Aggravation of ADAMTS and Matrix Metalloproteinase Production and Role of ERK1/2 Pathway in the Interaction of Osteoarthritic Subchondral Bone Osteoblasts and Articular Cartilage Chondrocytes — Possible Pathogenic Role in Osteoarthritis

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ABSTRACT. ***Objective.*** Degradative enzymes, such as A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and matrix metalloproteinases (MMP), play key roles in development of osteoarthritis (OA). We investigated if crosstalk between subchondral bone osteoblasts (SBO) and articular cartilage chondrocytes (ACC) in OA alters the expression and regulation of ADAMTS5, ADAMTS4, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13, and also tested the possible involvement of mitogen-activated protein kinase (MAPK) signaling pathway during this process.

Methods. ACC and SBO were isolated from normal and OA patients. An *in vitro* coculture model was developed to study the regulation of ADAMTS and MMP under normal and OA joint crosstalk conditions. The MAPK-ERK inhibitor PD98059 was applied to delineate the involvement of specific pathways during this interaction process.

Results. Indirect coculture of OA SBO with normal ACC resulted in significantly increased expression of ADAMTS5, ADAMTS4, MMP-2, MMP-3, and MMP-9 in ACC, whereas coculture of OA ACC led to increased MMP-1 and MMP-2 expression in normal SBO. Upregulation of ADAMTS and MMP under these conditions was correlated with activation of the MAPK-ERK1/2 signaling pathway, and addition of the MAPK-ERK inhibitor PD98059 reversed the overexpression of ADAMTS and MMP in cocultures.

Conclusion. These results add to the evidence that in human OA, altered bidirectional signals between SBO and ACC significantly influence the critical features of both cartilage and bone by producing abnormal levels of ADAMTS and MMP. We have demonstrated for the first time that this altered crosstalk was mediated by the phosphorylation of MAPK-ERK1/2 signaling pathway.
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Key Indexing Terms:
OSTEOARTHRITIS CHONDROCYTES OSTEOBLASTS
CELL INTERACTIONS MATRIX METALLOPROTEINASE
MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY

Some of the key pathophysiological features of osteoarthritis (OA) joints include abnormal subchondral bone metabolism and degeneration of the articular cartilage. It has been proposed that the changes in the underlying subchondral bone have a profound effect on the initiation of cartilage degeneration[1]. At the cellular level, the influence of OA subchondral bone osteoblasts (SBO) alters the phenotypic gene expression of articular cartilage chondrocytes (ACC) in a coculture model[2,3,4], and further, a strong correlation has been found with articular cartilage changes and abnormal subchondral bone remodeling in OA[5,6]. These findings provide evidence that articular cartilage and subchondral bone influence each other's metabolism, leading to altered bidirectional cell signaling that results in OA pathogenesis. An ideal therapeutic approach would therefore be directed at regulating this altered cell crosstalk. In order to achieve this outcome, specific pathological cascades that are triggered during the interactions and the molecular mechanisms that govern these events should be identified and targeted.

The excessive cartilage degeneration and abnormal bone remodeling that characterize OA prompted us to investigate

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the potential involvement of aggrecanases, such as A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and matrix metalloproteinases (MMP), in the interaction between articular cartilage and subchondral bone in the OA joint. The loss of aggrecan, through the actions of aggrecanase enzymes, is a key event in early OA, and ADAMTS4 and ADAMTS5 are the major cartilage aggrecanases in humans[7]. Studies in mice show that deletion of ADAMTS5 protects against the development of OA and inflammatory arthritis, suggesting that ADAMTS5 plays a key role during OA development[8]. There is, on the other hand, strong evidence that MMP, in particular MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13, are some of the major enzymes involved in the degeneration of the articular cartilage in OA[9]. In addition, upregulated expression of MMP has been reported in OA SBO, relating to the abnormal osseous tissue remodeling[10]. It is well documented that ACC and SBO express abnormal levels of proteolytic enzymes in OA tissues, but relatively little is known about the exact mechanism behind this abnormal production.

Mitogen-activated protein kinase (MAPK) cascades, including ERK1/2, JNK, and p38, mediate a number of cell responses in both osteoblasts and chondrocytes. There is mounting evidence that activation of MAPK, in particular ERK1/2, contributes to the induction of expression of MMP by a number of extracellular stimuli in different cell types, including chondrocytes[11,12] and osteoblasts[13]. The aim of our study was to examine whether the interactions between OA SBO and ACC are critical in regulating the abnormal production of ADAMTS and MMP by applying an *in vitro* coculture model; and to investigate the potential molecular mechanism involved during this crosstalk of cells. MAPK-ERK1/2-specific inhibitor was used to delineate the pathway involvement during this interaction process.

MATERIALS AND METHODS

Reagents: cell culture. Dulbecco modified Eagle's medium (DMEM) and antibiotics (penicillin and streptomycin) were purchased from Gibco (Invitrogen, Mt. Waverley, Victoria, Australia), fetal bovine serum (FBS) was obtained from Thermo (In Vitro Technologies, Nobel Park, Victoria, Australia), and Trizol was from Invitrogen. The MAPK pathway selective inhibitor for MEK-ERK1/2, PD98059, was purchased from Calbiochem (Novabiochem, Alexandria, NSW, Australia). MMP-2 and MMP-9 ELISA assay kits were from Raybio systems (Bioscientific Pty. Ltd., NSW, Australia). Phospho ERK1/2, MMP-2, and MMP-9 antibodies were purchased from Cell Signaling Technology (Genesearch, Arundel, Queensland, Australia). Antibodies of ADAMTS4, ADAMTS5, MMP-1, and MMP-8 were from Abcam (Sapphire Bioscience Pty. Ltd., Redfern, NSW, Australia). MMP-3 and MMP-13 antibodies were purchased from Thermo Scientific Pty. Ltd. (Fremont, Australia).

Articular cartilage sample collection and phenotypic determination. Normal ACC were obtained from knee medial compartment tibial joint cartilage from tissue donors (n = 4) who were undergoing above-the-knee amputations due to traumatic injury. All normal samples were collected anatomically from the middle of knee. Normal patients were healthy adults (mean age 58.73 ± 2.76 yrs) with no clinical signs or symptoms of joint, metabolic, or hormonal diseases (e.g., osteoporosis). None of the patients was taking medications that might affect cartilage or bone metabolism. Patients selected for study had all ceased taking antiinflammatory medication at least 2 weeks prior to surgery. Patients with early-stage OA were excluded if the samples showed any evidence of cartilage changes, such as softening of hyaline articular cartilage, thinning and fibrous dislocation, ulcerations of the cartilage, or light sclerosis of the subchondral bone. OA ACC were sourced from the main defective area of the medial compartment knee tibial joint cartilage from patients undergoing total knee replacement surgery. All OA samples were collected anatomically from the middle of knee. The mean age of patients with OA (n = 5) was 65.20 ± 5.94 years. All radiographs were reviewed, and the patient samples were classified according to 2 categories, depending on the Mankin score. Mankin score = 0 indicated normal cartilage and a score > 3 indicated degenerative OA cartilage[14]. Chondrocytes from the cartilage tissues were isolated as described[15]. Briefly, cartilage was dissected into small pieces with a sterile scalpel, and washed several times with 1× phosphate buffered saline (PBS). Chondrocytes were released by digesting the tissues in 0.2% type II collagenase in high-glucose DMEM at 37°C for 16 h. The cell suspension was filtered through 70-μm meshes and centrifuged at 1000 × g for 10 min and resuspended in DMEM at a density of 2500 cells/cm^2. The phenotype stability of ACC used in our study was confirmed by analyzing the messenger RNA (mRNA) expression of type II collagen (COL2), type I collagen (COL1), and aggrecan (AGG). Early-passage (passage 0–1) ACC were used for the coculture studies.

Ethics approval was granted from the Queensland University of Technology and Prince Charles Hospital ethics committees and informed consent was given by all patients involved.

Subchondral bone sample collection and phenotypic determination. Tibial knee bone specimens were taken from within 5 mm of the subchondral bone plate. OA SBO (n = 5) were cultured from bone sourced from the medial compartment of tibial knee from the patients with advanced OA, as described above, where the cartilage was degraded and showing prominent subchondral bone sclerosis and density. Normal SBO (n = 4) were cultured from tibial knee bone collected from the trauma patients with no evidence of cartilage degeneration. Normal and OA bone pieces were consistently collected from the middle of load-bearing medial compartment knee. None of the normal patients had received antiinflammatory or bisphosphonate medication. The criteria for these diagnoses are those established by the American College of Rheumatology[16].

SBO were isolated according to the method described by Beresford, *et al*[17,18]. Briefly, bone was minced into small pieces with a sterile bone cutter, and then washed several times with 1× PBS, then placed in T25 flasks with a sterile forceps and air dried for 10 min in a laminar flow hood. Complete medium, consisting of high-glucose DMEM supplemented with 10% FBS and 50 U/ml penicillin and 50 μg/ml streptomycin, was added to the bone pieces and incubated at 37°C in a standard humidified incubator containing 5% CO_2/95% atmospheric air. Cells started to emerge from bone pieces after about 1 week. The bone cell phenotype was confirmed by determining the production of early bone markers alkaline phosphatase (ALP) and osteocalcin (OCN). All bone cell populations tested negative for the hematopoietic cell markers CD34 and CD45 (data not shown). Isolated SBO showed strong staining for alizarin red and positive expression for ALP and OCN under osteogenic induction media, confirming the osteogenic lineage of these cells. Only early passage SBO (passage 2–3) were used for coculture studies.

Coculture model. Indirect coculture was performed to test the effect of soluble factors. The coculture studies were performed as 1 of the following 4 different combinations. Combination 1: normal SBO with normal ACC; Combination 2: normal SBO with OA ACC; Combination 3: OA SBO with normal ACC; and Combination 4: OA SBO with OA ACC. A time-dependent study (24 h, 48 h, 72 h, and 96 h) was performed to determine the effect of cocultures on respective cell type ADAMTS and MMP production.

High-density ACC micromass culture. High-density micromass droplets were prepared as described[19]. Briefly, ACC were resuspended in growth media at a final cell density of 2.5×10^7 cells/ml and spotted as 10 μl/well droplets in 6-well culture plates and incubated at 37˚C in 1 ml chondrogenic medium [serum-free high-glucose DMEM supplemented with 10 ng/ml transforming growth factor-ß3 (Bio Scientific, Gymea, NSW, Australia), 10 nM dexamethasone, 50 mg/ml ascorbic acid, 10 mg/ml sodium pyruvate, 10 mg/ml proline, and an insulin-transferrin-selenium supplement (final concentration 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite, 0.5 mg/ml bovine serum albumin, and 4.7 μg/ml linoleic acid)] for 1 week before they were used for indirect coculture experiments.

Coculture of ACC micromasses with SBO conditioned medium. Early passage (passage 2) SBO from normal and OA subchondral bone were first differentiated for 1 week in osteogenic medium (DMEM, 10% fetal calf serum, 10 nM dexamethasone, 10 mM ß-glycero-phosphate, 50 μg/ml ascorbic acid), then the cells were washed with PBS and incubated in serum-free high-glucose DMEM containing only 50 U/ml penicillin and 50 μg/ml streptomycin for 48 h. The conditioned media from these flasks was collected and centrifuged at 1000 × g for 15 min and the supernatants mixed with an equal volume of fresh media (preincubated at 37˚C) with the same supplements to form conditioned media. During coculture experiments, differentiated normal and OA ACC micromasses prepared as described above were grown in serum-free conditioned media from either normal or OA SBO. As controls, ACC were cultured in the medium composition that was not incubated with SBO. Total protein from the ACC was extracted and levels of pERK1/2, ADAMTS proteases, and MMP were determined by Western blot analysis.

Coculture of SBO with ACC conditioned medium. Micromasses prepared as described were first differentiated in chondrogenic medium for 1 week[4,20], then incubated with serum-free high-glucose DMEM containing only 50 U/ml penicillin and 50 μg/ml streptomycin for 48 h. The medium used by normal and OA micromasses was collected and centrifuged at 1000 × g for 15 min and the supernatants mixed with an equal volume of fresh medium (preincubated at 37˚C) to form conditioned medium. During coculture experiments, SBO (8000 cells/cm^2) were differentiated in osteogenic induction medium for 1 week, then washed with PBS and incubated with serum-free conditioned medium from either normal or OA ACC micromasses. As controls, SBO were cultured in the medium composition that was not incubated with ACC. Total proteins from the SBO were extracted and the levels of pERK1/2 and MMP were determined by Western blot. A schematic of indirect coculture models used in this study is illustrated in Figure 1B.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qPCR was performed to confirm the phenotype of normal and OA ACC as described[4]. Briefly, total RNA was isolated with Trizol reagent (Invitrogen), DNase treated and column purified using an RNeasy mini kit (Qiagen Pty. Ltd., Victoria, Australia). Complementary DNA was synthesized using Superscript III (Invitrogen) from 1 μg total RNA following the manufacturer's instructions. PCR primers were based on cDNA sequences from the National Center for Biotechnology Information Sequence Database using the Primer Express® software and primer specificity was confirmed by BLASTN searches. qRT-PCR was performed on an ABI Prism 7000 Thermal Cycler (Applied Biosystems, Scoresby, Australia) with SYBR Green detection reagent. Briefly, 2 μl of cDNA, 20 pmol of gene-specific primers, and 10 μl of 1× Master Mix were used in a 20 μl reaction volume; each sample was performed in duplicates. Thermocycling conditions were as follows: 1 cycle of 10 min at 95˚C for activation of the polymerase, 40 cycles of 10 s at 95˚C, and 1 min at 60˚C for amplification. Dissociation curve analysis was carried out to verify the absence of primer dimers and/or nonspecific PCR products. Relative expression of the genes of interest was normalized against 18srRNA and GAPDH housekeeping genes by comparative cycle of threshold (Ct) value method. The difference between the mean C_t values of the gene of interest and the housekeeping gene is denoted ΔC_t, and the difference between ΔC_t and the C_t value of the calibrator sample is denoted $\Delta\Delta C_t$. The $\log_2 (\Delta\Delta C_t)$ gives the relative value of gene expression.

Determining involvement of MAPK pathways. Western blot analysis using

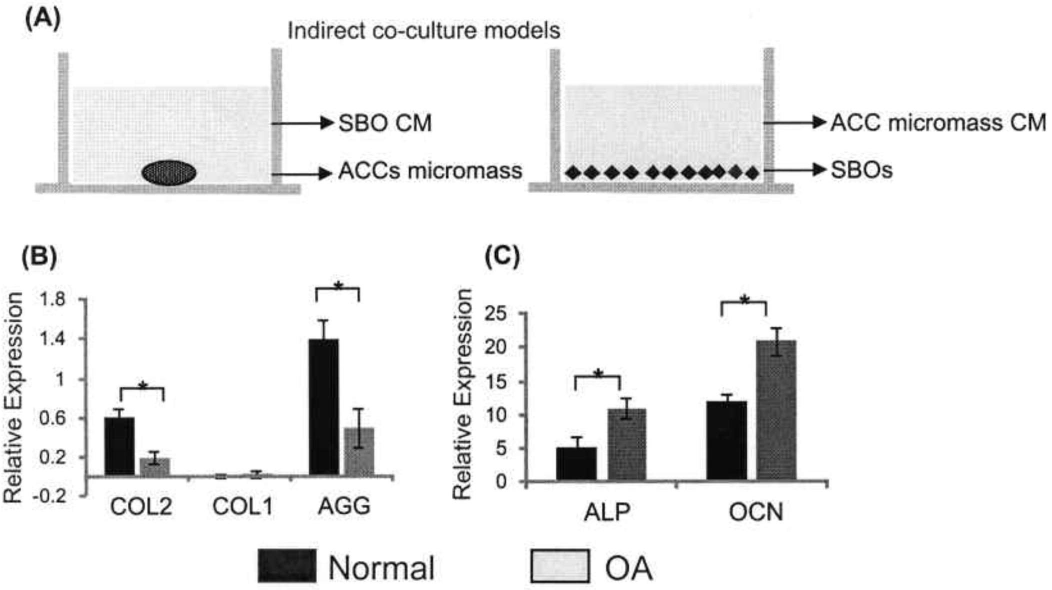

Figure 1. Characterization of articular cartilage chondrocytes (ACC) and subchondral bone osteoblasts (SBO). A. Coculture models in different combinations. SBO were cultured with conditioned media (CM) from ACC or vice versa. B. ACC phenotypic characterization assessed by gene expression of *COL2*, *COL1*, and *AGG* in normal (n = 4) and osteoarthritis (OA) ACC (n = 5). C. SBO phenotypic characterization assessed by the gene expression of *ALP* and *OCN* in normal (n = 4) and OA SBO (n = 5). Relative gene expression values are normalized to 18s and GAPDH housekeeping genes and shown as mean ± SD of $\Delta\Delta C_t$ values. *Significant difference between normal and OA ACC or SBO ($p < 0.05$).

antibodies against phosphorylated ERK1/2 was used to detect ERK signal activation in the indirect cocultured SBO and ACC. MAPK-mediated cellular interactions were further evaluated using PD98059, which selectively inhibits the ERK1/2 pathway. Briefly, the concentrated stock solution of the inhibitor was dissolved in dimethylsulfoxide (DMSO), and the cocultures incubated with or without PD98059. The final concentration of DMSO never exceeded 0.1% (vol/vol) and the same amount of DMSO vehicle was added to the control cultures. Pilot experiments showed that PD98059 at 10 μM was an optimal concentration for ERK1/2 inhibition; there was no observable change to cell proliferation at this concentration, nor any evidence of cytotoxicity, as assessed by lactose dehydrogenase assays (data not shown). All experiments were performed in triplicate.

Zymography. Conditioned media from normal and OA ACC/SBO were quantified before using them for cocultures using a micro-bicinchoninic acid (BCA) assay kit following the manufacturer's protocol (Thermo Scientific, Victoria, Australia) and the volume was adjusted to contain the same quantity of protein. After coculture, the gelatinolytic activity of serum-free conditioned medium was assessed by separating the proteins on 10% SDS-PAGE gel containing 1 mg/ml gelatine as substrate. The gels were washed for 30 min with 2.5% Triton X-100 and then incubated at 37˚C for 12–24 h in an incubation buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $CaCl_2$, and 50 mM NaCl. Gels were stained with coomassie brilliant blue and destained to visualize white bands against the blue background. Further verification was sought by incubating the gel in 10 mM EDTA (inhibitor of MMP).

Enzyme-linked immunosorbent assay. An ELISA was used to determine the amount of secreted MMP-2 and MMP-9 proteins in the conditioned medium of cocultured and non-cocultured cells. After determination of protein concentration, equal amounts of samples and standards (100 μl) were incubated in 96-well plates precoated with specific antibody overnight at 4ºC. After several washings, 100 μl biotinylated antibody was added and incubated 1 h at room temperature. The MMP secreted proteins were detected with horseradish peroxidase (HRP)-conjugated streptavidin solution. After washing, the amount of conjugate bound to each well was determined by addition of tetramethylbenzidine substrate. The reaction was quenched by adding a stop solution and optical density was measured immediately using a 96-well plate reader at 450 nm. The concentration of total MMP protein in each sample was extrapolated from a standard curve.

Western blot assay. Total cell lysates from cocultured and non-cocultured ACC and SBO were harvested by lysing cells with lysis buffer containing 1 M Tris HCl (pH 8), 5 M NaCl, 20% Triton X-100, 0.5 M EDTA, and a protease inhibitor cocktail (Roche, Castle Hill, Australia). Cell lysates were clarified by centrifugation and the protein concentration was determined by a BCA protein assay. A total of 10 μg protein was separated on a 12% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane, and blocked in 0.1% Tris-Tween buffer containing 5% nonfat milk. The membranes were incubated with primary antibodies at 1:1000 dilutions (for all the antibodies) overnight at 4ºC. After washing the membranes 3 times in TBS-Tween buffer they were incubated with a goat anti-mouse or goat anti-rabbit IgG-HRP-conjugated antibody at 1:2000 dilution for 1 h. Protein bands were visualized using ECL Plus^{TM} Western blotting detection reagents (Amersham Biosciences, Castle Hill, Australia) and exposed on radiographic film (Fujifilm, Stafford, Australia). Immunoblots were analyzed by densitometry using Image J software as described[6].

Immunohistochemistry. Cartilage tissues collected from OA patients were graded according to disease severity based on Mankin score. Immunohistochemistry for pERK expression in cartilage and subchondral bone was performed as described[4,6]. Briefly, tissue slices were dewaxed in xylene and dehydrated in ethanol. Endogenous peroxidases were blocked by incubation in 0.3% peroxide in methanol for 30 min after repeated washing in PBS. Sections were then incubated with proteinases K (Dako Multilink, Carpinteria, CA, USA) for 20 min for antigen retrieval. Next, all sections were treated with 0.1% bovine serum albumin with 10% swine serum in PBS. Sections were then incubated with optimal dilution of primary antibody overnight at 4˚C (p-ERK = 1:100; Gene Search Pty. Ltd., Queensland, Australia). Optimum concentration of antibody was determined by using a series of dilutions. Next day, sections were incubated with a biotinylated swine-anti-mouse, rabbit, goat antibody (Dako) for 15 min, and then incubated with HRP-conjugated avidin-biotin complex for 15 min. Antibody complexes were visualized by addition of buffered diaminobenzidine substrate for 4 min and the reaction was stopped by immersion and rinsing of the sections in PBS. Sections were lightly counterstained with Mayer's hematoxylin and Scott's blue for 40 s each, in between 3-min rinses with running tap water. Next, they were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene, and mounted with a coverslip using DePeX mounting medium. Controls for the immunostaining procedures included conditions where the primary antibody or the secondary antibodies (anti-mouse IgG) were omitted. An irrelevant antibody (anti CD-15) not present in the test sections was used as a control.

Statistical analysis. Each patient's normal ACC/SBO (n = 3) were cultured with conditioned media derived from 4 different patients' normal (n = 4) or OA (n = 4) ACC/SBO. In a parallel set of experiments, OA ACC/SBO (n = 3) were cultured with conditioned media derived from 4 different patients' normal (n = 4) or OA (n = 4) ACC/SBO. Results were presented as mean ± SD and are representative of at least 3 distinct experiments using ACC and SBO derived from 4 different donors. Repeated measures ANOVA with posthoc tests were used to assess statistical significance, where $p < 0.05$ was considered significant and n = number of donors.

RESULTS

ACC and SBO phenotype. Primary ACC grown in monolayer culture undergo a process of dedifferentiation within a few passages, which is characterized by a loss of *COL2* gene expression and upregulation of *COL1* gene expression. To ensure the phenotypic integrity of the ACC used in our study, the cells were cultured in micromass and assessed for expression of the cartilage-specific genes *AGG* and *COL2* and dedifferentiation marker *COL1*. The chondrogenic phenotype of ACC was confirmed by the robust expression of both *COL2* and *AGG* and low expression of *COL1* in all the patient samples collected. The expression of chondrogenic marker genes *COL2* and *AGG* was significantly downregulated in OA ACC compared with normal ACC ($p < 0.05$; Figure 1B). These results indicated that the OA ACC used in this study are phenotypically different from normal ACC. In addition, the expression of *ALP* ($p < 0.05$) and *OCN* ($p < 0.05$) was significantly upregulated in OA SBO compared to normal SBO. These results indicated that OA SBO had greater osteogenic potential than normal SBO and that the cells we used for the cocultures were phenotypically different (Figure 1C).

Phosphorylation status of MAPK-ERK1/2 in indirect cocultures of ACC and SBO. A time-course study (24, 48, 72, and 96 h) was first performed to determine the point of pERK1/2 activation in response to coculture conditions as described in Materials and Methods. ERK1/2 phosphorylation was detectible at 24 h, peaking at 72 h, and maintaining this level until 96 h (data not shown), suggesting that activation of pERK is chronic, as it seems not to be dephosphorylated after it was activated. This was the case for both SBO and ACC. The point at which MMP production was at its peak in response to conditioned media was also at 72 h;

this timepoint was therefore chosen for all subsequent experiments.

Effect of normal and OA SBO conditioned medium on ACC MAPK-ERK1/2 phosphorylation. Basal activation of pERK1/2 was greater in OA ACC compared to normal ACC, indicating that upregulation of this pathway may be biologically relevant to OA cartilage pathogenesis. When ACC were cultured in conditioned media from normal SBO, pERK1/2 levels in both normal and OA ACC remained unchanged. By contrast, pERK1/2 levels rose significantly in both normal and OA ACC when cultured with conditioned media from OA SBO (Figure 2A). Application of the ERK1/2 inhibitor PD98059 to the OA SBO conditioned medium reversed the ERK1/2 phosphorylation in normal ACC, returning it to normal levels (Figure 2B).

Effect of normal and OA ACC on SBO MAPK-ERK1/2 phosphorylation. As with OA ACC, OA SBO have a higher basal level of activated ERK1/2 compared to normal SBO, suggesting biological relevance of this pathway in OA subchondral bone pathogenesis. ERK1/2 phosphorylation increased in both normal and OA SBO when cultured with OA ACC conditioned medium. By contrast, SBO cultured with normal ACC conditioned medium did not show any changes of phospho-ERK1/2 expression (Figure 2C). PD98059 had the effect of decreasing ERK1/2 phosphorylation in normal SBO cultured with OA ACC conditioned medium (Figure 2D). These findings together indicate that the interactions of SBO and ACC isolated from OA joint bidirectionally activate the ERK1/2 signaling pathway.

Effect of coculture on expression of MMP-2 and MMP-9. An indirect coculture model was applied to determine whether interactions between SBO and ACC isolated from normal and OA tissue samples could result in differential activation of MMP-2 and MMP-9. Conditioned medium was collected after 72 h from cocultured and non-cocultured cells, and the presence of the bioactive proteases assessed by zymography and ELISA.

Zymography. Zymographic analysis of conditioned media revealed an increase of MMP-2 (72 kDa) proteolytic activity in OA ACC compared to normal ACC alone, indicating the pathological role of this MMP in OA pathogenesis. Culturing both normal and OA ACC with conditioned media from OA SBO resulted in hyperactivation of MMP-2 proteolytic activity compared to ACC cultured with conditioned media from normal SBO and controls. There was no observable MMP-9 activity in either normal or OA ACC; however,

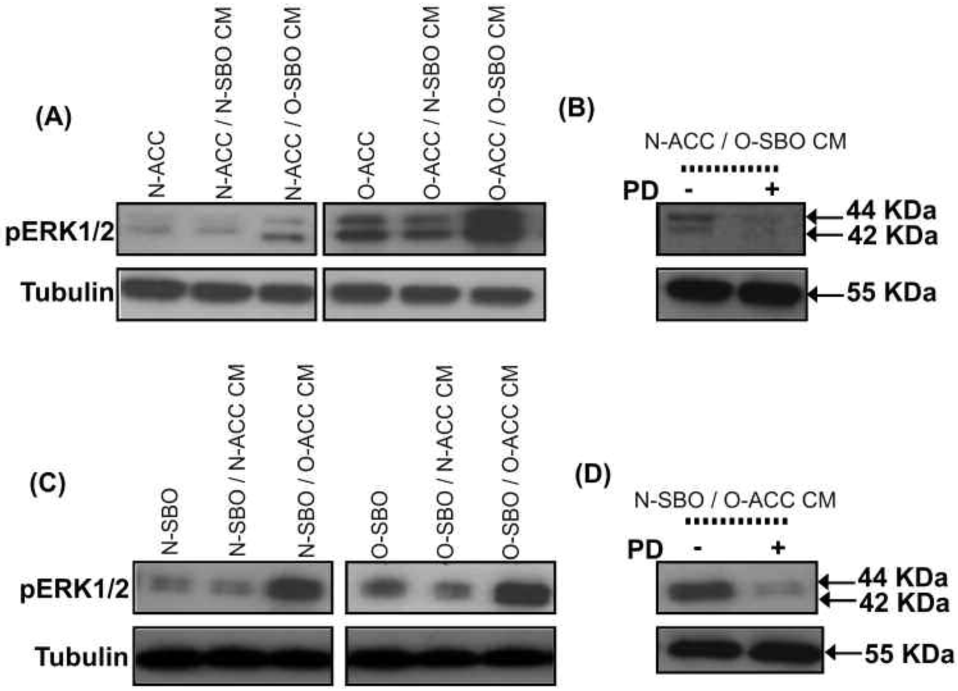

Figure 2. ERK1/2 phosphorylation status in indirectly cocultured articular cartilage chondrocytes (ACC) and subchondral bone osteoblasts (SBO). SBO and ACC were cultured 72 h in respective conditioned media (CM) combinations. A. Normal (N) and OA (O) SBO-conditioned media mediated ERK1/2 signaling changes in ACC. B. ERK1/2 phosphorylation in response to conditioned media from OA SBO was significantly reduced in normal ACC with the addition of 10 μM PD98059. C. Normal and OA ACC-conditioned media mediated ERK1/2 signaling changes in SBO. D. ERK1/2 phosphorylation in response to conditioned media from OA ACC was significantly reduced in normal SBO with the addition of 10 μM PD98059 (PD). Tubulin is shown as a loading control. Results are representative of experiments with cells from 4 different donors.

a band corresponding to pro MMP-9 (92 kDa) was induced in ACC upon culture with conditioned media from OA SBO. In contrast, the culture of ACC with normal SBO conditioned media showed no activation of MMP-9 (Figure 3A). Cultures were then performed in the presence of PD98059 to verify if ERK1/2 phosphorylation was involved in the activation of MMP-2 and MMP-9. The results showed a robust downregulation of MMP-2 and MMP-9 expression in OA ACC cultured in conditioned media from OA SBO, resulting from the inhibitory effects of PD98059 on ERK1/2 phosphorylation (Figure 3B). However, PD98059 showed no effect on OA ACC cultured with normal SBO or OA ACC alone (data not shown). On the other hand, OA ACC conditioned media had the effect of increasing the proteolytic activity of MMP-2 in SBO (Figure 3C). However, MMP-9 activity was not observed in either cocultured or non-cocultured conditions. The addition of PD98059 reversed the OA ACC conditioned media induced MMP-2 proteolytic activity in OA SBO (Figure 3D). However, PD98059 showed no effect on OA SBO cultured with normal ACC or OA SBO alone (data not shown). The enzyme activity was abolished by addition of EDTA to the developing buffer, proof that the induced enzyme belonged to the MMP family (data not shown).

ELISA results. Culturing ACC in conditioned media from normal SBO did not increase the expression of MMP-2 and MMP-9. By contrast, the total amount of secreted MMP-2 and MMP-9 rose significantly when ACC (both normal and OA) were cultured in conditioned media from OA SBO (Figure 4A, 4C). PD98059 suppressed the MMP-2 and MMP-9 production that was otherwise induced in normal ACC by OA SBO conditioned media (Figure 4B, 4D).

In addition, the results showed that culture of OA ACC conditioned media with SBO (both normal and OA) also led to increased MMP-2 expression, but this did not affect MMP-9 expression (Figure 4E, 4G). PD98059 reversed MMP-2 activity induced by OA ACC conditioned media in normal SBO (Figure 4F). On the other hand, inhibition of pERK showed no effect on MMP-9 expression in normal and OA SBO (Figure 4H).

Effect of indirect coculture of SBO and ACC on expression of ADAMTS proteases and MMP. ACC and SBO were cocultured indirectly in their respective conditioned media combinations for 72 hours. The purpose of this experiment was to determine the effects of secreted factors, between SBO and ACC, on the expression of ADAMTS5, ADAMTS4, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13.

Effect of normal and OA SBO conditioned media on ACC. The protein levels of ADAMTS4, ADAMTS5, MMP-2,

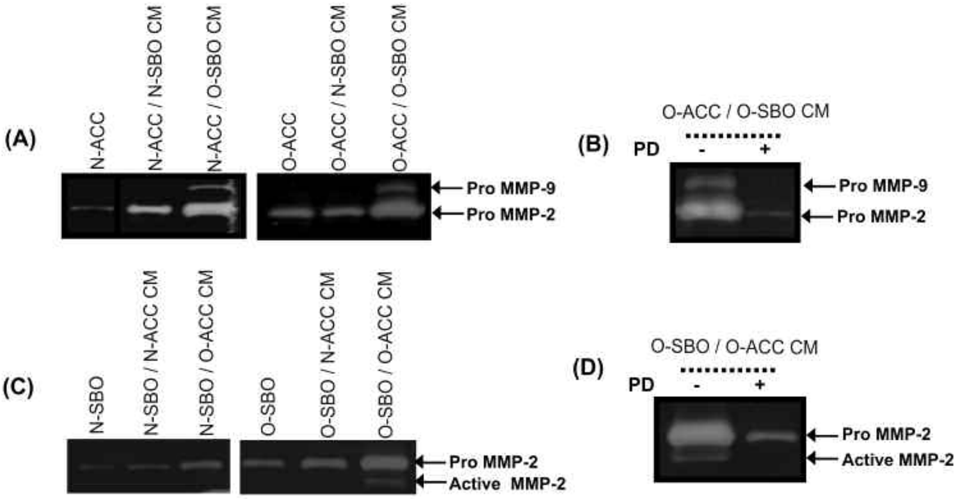

Figure 3. Stimulation of MMP-2 and MMP-9 activities assessed by zymography in non-cocultured versus cocultured cell-conditioned media (CM) and role of ERK1/2 pathway. A. Articular cartilage chondrocytes (ACC) were cultured in the presence or absence of normal and OA subchondral bone osteoblast (SBO)-conditioned media. After 72 h of culture, media were collected and zymography was performed to detect the presence of MMP-2 (72 kDa) and MMP-9 (92 kDa) bioactive gelatinase released by ACC in response to conditioned medium. D. OA ACC were cultured with conditioned media from OA SBO in the presence or absence of 10 μM PD98059 (PD). MMP-2 and MMP-9 induced by conditioned media from OA SBO were reversed in the presence of the inhibitor. C. SBO were cultured in the presence or absence of normal and OA ACC-conditioned media. After 72 h of culture, media were collected and zymography was performed to detect the presence of MMP-2 (72 kDa) and MMP-9 (92 kDa) gelatinase released by the SBO. B. OA SBO were cultured with conditioned media from OA ACC in the presence of 10 μM PD98059 (PD). MMP-2 induced by conditioned media from OA ACC was reversed in the presence of the inhibitor. One representative gel from experiments performed with 3 different patients is shown. White bands represent gelatin decomposed by gelatinase.

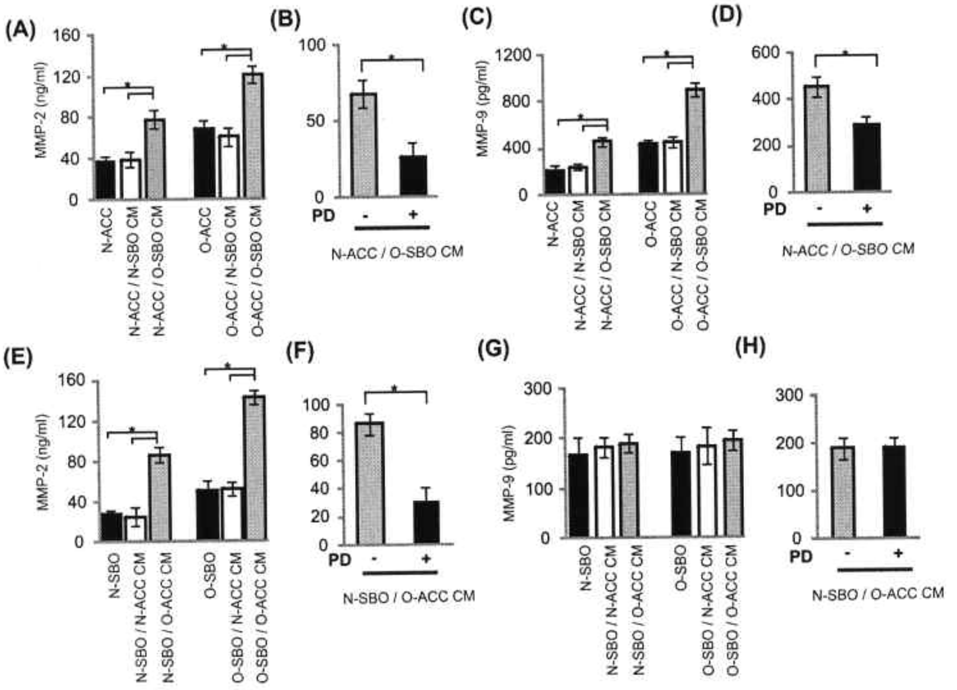

Figure 4. Quantitative measurement of MMP-2 and MMP-9 levels in conditioned media (CM) of non-cocultured and cocultured SBO and ACC by ELISA. A and C. ACC were cultured in the presence or absence of normal and OA SBO-conditioned media (CM) for 72 h. After 72 h, levels of MMP-2 and MMP-9 were measured by ELISA. Levels of both MMP-2 and MMP-9 were significantly increased when ACC (both normal and OA) were cultured in the presence of OA SBO-conditioned media. B and D. MMP-2 and MMP-9 expression in normal ACC cultured in conditioned media from OA SBO decreased with the addition of 10 μM PD98059 (PD). E and G. SBO were cultured in the presence or absence of normal and OA ACC-conditioned media for 72 h. After 72 h, levels of MMP-2 and MMP-9 were measured by ELISA. Levels of MMP-2 but not MMP-9 were significantly increased when SBO (both normal and OA) were cultured in the presence of OA ACC-conditioned media. F and H. MMP-2 expression induced by OA ACC-conditioned media was reduced with the addition of 10 μM of PD in normal SBO. Results are expressed as mean ± SD of experiments performed with cells from 4 different donors. *Significant difference ($p < 0.05$).

MMP-3, and MMP-9 remained stable in both normal and OA ACC when cultured in conditioned media from normal SBO. By contrast, the levels of ADAMTS4 (Figure 5A, 5B), ADAMTS5 (Figure 5A, 5C), MMP-2 (Figure 5A, 5E), MMP-3 (Figure 5A, 5F), and MMP-9 (Figure 5A, 5H) increased significantly in both normal and OA ACC when cultured with conditioned media from OA SBO, giving evidence that secreted factors from OA SBO affect the expression of ADAMTS proteases and MMP in both normal and OA ACC. The expression of MMP-1 (Figure 5A, 5D), MMP-8 (Figure 5A, 5G), and MMP-13 (Figure 5A, 5I) remained unaltered in response to OA SBO conditioned media compared to controls, although the expression of these proteins was considerably higher in OA ACC compared to normal ACC.

Effect of normal and OA ACC conditioned medium on SBO. The results showed that OA ACC conditioned media, but not normal ACC conditioned media, had significantly increased expression of MMP-1 (Figure 6A, 6D) and MMP-2 (Figure 6A, 6E) proteins in SBO (both normal and OA). There was, however, no significant difference in ADAMTS and other MMP tested in cocultured SBO versus non-cocultured SBO (Figure 6B, 6C, 6F, 6G, 6H, 6I).

Effect of PD98059 on expression of ADAMTS proteases and MMP in cocultures. PD98059 strongly inhibited the effects of conditioned media from OA SBO on normal ACC (Figure 7A), evident by downregulation of proteins such as ADAMTS4, ADAMTS5, MMP-2, MMP-3, and MMP-9. However, the application of PD98059 on ACC alone and ACC cultured with normal SBO showed no discernible effect on expression of ADAMTS and MMP (data not shown). This is an indication that ERK1/2 activation by OA SBO conditioned medium may be responsible for the abnormal ADAMTS and MMP production by ACC.

In a similar fashion, PD98059 strongly inhibited changes induced by OA ACC conditioned media in normal SBO (Figure 7B), which was evident by downregulation of MMP-1 and MMP-2. These effects were not seen in SBO cultured in control medium or in conditioned media from normal ACC (data not shown). This is an indication that

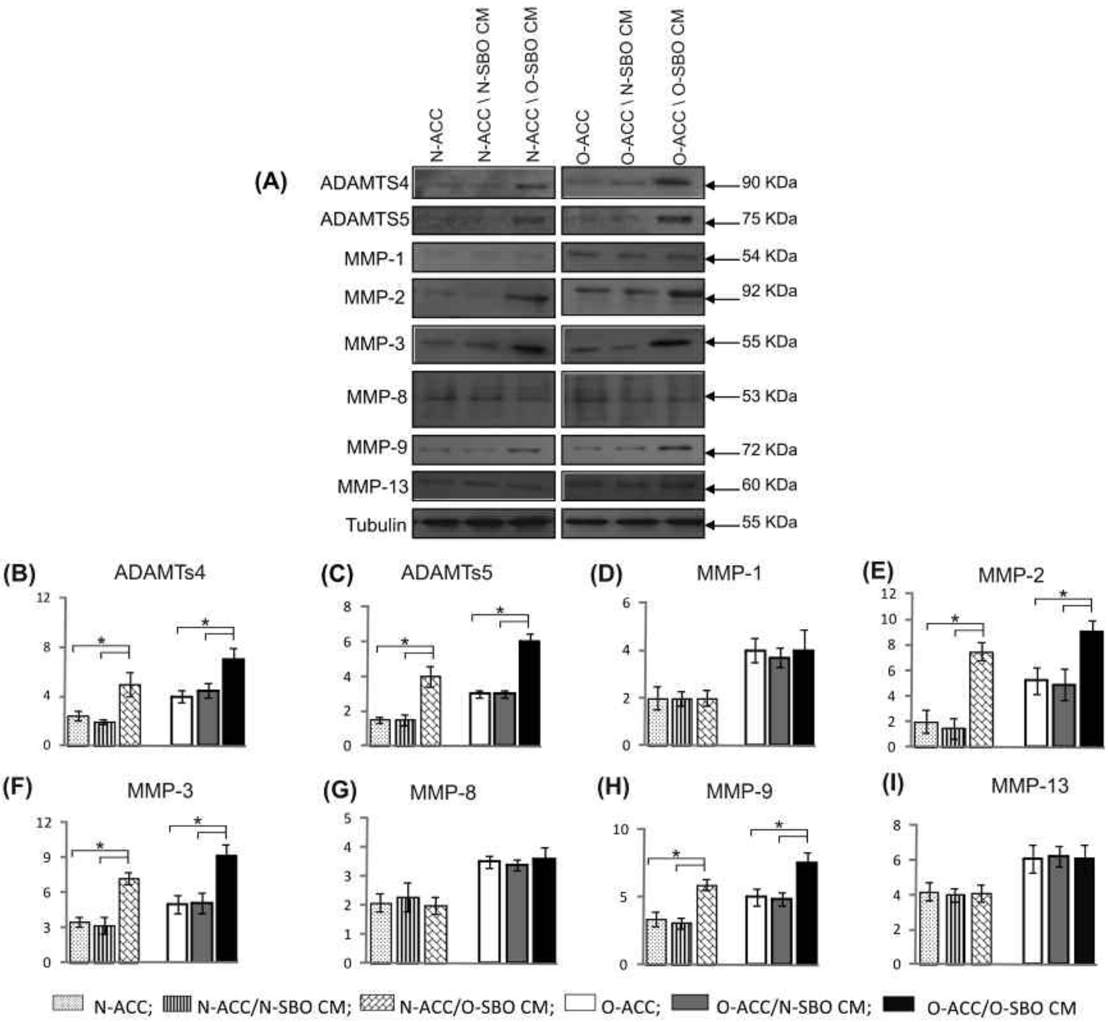

Figure 5. The effect of normal and OA SBO-conditioned media (CM) on ACC MMP and ADAMTS protein expression. A. Normal (N) and OA (O) SBO-mediated ADAMTS and MMP protein expression determined by Western blot. OA SBO-conditioned media has significantly increased ADAMTS4, ADAMTS5, MMP-2, MMP-3, and MMP-9 protein expression in ACC. Tubulin is shown as a loading control. B to I. Quantification of band density was performed using Image J software. Results are expressed as mean ± SD of 4 experiments with cells from 4 different donors after normalization of values with tubulin. Results are pooled and shown as mean ± SD. *p < 0.05.

ERK1/2 activation by OA ACC conditioned medium may be responsible for the MMP1 and MMP2 regulations in SBO.

Immunohistochemical analysis of pERK1/2 expression in OA cartilage and subchondral bone. Phospho-ERK1/2 appeared to localize to the nucleus in the majority of chondrocytes in the deeper layers of severely damaged cartilage that were closer to the subchondral bone (Figure 8A, 8B, 8C). pERK expression was also increased in the OA subchondral bone tissue compared to mild and moderate OA subchondral bone. These results provide the clinical relevance of this pathway to the *in vivo* situation and possible evidence of communication-induced expression (Figure 8D, 8E, 8F).

DISCUSSION

Our *in vitro* study is the first to provide evidence of the mechanism underlying the cycle between subchondral bone and cartilage that might lead to joint failure during development of OA. Signals from OA SBO stimulated ADAMTS5, ADAMTS4, MMP-2, MMP-3, and MMP-9 in ACC. In turn, OA ACC stimulated the MMP-1 and MMP-2 activity in SBO. The study further demonstrated that the bidirectional interaction was mediated by the phosphorylation of ERK1/2 signaling pathway.

A primary event in the destruction of cartilage in arthritic diseases is the loss of aggrecan from the extracellular matrix of articular cartilage. During aggrecan breakdown,

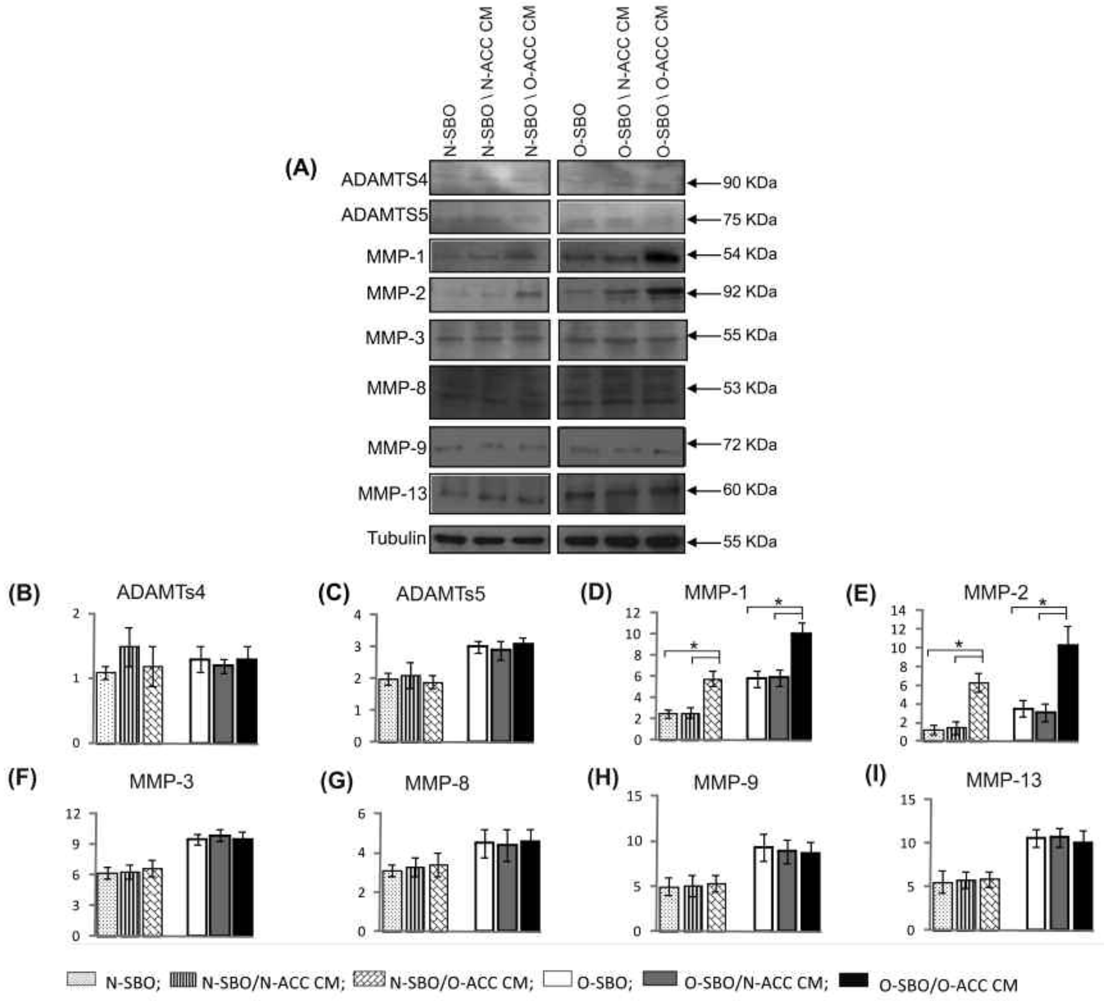

Figure 6. The effect of normal and OA ACC-conditioned media (CM) on SBO MMP and ADAMTS protein expression. A. Normal (N) and OA (O) ACC-mediated ADAMTS and MMP protein expression was determined by Western blot. OA ACC-conditioned media have significantly increased MMP-1 and MMP-2 protein expression in SBO. Tubulin is shown as a loading control. B to I. Quantification of band density was performed using Image J software. Results are expressed as mean ± SD of 4 experiments with cells from 4 different donors after normalization of values with tubulin. Results are pooled and shown as mean ± SD. $^*p < 0.05$.

cleavage sites are used that reside within the interglobular domain of the aggrecan core protein. The Asn341-Phe342 bond is cleaved by members of the MMP family, whereas the second of the 2 cleavage sites, the Glu373-Ala374 bond, is cleaved by members of the ADAMTS family. Both ADAMTS4 and ADAMTS5 have been shown to readily cleave aggrecan at this aggrecanase site[21,22]. This study identified that OA SBO secreted factors increased both ADAMTS4 and ADAMTS5 that could be responsible for increased aggrecan catabolism. This is significant since ADAMTS4 and ADAMTS5 are both reported to play an important pathological role in OA cartilage[23,24]. The extracellular matrix of articular cartilage also consists of type II collagen, proteoglycans, minor collagens (types V, VI, IX, X, and XI), and other noncollagenous matrix proteins. Abnormal expression of MMP-1, MMP-2, MMP-8, MMP-9, and MMP-13 are capable of cleaving the triple-helical domain of collagens, including type II, and they therefore play a decisive role in cartilage degradation[25,26]. We found that OA SBO secreted factors influenced the protein expression of MMP-2, MMP-3, and MMP-9, indicating a pathological proteolytic network underlying OA development. Our results also confirm the previous observation that factors secreted from OA SBO induce MMP-3 in ACC[2]. MMP-3 cleaves the telopeptide regions of noncollagen domains of types IX and XI collagen, and increased MMP-3 results in breakdown of the collagen network and is a feature of early OA[27].

Further, we observed that OA ACC had a greater basal expression of ADAMTS4, ADAMTS5, MMP-2, MMP-3, and MMP-9 compared to normal ACC. It is quite possible that OA ACC are already primed, having undergone changes

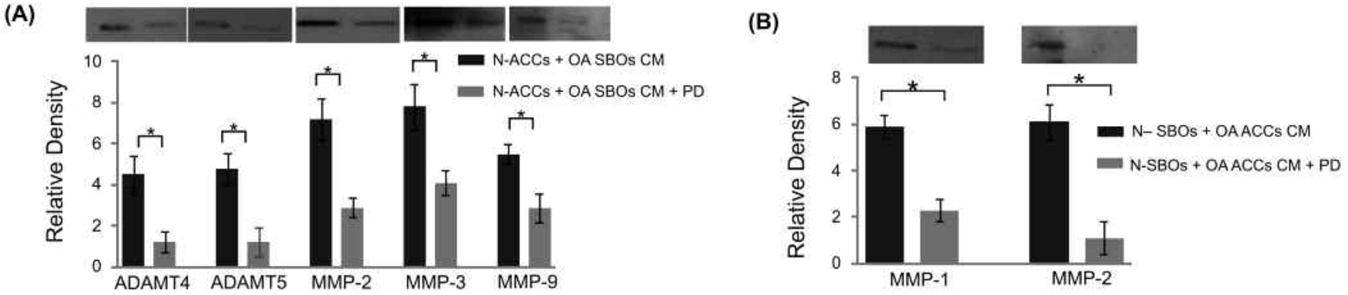

Figure 7. Effect of ERK pathway inhibition on ADAMTS and MMP in cocultures. A. OA SBO-conditioned medium (CM)-induced ADAMTS4, ADAMTS5, MMP-2, MMP-3, and MMP-9 protein expression was significantly reduced in normal ACC with addition of 10 μM PD98059 (PD) to the cultures. Quantification of band density was performed using Image J software. PD had no discernible effect on ADAMTS and MMP expression in ACC cultured in normal medium or CM from normal SBO (data not shown). B. OA ACC-conditioned media-induced MMP-1 and MMP-2 expression was significantly reduced with the addition of 10 μM PD in normal SBO. There was no discernible effect from PD on ADAMTS and MMP expression in SBO in normal medium or CM from normal ACC (data not shown). Densitometry was performed using Image J software. Results are expressed as the mean ± SD of 4 experiments with cells from 4 different donors after normalization of values with tubulin. Results are pooled and shown as mean ± SD. $^*p < 0.05$.

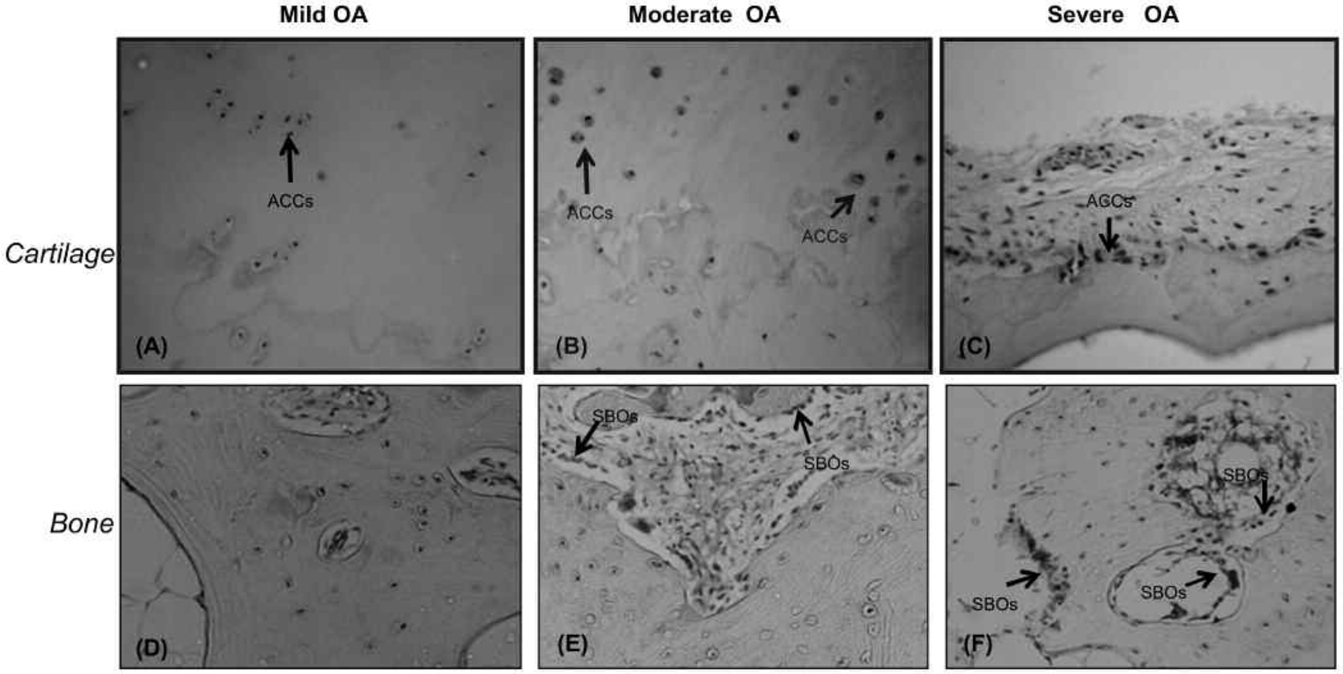

Figure 8. Immunohistochemical staining of pERK in cartilage and subchondral bone. A-C. Osteochondral sections from patients with OA were graded according to disease severity based on Mankin score. Upregulation of ERK phosphorylation was observed mostly in the deep layers of severely damaged OA cartilage compared to mildly and moderately damaged cartilage. D-F. pERK expression was also increased in severely damaged OA subchondral bone tissue compared to mildly and moderately damaged bone tissue samples. Images are representative of samples from 5 different patients. SB: subchondral bone; ACC: articular cartilage chondrocytes; SBO: subchondral bone osteoblasts. Scale bar = 200 μm.

in vivo in response to nearby SBO in the OA lesion, or other factors that are independent of SBO. Interestingly, similar to normal ACC, the expression of ADAMTS4, ADAMTS5, MMP-2, MMP-3, and MMP-9 enzymes increased even further in response to OA SBO conditioned media. These results suggest that OA ACC may not have reached plateau expression in response *in vivo* to nearby OA SBO or in response to other factors at *in vivo* level, and therefore the addition of OA SBO conditioned media further increased the expression of those enzymes. In contrast to these findings, although increased expression of MMP-1, MMP-8, and MMP-13 was seen in the OA ACC compared to normal ACC, this increase could not be attributable to secreted factors from OA SBO conditioned media. This observation suggests that perhaps OA SBO secreted factors were not the only mediators responsible for abnormal production of MMP in ACC; other mechanisms or pathways must therefore be responsible for their dysregulated expression. Each

MMP gene has a unique promoter that contains various transcription factor binding sites[28]. For example, it is well known that the MMP-2, MMP-9, and MMP-3 genes possibly act through the AP-1 site. However, the AP-1 site is not sufficient to drive transcription of the MMP-13 and MMP-1[28,29]. This difference may arise because formation of AP-1 complex takes place through different signaling pathways that form heterogeneous complexes that bind to the AP-1 site with different affinities[30]. This suggests that OA SBO were unable to secrete the factors required to drive the promoter for MMP-1, MMP-8, and MMP-13. It is known that a variety of OA factors such as hypoxia[31], altered biomechanics[32], and physical variables such as obesity induced altered adipokine profile[33] either alone or together can act cooperatively to increase the observed altered levels of MMP-1, MMP-8, and MMP-13 in OA ACC compared to normal ACC.

We previously reported that OA SBO significantly reduced COL2 and aggrecan expression in ACC[4]. Using a coculture model, Sanchez, *et al* demonstrated that sclerotic osteoblasts induced a marked deregulation of chondrocyte metabolism, characterized by decreased aggrecan synthesis[3]. Another study demonstrated that sclerotic osteoblasts, but not nonsclerotic osteoblasts, increased MMP-3 and MMP-13 in chondrocytes[2]. However, in our study we found that OA SBO stimulated only MMP-3, with no effect on MMP-13. The discrepancy in results could be attributed to many factors including variations in culture protocols. These observations, together with the current findings, suggest that OA SBO influence ACC by suppressing anabolism and promoting catabolism. This notion is supported by the fact that normal SBO, when cocultured with ACC, do not elicit the same effects, which is most likely how SBO and ACC interact to maintain the joint homeostasis under normal conditions. However, to date, the reciprocal effects OA ACC have on SBO metabolism and possible signaling pathways involved during this altered crosstalk have not been identified.

Of note, when cultured in conditioned media from OA ACC, there was a significant increase in MMP-1 and MMP-2 activity in both normal and OA SBO. The mechanisms for the modulation of osteoblast phenotype by MMP expression and activity remain unclear, but one can propose hypotheses based on what is known. For example, a sequential evaluation of subchondral bone changes in an OA animal model points to a predominance of bone formation in the more advanced late stages of the disease, whereas bone resorption is favored during remodeling in the early phases[34]. Given that MMP-1 and MMP-2 are some of the principal proteases capable of degrading the bone matrix[35], the increased production of these proteases by SBO, prompted by their interaction with OA ACC, suggests this is a pivotal mechanism underlying the elevated bone remodeling that leads to bone sclerosis. Further, there is evidence of elevated levels of common osteogenic markers in OA SBO compared to SBO isolated from normal controls[36]. MMP-2, for example, is developmentally regulated during *in vitro* osteoblast differentiation[37], and is also regulated *in vitro* by factors implicated in controlling bone tissue turnover[38]. It therefore seems likely that these proteases are involved in the enhanced osteoblast and osteoclast activity that is a typical feature in OA bone. In contrast to MMP-1 and MMP-2, both of which were increased in SBO exposed to conditioned media from OA ACC, the expression levels of MMP-9 were unaffected by conditioned media of OA ACC, although the basal MMP-9 levels in OA SBO were greater than those in normal SBO. It is possible that MMP-9 is regulated by autocrine factors from the SBO, and therefore its effect is independent of the interactions with OA ACC.

It is still not known what mechanisms govern the increased expression of ADAMTS and MMP in the interaction between OA SBO and OA ACC. Our intention was to elucidate whether MAPK-ERK signaling pathway is modulated by cocultures. The phosphorylation of ERK1/2 started to overexpress in response to the conditioned media starting from 24 hours, reached its peak at 72 hours, and become stabilized (data not shown) in response to OA ACC/OA SBO conditioned media. These results suggest that the activation of ERK was chronic and did not dephosphorylate after it was activated. Duration of ERK activation, whether the expression is transient or stable, depends on the characteristics of the stimuli and nature of the molecular mechanisms and of the cells involved[39]. Constitutive ERK activity regulates mRNA stability, providing sustained RNA levels for translation, and therefore can lead to permanent tissue damage, unlike the transient changes, which are temporary[40,41]. As demonstrated by our immunostaining results, ERK is clearly upregulated in the subchondral bone and cartilage tissues in severe OA, confirming that ERK activation is stable in the progression of disease.

MAPK-ERK act on the promoter regions of inducible MMP genes depending on the nature of the extracellular stimuli[30], and our results showed clear evidence of increased phosphorylation of the ERK1/2 pathway in ACC when these cells were exposed to conditioned media from OA SBO. Similarly, SBO exposed to conditioned media from OA ACC showed increased ERK1/2 phosphorylation in these cells. From these results one can infer the presence of bidirectional crosstalk between OA SBO and OA ACC through ERK1/2 signal activation, and that this and other pathways may be involved in regulating the abnormal ADAMTS and MMP levels seen in our indirect coculture method. By suppressing the ERK1/2 pathway with the specific inhibitor PD98059, we demonstrated a complete attenuation of conditioned media-induced production of ADAMTS5 and MMP, lending support to the pathophysiological role of this pathway in the nexus between OA SBO and ACC. These results are consistent with findings that suggest targeting MAPK pathways, ERK1/2 in particular, may be a means of reducing MMP expression in a variety of cells[42], a finding that has also been demonstrated in an OA animal model[43]. Indeed, MAPK have been shown to be activated in OA cartilage and there is evidence that ERK1/2 play a key role in cartilage destruction[44,45,46]. We also observed that the ERK1/2 pathway was expressed in significantly higher levels in OA cartilage and bone compared to normal tissues, evidence of the relevance of these pathways to the pathophysiology of OA.

Recent data support the view that cartilage and bone can communicate across the calcified tissue barrier[47]. Studies have demonstrated the presence of connections such as microcracks[48], vascular channels[49], and neovascularization between subchondral bone and cartilage, giving rise to hypotheses that mediators produced by subchondral bone or vice versa may pass through these channels, thereby directing cell-to-cell interactions[50]. Moreover, it has been suggested that the products derived from subchondral bone or cartilage are readily secreted into the joint space, as evidenced by their detection in synovial fluid. Therefore, it is likely that the molecules that influence the cartilage or bone will gain access to each other through the synovial fluid[51].

The putative soluble and transcription regulators of the ERK1/2 signaling pathway in the OA SBO-ACC cocultures compared to normal SBO-ACC cocultures remain unknown. The MAPK-MMP pathways are most likely transcriptionally regulated by classic mediators such as tumor necrosis factor-α (TNF-α)[52], vascular endothelial growth factor (VEGF)[53], transforming growth factor-ß3 (TGF-ß3)[54], and interleukins[42], all of which can activate a pleiotropic cascade of signaling pathways. For example, studies have shown that levels of interleukin 6 (IL-6)[55], IL-1ß[56], TNF-α[57], IL-1α[42], and macrophage inflammatory protein-1[58] are significantly higher in patients with OA. Activation of cells by these cytokines is mediated by binding to specific cell membrane receptors, triggering the activation of a number of complex intracellular signaling pathways. Among these, IL-6[59], IL-1, and TNF-α cytokines act through the MAPK-ERK signaling pathway, and this is required for the release of MMP, as shown in *in vitro* studies[60]. It is also reported that the production of chemokine stromal cell-derived factor (SDF)-1 is significantly higher in OA patients, and SDF-1α acts through CXCR4 to activate ERK and the downstream transcription factors (c-Fos and c-Jun), resulting in the activation of AP-1 on the MMP-13 promoter, contributing to cartilage destruction during OA[61]. Similarly, in OA subchondral bone overproduction of IL-6[36], leptin[62], and growth factors such as insulin-like growth factor-1[63] can stimulate activation of ERK1/2, resulting in the abnormal OA subchondral bone osteoblast phenotype. It is possible that one or more of these factors or other unknown factors are induced by the conditioned medium from OA-derived SBO and ACC, giving rise to a positive feedback of the ERK1/2 pathway, which in turn triggers the MMP-mediated degenerative changes in the OA joint. A proteomics study is currently under way to identify soluble factors that may be involved in the modulation of MAPK in the interaction between ACC and SBO that leads to progression of OA.

Our *in vitro* study is the first to provide direct insight into the mechanisms underlying the cycle between subchondral bone and cartilage in the development of OA. It seems likely that bidirectional ERK1/2 cell signaling activation in the OA bone-cartilage unit may initiate catabolic cues with a role in disease progression. Therapeutic strategies to combat this interaction with pharmacological ERK1/2 inhibitors may be effective in reducing OA-associated joint damage.

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