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ERK1/2 and p38 in the regulation of hypertrophic changes of normal articular cartilage chondrocytes induced by osteoarthritic subchondral osteoblasts

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Abstract

Objective: Earlier studies have reported the influence of subchondral bone osteoblasts (SBOs) on phenotypical changes of articular cartilage chondrocytes (ACCs) during osteoarthritis (OA) development. The molecular mechanisms involved during this process still remain elusive, in particular the signal transduction pathways. The aim of this study was to investigate in vitro effects of OA SBOs on the normal ACCs phenotypical changes, and to unveil the potential involvement of mitogen activated protein kinase (MAPK) signaling pathways during this process.

Methods: Normal and arthritic cartilage and bone samples were collected for the isolation of ACCs and SBOs. Direct and indirect co-culture models were applied to study chondrocyte hypertrophy under the influence of OA SBOs. Mitogen-activated protein kinases in the regulation of the cell-cell interactions were monitored by phosphorylated antibodies and relevant inhibitors.

Results: Our results showed that OA SBOs lead to increased hypertrophic gene expression and matrix calcification in ACCs by means of both direct and indirect cell-cell interactions. For the first time, we demonstrated that OA SBOs suppressed p38 phosphorylation and induced ERK1/2 signal phosphorylation in co-cultured ACCs. The ERK1/2 pathway inhibitor, PD98059, significantly attenuated hypertrophic changes induced by conditioned media from OA SBOs and the p38 inhibitor, SB203580, resulted in the upregulation of hypertrophic genes in ACCs.

Conclusion: This study suggests that the pathological interaction of OA SBOs and ACCs is mediated via the activation of ERK1/2 phosphorylation and deactivation of p38 phosphorylation, resulting in ACCs undergoing hypertrophic differentiation.
**Introduction**

Explanations as to the aetiology of osteoarthritis (OA) has long focussed on the destruction of articular cartilage, the activating factors of which were thought to be triggered as a result of repetitive loading (1, 2). Cartilage pathology in OA is associated with changes of cellular phenotype of articular chondrocytes to a state of terminal differentiation (3, 4). However, the long term molecular events that are responsible for this transition are not well understood.

Recent studies suggest that the subchondral bone plays a major role in OA cartilage changes, an indication of active communication taking place between the subchondral bone and the cartilage in the progression of OA (5, 6). Bone anabolic factors, such as osteocalcin (OC), osteopontin (OPN), and alkaline phosphatase (ALP) are all upregulated in OA subchondral bone osteoblasts (SBOs) compared to normal SBOs supporting the notion of dysfunction of osteoblast behaviour (7-9). It has been shown in OA animal models that a thickening of subchondral bone precedes cartilage changes (10, 11), and it has further been demonstrated that *in vivo* factors produced by OA SBOs increase glycosaminoglycan (GAG) release from the cartilage (12) and can influence cartilage specific gene expression (13). It was demonstrated by the application of a co-culture model of bovine explant subchondral bone and cartilage, that excision of subchondral bone from articular cartilage resulted in increased chondrocytes death, thus demonstrating the important role of subchondral bone in maintaining joint homeostasis (14). However, the molecular mechanisms, and in particular signaling pathways, by which normal and OA SBOs regulate articular cartilage phenotype remain unknown.
Activation of the three major classes of mitogen activated protein kinases (MAPKs) - extracellular signal–regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK - has been detected in chondrocytes (15). MAPKs are known to be responsible for the conversion of vast number of extracellular stimuli into specific cellular responses, including chondrocyte proliferation and differentiation (16, 17). The requirement of MAPK signaling pathways, in particular p38 and ERK1/2, during various phases of endochondral ossification has also been demonstrated in several studies (18, 19). MAPK signaling pathway has been shown to play a distinct role in aspects of cartilage biology such as cartilage matrix synthesis and homeostasis (20, 21). The role of MAPK signaling in skeletal development and in the biology of cartilage, points towards a possible association of altered MAPK signaling and OA. Indeed, alterations in these signaling pathways are reported to play a prominent role in chondrocyte dysfunction as a part of OA pathogenesis and disease progression (22). Since the OA SBOs are reported to alter the cartilage phenotype, it is possible that these alterations in ACCs may occur via MAPK regulation. However, there are no studies to date that have explored the role of MAPKs signaling factors in the cell-cell interactions of SBOs and ACCs. The present study was to investigate MAPKs signaling pathways in the hypertrophic changes of normal ACCs induced by OA SBOs using both direct and indirect co-culture systems.

**Methodology**

**Articular cartilage sample collection and phenotypic determination:** Ethics approval for this project was granted from the Queensland University of Technology and Prince Charles Hospital Ethics Committees and informed consent was given by
all patients involved. OA ACCs (n=5) were sourced from the main defective area of the medial compartment cartilage showing degenerative changes. The average age of OA patients used in this study was 65.20 ± 5.94. Normal ACCs (n=3) were obtained from trauma patients, where knee tissue was available. Normal patients were healthy adults aged between 53.56 ± 10.76 years old, with no clinical signs or symptoms of joint, metabolic or hormonal diseases (osteoporosis), and with no history of medication that might affect cartilage or bone metabolism. To eliminate early OA symptoms, samples showing any evidence of cartilage changes were excluded. These changes included softening of the hyaline articular cartilage; thinning and fibrous dislocation; ulcerations of the cartilage; and light sclerosis of the subchondral bone. Cartilage was classified according to Mankin score (23) based on saffranin-O and H&E histology staining. Chondrocytes from the cartilage tissues were isolated following a method described previously (24). Only early passage ACCs (P0 to P2), showing a strong expression of type II collagen (COL2) and aggrecan (AGG) were used for subsequent experiments.

Subchondral bone sample and phenotypic determination: Bone specimens were taken within 5 mm of the subchondral bone plate. OA SBOs (n=5) were sourced from the weight bearing sites, where the cartilage was degraded and showed prominent subchondral bone erosion and density, from patients with advanced OA and undergoing primary total knee replacement surgery. The average age of OA patients used in this study was 65.20 ± 5.94. Normal SBOs (n=3) were collected from patients undergoing surgery for fracture repair, with no evidence of bone erosion or the cartilage degeneration, as judged by criteria established by the American College of Rheumatology (25). Normal patients were healthy adults aged 53.56 ± 10.76 years
old, with no clinical signs or symptoms of joint, metabolic or hormonal diseases (osteoporosis) or taking medication that might affect cartilage or bone metabolism. After removing the overlaying cartilage, SBOs were isolated according to a methodology described by Beresford (26, 27). Isolated normal and OA SBOs were differentiated in the osteogenic medium (supplemented with 10% foetal bovine serum (FBS) (In Vitro Technologies, Nobel Park, VIC, Australia), 50 u/mL penicillin, 50 µg/mL streptomycin, 10nM dexamethasone, 10mM β-glycero-phosphate, 50µg/mL ascorbic acid) for the characterization of bone cell phenotype determined by the expression of the bone markers of alkaline phosphatase (ALP) and osteocalcin (OC), as well as the staining by 1% alizarin red solution after 2 weeks of osteogenic induction.

**Chondrocyte pellet culture:** Cell culture systems known to preserve the chondrocyte phenotype were used in the co-culture studies. 2x10⁵ cells of ACCs were resuspended in a serum free chondrogenic media (serum-free medium-high glucose DMEM (Invitrogen, Mt Waverley, VIC, Australia) supplemented with 10 ng/mL transforming growth factor-β3 (Bio Scientific, Gymea, NSW, Australia), 10nM 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) and centrifuged at 600 x g for 20 min to form a pellet. Pellets were allowed to differentiate for two weeks in 3 dimensional conditions in 15 mL Falcon tubes, in chondrogenic medium which was replenished every 2 to 3 days. After 2 weeks of chondrogenesis ACCs pellets were co-cultured with normal or OA SBOs as described below.
High density micromass culture: High density micromass droplets were prepared as described previously (18). Briefly, following trypsinization the ACCs were resuspended in growth media at a final cell density of $2.5 \times 10^7$ cells/mL and spotted as 10 μL/well droplets in 24 well culture plates and incubated at 37°C for 2 hr to allow the cell attachment to the plate. Micromasses were cultured for 1 week in chondrogenic media. After 1 week, micromasses containing ACCs were cultured with the conditioned media generated from normal or OA SBOs as described below.

Direct co-culture: ACC pellets were prepared as described above and placed directly upon the monolayer of normal or OA SBOs (75,000 cells/well) in the 24 well plates and co-cultured for further 2 weeks in the high glucose DMEM supplemented with 1% FCS, 0.5% glutamine, 50 u/mL penicillin, 50 μg/mL streptomycin 50μM ascorbic acid, 10nM dexamethasone, and 10 mM β-glycerophosphate. After 14 days of co-culture, the ACC pellets were washed three times in PBS and fixed in 4% paraformaldehyde for 10 min and stained with 1% alizarin red or 0.5 % alcian blue to assess the effect of normal or OA SBOs on ACCs matrix deposition. RNA and protein was also extracted from some of the pellets. The culture system selected for this co-culture study was modified from the previously described protocols of formation of a chondro-osseous rudiment in micromass cultures (28).

Indirect co-culture: Preparation of SBOs conditioned medium (CM): Passage 2, SBOs from normal and OA subchondral bone (2.5×105 cells) were cultured in high glucose DMEM supplemented with 1% FCS, 0.5% glutamine, 25 units/mL penicillin, 25 μg/mL streptomycin 50 μM ascorbic acid, 10nM dexamethasone, and 10 mM β-
glycerophosphate in 25 cm$^2$ flasks for 2 days. The media from these flasks was collected and centrifuged at 1000 × g for 15 min and the supernatants were transferred to fresh tubes and mixed with an equal volume of fresh (preincubated in 37°C in the incubator) media with the same supplements to form CM. During co-culture experiments, ACCs micromasses, prepared as described above, were grown for 1 week in CM from either normal or OA SBOs. The control ACCs were cultured in the same above medium composition, which was not incubated with SBOs. Media was replenished for every 2 days. At the end of co-culture period, protein and total RNA was harvested from the ACCs and some cells were fixed with 4% paraformaldehyde and stained with alizarin red and alcian blue to assess extracellular matrix deposition.

**Detection of MAP kinase activation:** The MAP kinase mediated cellular interactions was evaluated by using the MAP kinase inhibitors SB203580 (Novabiochem, Alexandria, NSW, Australia) and PD98059 (Novabiochem) for p38 and ERK1/2 pathway inhibition respectively. ACC micromasses were incubated with or without the MAPK inhibitors in CM prepared from normal or OA SBOs. The stock solutions of each inhibitor were dissolved in DMSO; the final concentration of DMSO not exceeding 0.1% (v/v). An equal amount of DMSO vehicle was added to control ACCs. Medium was replenished every 2 days. The optimal concentration for inhibition in ACCs was found to be 10 μM for ERK1/2 and 5 μM for p38. At these concentrations there was no evidence of cytotoxicity, nor was cell proliferation influenced by the addition of the inhibitors. All experiments were performed in triplicates.
RNA Extraction and Quantitative RT-PCR (qRT-PCR): Total RNA was isolated with TRIZOL reagent (Invitogen), DNase treated and column purified using an RNeasy Mini Kit (QIAGEN Pty Ltd, VIC, Australia). Complementary DNA was synthesized using Superscript III (Invitrogen) from 1µg total RNA following the manufacturer’s instructions. PCR primers were designed based on cDNA sequences from the NCBI Sequence database using the Primer Express® software and the primer specificity were confirmed by BLASTN searches. RT-qPCR was performed on an ABI Prism 7000 Thermal Cycler (Applied Biosystems, Scoresby, VIC, Australia) with SYBR Green detection reagent. Briefly, 2 µL of cDNA, 20 pmol of gene-specific primers and 10 μL of 1x Master Mix were used in a 20 μL reaction volume; each sample was performed in duplicates. The thermo cycling conditions were as follows: 1 cycle of 10 min at 95°C for activation of the polymerase, 40 cycles of 10 sec 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 non-specific PCR products. The relative expression of the genes of interest was normalized against housekeeping genes of GADPH and 18s.

Western blot: Total protein lysates were harvested by lysing the cells with a lysis buffer containing 1 M Tris HCl (pH 8), 5 M NaCl, 20% Triton X140, 0.5 M EDTA and a protease inhibitor cocktail (Roche, Castle Hill, Australia). The cell lysate was clarified by centrifugation and the protein concentration determined by a bicinchoninic acid protein assay (Sigma, Castle Hill, Australia). Ten microgram of protein was separated by electrophoresis on a 12 % sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with a Tris-Tween buffer containing 5% non-fat milk. The membranes were incubated with
primary antibodies against phospho-p38 (1:1000, Genesearch, Arundel, Australia), phospho-ERK1/2 (1:2000, Quantum Scientific, Murarrie, Australia) and tubulin (1:5000, Quantum Scientific) overnight at 4°C. After washing the membranes three times in TBS-Tween buffer they were incubated with anti-rabbit secondary antibody at 1:2000 dilutions for 1 hr. The protein bands were visualized using the ECL Plus™ Western Blotting Detection Reagents (Amersham Biosciences, Castle Hill, Australia) and exposed on X-ray film (Fujifilm, Stafford, Australia). Immunoblots were analysed by densitometry using Image J software.

Statistical analysis: Each normal ACC co-cultured with either normal SBOs (n=3) or OA SBOs (n=3) and the study was repeated in three normal ACCs. Results were presented as a mean ± SD. The relative expression is the mean of three combinations of chondrocytes and osteoblasts in the co-culture studies. Repeated measures ANOVA with post hoc tests were used to assess statistical significance, where p ≤ 0.05 was considered significant.

Results
Expression of chondrogenic and hypertrophic genes in normal and OA ACCs: The cell proliferation study indicated that there was no difference in cell numbers between normal and OA ACC pellet culture (data not shown). The mRNA expression of chondrogenic and hypertrophic marker genes was compared between normal and OA ACCs. The expression of CBFA1 (p≤0.05), COL10 (p≤0.05) and ALP (p≤0.05) was all significantly upregulated in OA ACCs compared to normal ACCs, whereas the expression of COL2 and AGG expression (p≤0.05) was significantly down regulated in OA ACCs compared to normal ACCs (Fig 1A). These results indicated
that OA ACCs had greater potential to undergo hypertrophic differentiation compared to normal ACCs. With respect to MAPK phosphorylation, it was observed that the p38 phosphorylation was down regulated in OA ACCs in comparison to normal ACCs (Fig 1B). On the other hand ERK1/2 phosphorylation was significantly up regulated in OA ACCs compared with normal ACCs (Fig 1C).

**Hypertrophic differentiation of normal ACCs in the co-culture with OA SBOs**

*Direct co-culture*: No difference in the cell proliferation rates was observed in SBOs after 14 days culture (data not shown). At day 14 of co-culture GAG matrix deposition was lower in the co-culture groups compared to non co-cultured ACCs. There was slightly lower staining intensity in the co-culture with OA SBOs compared with normal SBOs (Fig 2A, upper panel). On the other hand, mineralization in the ACCs pellets was significantly enhanced in ACCs co-cultured with OA SBOs compared to non co-cultured ACCs and ACCs co-cultured with normal SBOs (Fig 2A, middle panel). The expression of COL2 immunostaining was decreased in the co-culture groups compared to non co-cultured ACCs pellets. Furthermore, the expression of COL2 in ACCs was significantly decreased in the presence of OA SBOs compared to normal SBOs (Fig 2A, lower panel). At the gene expression level, cartilage specific genes such as COL2 and AGG were significantly lower in ACCs co-cultured with OA SBOs compared to ACCs co-cultured with normal SBOs and ACCs alone (Fig 2B). These observations were further validated by the mRNA expression of hypertrophy and mineralization marker genes in ACCs pellets. The results from RT-qPCR indicated that OA SBOs induced a significant upregulation of mineralization and hypertrophic markers such as COL10, ALP and CBFA1 compared to co-culture group containing normal SBOs and ACC pellets alone (Fig 2C).
**Indirect co-culture:** When ACC micromasses were cultured with normal or OA SBO CM, alcian blue staining revealed that cartilage matrix deposition was attenuated by both normal and OA SBOs CM; however, the cartilage matrix loss was more prominent in the case of OA SBOs CM (Fig 3A, upper panel). Conversely, matrix mineralization was greater in ACC micromasses grown for 7 days in OA SBOs CM as demonstrated by alizarin red staining (Fig 3A, lower panel). The induction of cartilage specific genes $COL2$ and $AGG$ was significantly downregulated in the presence of both normal and OA SBOs CM compared to ACCs cultured alone, although this decrease was more prominent in ACCs grown in the presence of OA SBOs CM (Fig 3B). By contrast, the expression of cartilage hypertrophy markers $CBFA1$, $COL10$ and $ALP$, were significantly upregulated in the presence of OA SBOs CM (Fig 3C) compared to both normal SBOs CM and control groups.

**Phospho p38 and pERK1/2 kinase signaling pattern in the co-cultured ACCs**

The effect of normal and OA SBOs on the ACCs was assessed with respect to alteration of MAPK signaling cascade in both direct and indirect co-culture systems. The results showed that the phosphorylation of ERK1/2 was significantly augmented when ACCs were co-cultured with OA SBOs, in both the direct and indirect co-culture models, compared to non co-cultured ACCs and ACCs co-cultured with normal SBOs. p38 phosphorylation, on the other hand, was considerably downregulated in the ACCs co-cultured with normal SBOs compared to ACCs alone. Nonetheless, the co-culture of ACCs with OA SBOs led to a complete attenuation of p38 phosphorylation. These results suggest that upregulation of ERK1/2 and downregulation of p38 phosphorylation are involved in the interaction between ACCs and SBOs, which in turn leading to ACCs hypertrophic changes (Fig 4A-D).
Addition of ERK1/2 inhibitor PD98059 reversed the ACCs phenotypic changes induced by OA SBOs CM

Incubation with ERK1/2 inhibitor PD98059 in ACCs cultured with OA SBOs CM had the effect of decreasing the phospho-ERK1/2 and increasing the expression of phospho-p38 in a concentration dependent manner (Fig 5A). ACCs alone did not show significant changes in response to the addition of PD98059, an indication that the observed effects were specific to OA SBO CM. RT-qPCR showed that use of PD98059 reversed the expression of hypertrophic gene expression of CBFA1, COL10 and ALP in ACCs micromasses cultured in the presence of OA SBOs CM (Fig 5B). By contrast, the expression of COL2 and AGG was upregulated by addition of PD98059 (Fig 5C).

Inhibitor of p38, SB203580, induced hypertrophic gene expression in ACCs cultured in normal SBOs CM

Inhibition of the p38 phosphorylation by SB203580 led to reduction of p38 activity and activated ERK1/2 phosphorylation in ACCs cultured in normal SBO CM (Fig 6A). In the presence of SB203580, the hypertrophic markers of CBFA1, COL10, and ALP were significantly enhanced, whereas, chondrogenic markers of COL2 and AGG were downregulated in ACCs in the presence of normal SBOs CM (Fig 6C&D). These results indicate the use of SB203580 could significantly shift of ACCs towards a more hypertrophic phenotype. Similar results were also obtained in normal ACCs cultured alone (data not shown).

Discussion
In this study we have demonstrated the importance of MAPK signaling pathways as the means by which OA SBOs induce altered ACCs phenotypic changes, and provide some insight into the cross-talk taking place between the p38 and ERK1/2 signaling pathways during this pathological interaction process.

It was observed that ACCs isolated from OA patients produced significantly greater levels of the *CBFA1*, *COL10* and *ALP* mRNA when compared to ACCs isolated from healthy patients. This finding indicates that the OA ACCs possessed greater potential to undergo hypertrophic differentiation, results which corroborate previous work comparing normal and OA ACCs (29, 30).

Applying an *in vitro* indirect co-culture model, Sanchez et al demonstrated that the sclerotic OA SBOs decreased cartilage specific gene expression such as *SOX9* and *COL2*. They also showed that inhibitors of hypertrophic differentiation such as pTHrP and PTH-R were significantly downregulated in ACCs co-cultured with OA SBOs (13). These findings are evidence that OA SBOs can decrease the inhibitors of hypertrophic differentiation leading to a subsequent mineralized matrix deposition in cartilage. In the present study, using both direct and indirect co-culture methods, we showed that OA SBOs increased both hypertrophic gene expression and matrix mineralization. Interestingly, hypertrophic changes are followed by a simultaneous decrease of the chondrocyte specific phenotype. A characteristic change of OA is an upregulation of hypertrophy and mineralization related markers (4) and a downregulation of chondrocyte specific markers (COL2 and AGG) in articular cartilage (31). The observations in our study suggest that the interaction of OA SBOs may lead to these typical hypertrophic changes in ACCs. It has been reported that the
transition of ACCs to hypertrophic changes contributes to the activation of matrix metallo proteinases (MMP), which precedes cartilage degeneration (32, 33), indicating that the phenotypic conversion of ACCs to hypertrophy is pathological for the health and integrity of articular cartilage leading to its degeneration.

However, the reasons as to why OA SBOs seem to induce these altered ACCs phenotype remain unclear; there are nevertheless several potential pathways responsible. Both our own studies (data not shown), and that from other groups, have demonstrated that OA SBOs produce abnormal levels of osteogenic markers, growth factors and cytokines. Specifically, increased production of growth factors such as insulin like growth factor (IGF)-1 (34), and transforming growth factor (TGF)-β (35) are reported in OA SBOs s. Among these factors IGF-1 is implicated in inducing cartilage hypertrophic changes in growth plate chondrocytes (36, 37). In addition, it has been reported that OA SBOs produce abnormal levels of cytokines such as interleukin (IL)-1 & -6 (35), tumor necrosis factor (TNF) and MMP-13 (38, 39), the factors that have the ability to activate a diverse array of signaling pathways in cartilage hypertrophy. Therefore, it is possible that the secreted bio-molecules from OA SBOs either individually or co-operatively communicate with ACCs there by mediating the inducing phenotype changes of ACCs. Further studies are required to delineate the soluble factors from OA SBOs that are responsible for triggering hypertrophic changes of ACCs in OA.

Among signaling factors, the MAPK subtypes ERK1/2 and p38 play a key role in the signaling process of chondrocyte cellular differentiation and homeostasis depending on the nature of extracellular stimuli (15, 40). This knowledge prompted us to
investigate MAPK signaling in the context of what influence normal and OA SBOs have on the differentiation of ACCs. This is the first study of its kind to report that OA SBOs induce ERK1/2 phosphorylation and suppress p38 phosphorylation in ACCs, indicating that the alterations of these pathways accompany ACC pathological phenotypic changes. Indeed, we have demonstrated that the basal levels of ERK1/2 phosphorylation increased and p38 decreased in OA ACCs compared to normal ACCs, an indication of the pathological relevance of these pathways in OA pathogenesis.

When the influence of ERK1/2 phosphorylation is blocked by an inhibitor, p38 was activated in ACCs grown in the presence of OA SBO conditioned media. The application of ERK1/2 inhibitor in OA SBOs CM, reversed ACCs hypertrophy and there was a return to the chondrogenic phenotype of ACCs. This observation implies that OA SBOs induced altered phenotypic changes in ACCs via a deactivation of p38 and an activation of the ERK1/2 phosphorylation. This notion was further supported by results showing that when p38 was neutralized by an inhibitor in ACCs co-cultured with normal SBOs, ERK1/2 phosphorylation was augmented and a weakening of chondrogenic gene expression and increase of hypertrophic gene expression was observed. Together this data indicates that OA SBOs decrease p38 phosphorylation and increase ERK1/2 activity, with a resulting reduced chondrogenic phenotype and increased hypertrophic phenotype. MAPKs are regulated at several levels, including kinase-kinase and kinase-substrate interactions, and inhibition of cross-talk/output by the MAPKs themselves (41, 42). The activities of p38 are primarily governed by extensive cross-talk with ERK1/2, a process that involves protein phosphatase, resulting in a reciprocal bidirectional equilibrium between ERK1/2 and p38.
phosphorylation, where an increase in p38 activity suppresses activation of ERK1/2 and vice versa (43). Such cross-talk appears to play a role in OA SBOs regulated ACCs phenotype, the existence of which has been shown in chondrocytes. For example, the opposing roles of ERK1/2 and p38 have been demonstrated in chondrogenesis regulation (44). The finding that the ERK1/2 activation increased the hypertrophic differentiation in ACCs, are consistent with the study showing the strong activation of ERK1/2 pathway in hypertrophic zone of the growth plate (45). Furthermore, it has been demonstrated that the inhibition of ERK1/2 delayed hypertrophic differentiation in growth plate chondrocytes during endochondral ossification (46).

It is possible that components of the p38 and ERK1/2 pathways may interact directly in the transcriptional complex. The intermediate p38 and ERK1/2 pathway substrates involved in these interaction are not known, but it is interesting that PD98059 (anti ERK1/2) significantly reduced the expression of the transcription factor CBFA1, whereas SB203580 (anti p38) activated this transcription factor. During early skeletogenesis chondrocyte hypertrophy is stimulated through the expression of CBFA1 in prehypertrophic chondrocytes, most likely by upregulation of COL10 expression (47). Continuous expression of CBFA1 in chondrocytes induces hypertrophic differentiation and endochondral ossification, suggestive of an important role of this transcription factor in triggering hypertrophic changes (48). It is therefore possible that OA SBOs induced altered phenotypic changes are triggered in ACCs via the activation of MAPK-CBFA1 pathway.
Conclusions: This study demonstrated that OA SBOs could induce the activation of ERK 1/2 and deactivation of p38 in ACCs resulting hypertrophic changes of chondrocytes. These data provide an insight into MAPK signaling pathways involved in the molecular mechanisms of osteoarthritis pathogenesis, which may have significant clinical implications.

Acknowledgment

We would like to acknowledge the financial support from the Prince Charles Hospital Research Foundation to the project.
Legends

Figure 1: Characterization of micromass culture of normal and OA ACCs

(A) The mRNA expression of chondrogenic and hypertrophic marker genes was compared between normal (n=3) and OA ACC (n=5) micromasses after 7 days culture in hypertrophic differentiation media containing high glucose DMEM supplemented with 1% FCS, 0.5% glutamine, 50 u/mL penicillin, 50 µg/mL streptomycin 50µM ascorbic acid, 10nM dexamethasone, and 10 mM β-glycerophosphate. The expression of CBFA1, COL10 and ALP was significantly upregulated in OA ACCs compared to normal ACCs. However, the expression of COL2 and AGG was significantly decreased in OA ACCs. Results are shown as mean ± SD. *: p≤0.05. (B-C) Western blot analysis was performed to determine phosphorylation changes of p38 (B) & ERK1/2 (C). Tubulin was used as a loading control. The figures are representative of protein bands from three separate experiments. * represents a significant difference (p<0.05).

Figure 2: Effects of normal and OA SBOs on ACCs matrix deposition and gene expression in direct co-culture.

(A) Cartilage matrix including GAG stained by alcian blue and COL2 was significantly decreased and mineralization matrix stained by Alizarin red was significantly increased in ACC pellets co-cultured with OA SBOs. ACC pellets grown alone acted as controls (n=3). (B-C) Chondrogenic markers of COL2 and AGG were downregulated, but hypertrophic markers of CBFA1, COL10, and ALP were significantly upregulated in ACCs co-cultured with OA SBOs. mRNA levels were normalized against GAPDH and 18s and the relative expression is presented. Results
are shown as mean ± SD from three combination studies of normal ACC pellets co-cultured with OA SBOs. * represents a significant difference (p<0.05).

**Figure 3: Effects of normal and OA SBOs conditioned media (CM) on normal ACCs matrix deposition and gene expression in indirect co-culture.**

**(A)** ACCs were cultured with CM from normal or OA SBOs and control ACC micromasses were cultured in non-CM. GAG deposition decreased and matrix mineralization increased in the ACCs cultured with OA SBOs CM after 7 days. **(B&C)** Expression levels of \( \text{COL2} \) and \( \text{AGG} \) decreased and \( \text{CBFA1}, \text{COL10}, \) and \( \text{ALP} \) level increased by quantitative PCR after culturing ACC micromasses for 7 days in OA SBOs CM. Results are shown as mean ± SD from three combination studies of ACCs cultured with CM. * represents a significant difference (p<0.05).

**Figure 4: MAPK signaling pattern in the direct and indirect co-cultures of ACCs**

**(A)** ACC pellets were co-cultured directly with normal or OA SBOs monolayer. After 14 days the ACCs pellet protein was isolated and increased phospho-ERK1/2 and decreased phopho-p38 were noted in the co-culture with OA SBOs. ACC pellets cultured alone were used as control. Tubulin was used as a loading control. **(B)** ACCs micromasses were cultured in the presence or absence of CM from normal or OA SBOs for 7 days. Increased phospho-ERK1/2 and decreased phopho-p38 were detected. Band density quantification was performed using Image J software for phospho-ERK1/2 (D) and phospho-p38 (E). Each value represents a protein bands from three separate experiments. The mean ± SD is shown from three combination studies of ACC pellets co-cultured with SBOs. * represents a significant difference (p<0.05).
Figure 5: Use of PD98059 reversed the expression of hypertrophic genes in ACCs induced by OA SBOs conditioned media (CM) (A) ACCs micromasses were cultured in CM from OA SBOs with or without the ERK1/2 inhibitor PD98059 at different concentrations. After 7 days, total cell protein was isolated from ACCs micromasses and phosphorylation changes in ERK1/2 and p38 were measured. PD58059 decreased the levels of ERK1/2 in a concentration dependent manner, with a co-contaminant increase of p38 levels. (B-C) The mRNA expression of COL2 and AGG in ACCs micromasses was enhanced with addition of ERK1/2 inhibitor PD98059 upon culturing with OA SBOs CM, but CBFA1, COL10, and ALP mRNA was downregulated retrospectively. mRNA levels were normalized against GAPDH and 18s and the relative expression is shown. Results were representative of three combination studies of ACCs micromasses cultured in CM from OA SBOs. * represents a significant difference (p<0.05).

Figure 6: Use of SB203580 induced the expression of hypertrophic genes in ACCs cultured with normal SBOs conditioned media (CM). (A) ACCs micromasses were cultured in CM from normal SBOs with or without the p38 inhibitor, SB203580. After co-culture for 7 days, total cell protein was isolated from ACCs and changes to p38 and ERK1/2 phosphorylation was measured. Addition of SB203580 in culture media showed inhibitory effect on p38 phosphorylation, but enhanced pERK1/2 activity. (B-C) SB203580, decreased the expression of COL2 and AGG, but increased CBFA1, COL10, and ALP mRNA level in ACCs micromasses after co-culturing with normal SBOs. mRNA levels were normalized against GAPDH and 18s and the relative expression is shown. Results were representative of three
combination studies of ACCs micromasses cultured in CM from normal SBOs. * represents a significant difference (p<0.05).
References:

Figure 1: Characterization of micromass culture of normal and OA ACCs

(A) The mRNA expression of chondrogenic and hypertrophic marker genes was compared between normal (n=3) and OA ACC (n=5) micromasses after 7 days culture in hypertrophic differentiation media containing high glucose DMEM supplemented with 1% FCS, 0.5% glutamine, 50 u/L penicillin, 50 µg/mL streptomycin 50µM ascorbic acid, 10nM dexamethasone, and 10 mM β-glycerophosphate. The expression of CBFA1, COL10 and ALP was significantly up regulated in OA ACCs compared to normal ACCs. However, the expression of COL2 and AGG was significantly decreased in OA ACCs. Results are shown as mean ± SD. *: p≤0.05.

(B-C) Western blot analysis was performed to determine phosphorylation changes of p38 (B) & ERK1/2 (C). Tubulin was used as a loading control. The figures are representative of protein bands from three separate experiments. * represents a significant difference (p<0.05).
Figure 2: Effects of normal and OA SBOs on ACCs matrix deposition and gene expression in direct co-culture.

(A) GAG stained by alcian blue and COL2 was significantly decreased and mineralization matrix stained by Alizarin red was significantly increased in ACC pellets co-cultured with OA SBOs. ACC pellets grown alone acted as controls (n=3). (B-C) Chondrogenic markers of COL2 and AGG were downregulated and hypertrophic markers of CBFA1, COL10, and ALP were significantly upregulated in ACC pellets co-cultured with OA SBOs. mRNA levels were normalized against GAPDH and 18s and the relative expression is presented. Results are shown as mean ± SD from three combination studies of normal ACC pellets co-cultured with OA SBOs. * represents a significant difference (p<0.05).
Figure 3: Effects of normal and OA SBOs conditioned media (CM) on normal ACCs matrix deposition and gene expression in indirect co-culture.

(A) ACCs were cultured with CM from normal or OA SBOs and control ACC micromasses were cultured in non-CM. GAG deposition decreased and matrix mineralization increased in the ACCs cultured with OA SBOs CM after 7 days. (B&C) Expression levels of COL2 and AGG decreased and CBFA1, COL10, and ALP level increased by quantitative PCR after culturing ACC micromasses for 7 days in OA SBOs CM. Results are shown as mean ± SD from three combination studies of ACCs cultured with CM. * represents a significant difference (p<0.05).
Figure 4: MAPK signaling pattern in the direct and indirect co-cultures of ACCs

(A) ACC pellets were co-cultured directly with normal or OA SBOs monolayer. After 14 days the ACCs pellet protein was isolated and increased phospho-ERK1/2 and decreased phospho-p38 were noted in the co-culture with OA SBOs. ACC pellets cultured alone were used as control. Tubulin was used as a loading control.

(B) ACCs micromasses were cultured in the presence or absence of CM from normal or OA SBOs for 7 days. Increased phospho-ERK1/2 and decreased phospho-p38 were detected. Band density quantification was performed using Image J software for phospho-ERK1/2 (D) and phospho-p38 (E). Each value represents a protein bands from three separate experiments. The mean ± SD is shown from three combination studies of ACC pellets co-cultured with SBOs. * represents a significant difference (p<0.05).
Figure 5: Use of PD98059 reversed the expression of hypertrophic gene in ACCs induced by OA SBOs conditioned media (CM). (A) ACCs micromasses were cultured in CM from OA SBOs with or without the ERK1/2 inhibitor PD98059 at different concentrations. After 7 days, total cell protein was isolated from ACCs micromasses and phosphorylation changes in ERK1/2 and p38 were measured. PD98059 decreased the levels of ERK1/2 in a concentration dependent manner. (B-C) The mRNA expression of COL2 and AGG in ACCs micromasses was enhanced with addition of ERK1/2 inhibitor PD98059 upon culturing with OA SBOs CM, but CBFA1, COL10, and ALP mRNA was downregulated retrospectively. mRNA levels were normalized against GAPDH and 18s and the relative expression is shown. Results were representative of three combination studies of ACCs micromasses cultured in CM from OA SBOs. * represents a significant difference (p<0.05).
Figure 6: Use of SB203580 induced the expression of hypertrophic gene in ACCs cultured with normal SBOs conditioned media (CM).
(A) ACCs micromasses were cultured in CM from normal SBOs with or without the p38 inhibitor, SB203580. After co-culture for 7 days, total cell protein was isolated from ACCs and changes to p38 and ERK1/2 phosphorylation was measured. Addition of SB203580 in culture media inhibited p38 phosphorylation.
(B-C) SB203580 decreased the expression of COL2 and AGG, but increased CBFA1, COL10, and ALP mRNA level in ACCs micromasses after co-culturing with normal SBOs. mRNA levels were normalized against GAPDH and 18s and the relative expression is shown. Results were representative of three combination studies of ACCs micromasses cultured in CM from normal SBOs. * represents a significant difference (p<0.05).