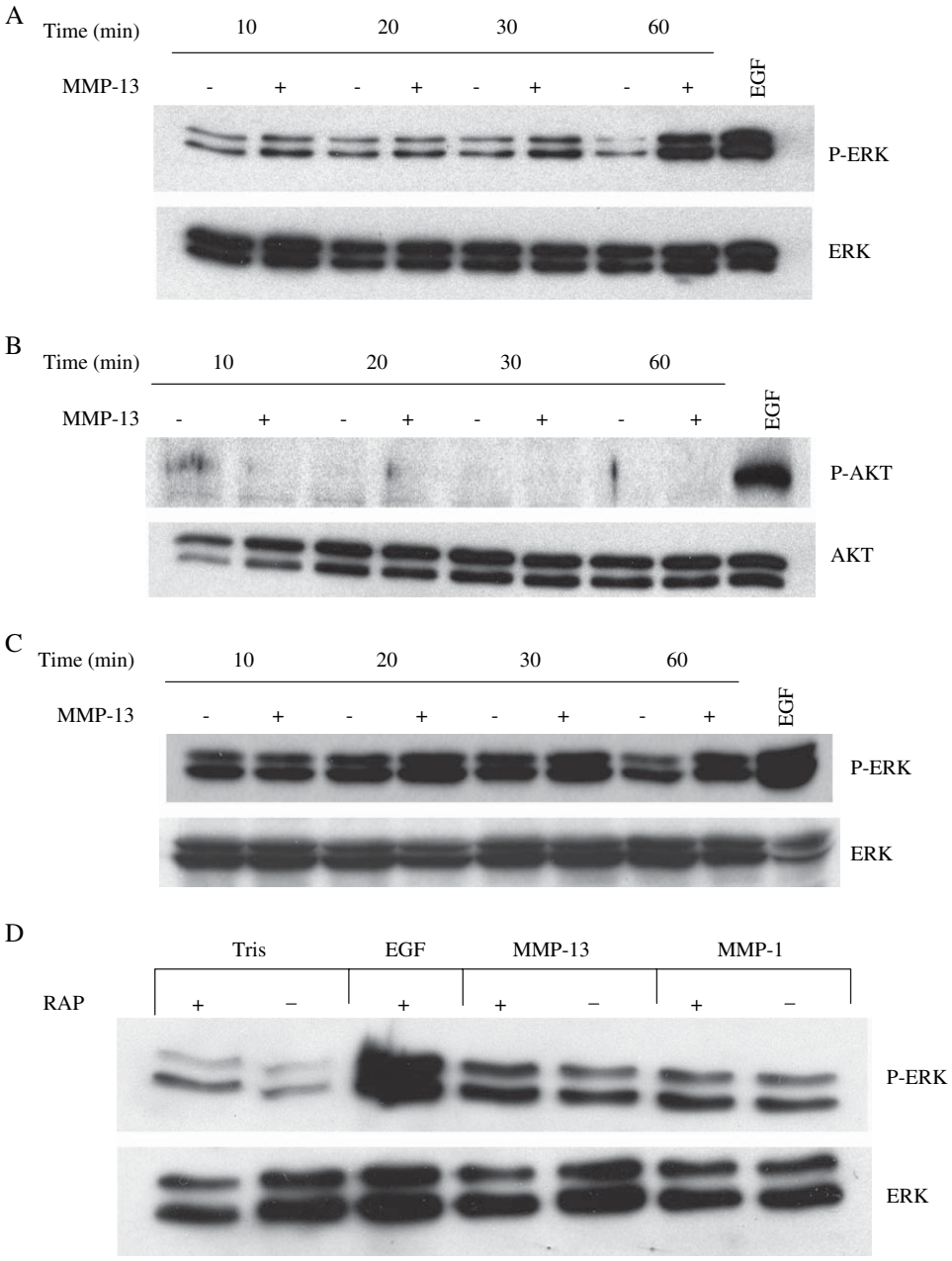

Fig. 6. Treatment of rabbit chondrocytes or MEF1 cells with MMP-13 results in phosphorylation of ERK1/2 but not Akt and involves the MMP-13 specific receptor. Rabbit chondrocytes (A, B and D) or MEF1 cells (C) were serum starved for 24 h, then treated for the indicated times with or without 50 nM MMP-13, hMMP-1 in the presence or the absence of 5 mM RAP or 10 ng/ml EGF as indicated. Total cell protein (10 μg) was separated on a 10% SDS-PAGE gel, electrotransferred to PVDF membrane and immunoblotted with either P-ERK1/2 (A, C and D) or P-Akt (B) antibodies. Following detection the respective blots were stripped and re-probed with ERK1/2 (A, C and D) or Akt (B) antibody as indicated.

Discussion

In this report we characterize the MMP-13 receptor endocytotic system in rabbit chondrocytes, and identify that the presence of the enzyme can also influence phosphorylation of intracellular ERK1/2. The presence of this receptor system has previously been documented in normal human chondrocytes and shown to be impaired in chondrocytes from patients with OA[16]. While a ready supply of human chondrocytes from patients with OA can be obtained from joint replacement surgery, it is difficult to find sufficient samples of normal human articular cartilage. Thus to fully characterize the MMP-13 endocytotic-receptor system in chondrocytes we have used rabbit chondrocytes, which have been extensively used in previous chondrocytic studies and can be harvested on demand.

The MMP-13 endocytotic-receptor system was first identified in osteoblastic cells and has subsequently been found to exist in fibroblasts[15], synoviocytes and chondrocytes[16]. In the osteoblast and fibroblast this system contains two components, an unknown high affinity receptor essential for MMP-13 to bind to the cells, and the low affinity LRP1,

which is required for the internalization of MMP-13[15]. The present studies in rabbit chondrocytes have also identified that there are multiple receptors in the MMP-13 endocytotic-receptor system. Similar to the rat osteoblastic and human chondrocytic studies, the high affinity receptor on rabbit chondrocytes has a K_d in the nanomolar range ($K_d = 5$, 13.7 and 12.5 nM, respectively). Similarly the low affinity receptor identified on the rabbit chondrocytes had a dissociation constant of 58.6 nM which is slightly lower, but comparable to that reported for the human chondrocytes, 101 nM. Interestingly, the number of receptors found on the rat osteoblastic cells and rabbit chondrocytes are lower than those reported in the human chondrocytes and this may indicate that this system varies between different species.

Alternatively, the higher receptor numbers identified on the human cells may be reflective of the specificity of the MMP-13 endocytotic-receptor system which also seems to vary slightly between species. In the rat osteoblastic cells, this system was highly specific with only MMP-13 competing for binding to the osteoblasts. No specific binding was identified on rat osteoblastic cells when [^{125}I]-MMP-1 and -2 were used[15]. In these current studies on rabbit chondrocytes only MMP-1 was able to compete for binding with [^{125}I]-MMP-13, but to a lesser extent than MMP-13, while none of the other collagenases (MMP-8), gelatinase B (MMP-2) or stromelysin (MMP-3) competed with MMP-13 for binding, indicating that this system is very specific for MMP-13 in rabbit as well as rodent cells. Interestingly, in the human chondrocytes, the MMP-13 endocytotic-receptor system is not completely specific for MMP-13, since both MMP-1 and MMP-3 were able to partially compete for binding with [^{125}I]-MMP-13[16]. This suggests that the specificity of this mechanism varies with different animal species. The increased number of receptors found on the human cells may reflect the fact that the MMP internalization mechanism endocytoses MMPs other than MMP-13 in human cells.

Despite the potential difference in the unknown high affinity receptor in the MMP-13 endocytotic-receptor system, the low affinity receptor previously identified as LRP1[15] is a consistent component of the system for all cells studied. The LRP1 receptor previously identified in the osteoblastic cells and fibroblasts is strongly expressed in the rabbit chondrocytes and inhibiting its ligand binding functions with RAP dramatically reduces internalization of MMP-13. This indicates that the LRP1 component of the MMP-13 endocytotic-receptor system is conserved in rabbits, rodents and humans.

The observation that external treatment with MMP-13 can elicit phosphorylation of ERK1/2 is interesting in light of the effects that activation of this pathway can have on chondrocytes. The ERK1/2 signalling pathway has been shown to be a negative regulator of cartilage-specific gene expression in the embryonic limb mesenchyme during chick development[29]. ERK1/2 phosphorylation also induces dedifferentiation of articular chondrocytes grown in monolayers[30], while down-regulation of ERK1 is required for redifferentiation of articular chondrocytes[31] which indicates that this signal transduction pathway also has a role in regulating the adult chondrocyte phenotype. In addition EGF-induced ERK1/2 phosphorylation is involved in the regulation of chondrocytic expression of Cox-2 and PGE_2 production by the chondrocyte[32]. PGE_2 is a pro-inflammatory cytokine found in large quantities in arthritic joints and has been shown to contribute to joint inflammation and the associated bone erosion[33,34]. Thus, the ERK1/2 signalling pathway is a pivotal cascade in the chondrocyte and its activation prevents maintenance of the chondrocyte phenotype and induces the development of a deleterious cytokine environment. The fact that MMP-13 is increased in OA and can cause activation of the ERK1/2 pathway suggests that in addition to regulating the integrity of the extracellular matrix this protease may also regulate the chondrocyte phenotype and cytokine production profile through the ERK1/2 pathway in OA.

LRP1 is a member of the LDL receptor superfamily and is known for its ability to bind and internalize a diverse group of ligands including proteases[35,36], protease inhibitor complexes[37,38] and lipoproteins[39]. Following internalization, the ligand and LRP1 are uncoupled in cellular endosomes and the ligands proceed to lysosomes for degradation while LRP1 is recycled back to the plasma membrane. In addition to their role in regulating availability of extracellular ligands the LDL receptor family members can also signal into the cell. The very low-density lipoprotein receptor and the apoE receptor are involved in the cortical development transducing signals mediated by the ligand reelin[40,41]. LRP1 has also been implicated in cellular functions other than ligand internalization, for example the ligand alpha 2 macroglobulin induces calcium influx in neurons when it binds to LRP1[42]. In addition LRP1 can undergo tyrosine phosphorylation[23] and it has subsequently been shown that this occurs indirectly through the PDGFR and Src family kinases[43,44]. Despite these observations it was only recently that the importance of LRP1 signalling has been shown to have physiological significance. Boucher *et al.*[23] generated smooth muscle-specific LRP1 deficient mice which have an overexpression and subsequent activation of the PDGFR which results in an increased prevalence of cholesterol-induced atherosclerosis. Thus in this instance the presence of LRP1 regulates PDGFR signalling and as a consequence the cellular response. However, in the MMP-13 internalization system described here the LRP1 is essential for internalization of the MMP-13 protein but the process of internalization, and thus LRP1, is not required for phosphorylation of ERK1/2.

In summary these studies have characterized the MMP-13 endocytotic-receptor system and identified some potential differences between these mechanisms in rabbit, rodent and human cells. The isolation of the specific MMP-13 receptor is currently in progress and identification of this receptor will allow for the detailed assessment of the differences in the rabbit, rodent and human systems as well as the specific receptor's involvement in the MMP-13-stimulated phosphorylation of ERK1/2. The ability of MMP-13 to activate intracellular ERK1/2 has many consequences for chondrocytes and further studies are required to elucidate the physiological and pathological consequences of MMP-13-induced phosphorylation of ERK1/2 in chondrocytes.

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