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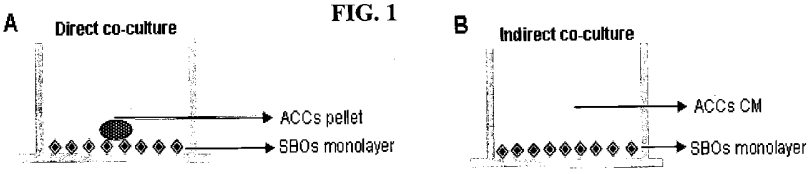
(54) **Title**: METHODS AND COMPOSITIONS FOR THE TREATMENT OF OSTEOARTICULAR DISEASE

FIG. 1



(57) **Abstract**: The present invention provides methods, and compositions for prophylactic and therapeutic treatment of a mammal (e.g. a human) suffering from an osteoarticular disease, such as osteoarthritis. More specifically, the present invention relates to the use of MAPK signalling pathway modulators, such as ERK 1/2 inhibitors, CFBA1 inhibitors and p38 activators, to modulate the pathological interactions that occur between subchondral bone cells and cartilage cells during the development and/or progression of osteoarticular diseases. Suitably, modulation of these interactions at least partly alleviates major symptoms associated with osteoarticular diseases and slows disease progression, thereby reducing the need for surgical intervention and anti-inflammatory medication.

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TITLE

METHODS AND COMPOSITIONS FOR THE TREATMENT OF OSTEOARTICULAR DISEASE

FIELD OF THE INVENTION

THIS INVENTION relates to treatment of bone and joint disease. More specifically, the present invention relates to the use of mitogen activated protein kinase (MAPK)-signalling pathway modulators, and compositions comprising the same, for the treatment of osteoarticular disease, particularly osteoarthritis.

BACKGROUND OF THE INVENTION

Osteoarthritis (OA) is a common musculoskeletal disorder that is particularly prevalent in persons above the age of 65. It is characterized by a progressive degeneration of the articular cartilage, and studies have shown that subchondral bone osteoblast metabolism is abnormally affected in OA (Lajeunesse *et al.*, 2003; Lajeunesse *et al.*, 2004).

The mechanisms responsible for the abnormal subchondral bone activity remain elusive and questions remain unanswered as to whether subchondral bone changes precede cartilage degeneration or *vice versa*, or may be run parallel with each other. In OA animal models, it has been shown that a thickening of subchondral bone precedes fibrillation of the cartilage (Carlson *et al.*, 1996; Anderson-MacKenzie *et al.*, 2005; Fahlgren *et al.*, 2003), whereas other studies suggest that the changes seen in the articular cartilage is in response to repeated shock to the joints which results in a biological activation and promotes remodelling of the subchondral bone (Chappard *et al.*, 2006; Oegema *et al.*, 1997).

Since the molecular mechanisms that contribute to the development and progression of degenerative joint conditions, including OA, have remained unknown, there is a lack of prognostic and therapeutic treatments available for these crippling conditions. Consequently, individuals suffering from osteoarthritis often rely on anti-inflammatory drugs that may cause gastrointestinal bleeding or damage internal organs, such as the liver and the kidneys.

SUMMARY OF THE INVENTION

The lack of information regarding the molecular mechanisms, and in

particular the signalling pathways, by which normal and osteoarthritic bone and cartilage cells interact to regulate articular cartilage differentiation has hampered early and effective treatment of osteoarticular diseases, including osteoarthritis.

Current methods of treatment of osteoarthritis are scarce and primarily focus on the administration of anti-inflammatory medication that alleviates pain. Thus, to facilitate the development of novel and efficient prophylactic and therapeutic treatments for osteoarthritis and related disorders, there is a need for new insight into the signalling pathways that are involved in osteoarthritis pathogenesis.

The present invention arises from the inventors' surprising discovery that pathological interactions between bone and cartilage cells during osteoarticular diseases, such as osteoarthritis, activate the MAPK (ERK1/2) signalling pathway and suppress the MAPK (p38) signalling pathway, resulting in increased cell differentiation and subsequent mineralization.

It is therefore proposed that modulation of the MAPK-signalling pathway using MAPK-signalling pathway specific modulators may at least partly reduce the symptoms associated with osteoarticular diseases, particularly osteoarthritis.

In a first aspect, the invention therefore provides a method of prophylactic and/or therapeutic treatment of an osteoarticular disease in a mammal, said method including the step of modulating a MAPK-signalling pathway of an osteoblast and/or a chondrocyte in said mammal, whereby (i) modulation of the MAPK-signalling pathway, associated with an interaction between osteoarthritic osteoblasts and normal chondrocytes, prevents or inhibits differentiation of a normal chondrocyte to an osteoarthritic phenotype; and/or (ii) modulation of the MAPK-signalling pathway, associated with an interaction between osteoarthritic chondrocytes with normal osteoblasts, prevents or inhibits differentiation of a normal osteoblast to an osteoarthritic phenotype; to thereby treat or prevent said osteoarticular disease.

In a second aspect, the invention provides a method of screening for, designing, engineering or otherwise producing a MAPK-signalling pathway modulator for use in treatment of an osteoarticular disease, said method including the steps of determining whether said MAPK-signalling pathway modulator can modulate a MAPK-signalling pathway in one or more cells and/or tissues that are

associated with an osteoarticular disease.

In a third aspect, the invention provides a pharmaceutical composition comprising one or more MAPK-signalling pathway modulators selected from the group consisting of: (i) a MAPK-signalling pathway modulator produced according to the method of the second aspect (ii) an ERK1/2 inhibitor, (iii) a CFBA1 inhibitor, and (iv) a p38 activator, and a pharmaceutically acceptable carrier, diluent or excipient, for use in the treatment of an osteoarticular disease or condition in a mammal.

In one embodiment, modulation of the MAPK-signalling pathway of the osteoarthritic osteoblast, and/or the normal chondrocyte, prevents or inhibits differentiation of the normal chondrocyte to the osteoarthritic phenotype.

In one particular embodiment, the MAPK-signalling pathway of the osteoarthritic osteoblast is modulated.

In another particular embodiment, the MAPK-signalling pathway of the normal chondrocyte is modulated.

In another embodiment, modulation of the MAPK-signalling pathway of the osteoarthritic chondrocyte and/or the normal osteoblast prevents or inhibits differentiation of the normal osteoblast to the osteoarthritic phenoptype.

In one particular embodiment, the MAPK-signalling pathway of the osteoarthritic chondrocyte is modulated.

In another particular embodiment, the MAPK-signalling pathway of the normal osteoblast is modulated.

Preferably said osteoarticular disease is selected from the group consisting of inflammatory rheumatism, metabolic arthropathy, degenerative rheumatism, rheumatoid arthritis, osteoarthritis, spondylarthritis, gout, chondrocalcinosis and arthrosis.

More preferably, said osteoarticular disease is osteoarthritis.

In one particular embodiment, said chondrocytes are, or comprise, articular cartilage chondrocytes (ACCs).

In another particular embodiment, said osteoblasts are, or comprise, subchondral bone osteoblasts (SBOs).

Typically, although not exclusively, MAPK-signalling pathway modulation reduces matrix mineralization and/or bone sclerosis.

Preferably, MAPK-signalling pathway modulation modulates the expression and/or activity of one or more molecules selected from the group consisting of ERK1/2, p38 and CFBA1.

Suitably, MAPK-signalling pathway modulation reduces the expression and/or activity of ERK1/2 and/or CFBA1. Preferably, the expression and/or activity of ERK1/2 and/or CFBA1 is reduced using an ERK1/2 inhibitor and/or a CFBA1 inhibitor.

Suitably, MAPK-signalling pathway modulation increases the expression and/or activity of p38 in cells such as ACCs. Preferably, the expression and/or activity of p38 is increased using a p38 activator.

As described herein, the term "*mammal*" includes and encompasses humans, domestic and farm animals, such as dogs, horses, cats, sheep, pigs, cows.

Preferably, the mammal is a human.

Throughout this specification, unless the context requires otherwise, the words "*comprise*", "*comprises*" and "*comprising*" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Diagrammatic representation of co-culture models. (A) In the direct co-cultures, ACCs pellets were prepared and placed directly upon the SBOs monolayer in different combinations as described in methods and materials. **(B)** In the indirect co-cultures, ACCs conditioned media (ACCs CM) were prepared and cultured with SBOs as described in methodology.

Figure 2: Expression of osteogenic genes in SBOs. The mRNA expression of osteogenic marker genes was compared between normal and OA SBOs after 14 days in differentiation media. The expression of *CBFA1*, *ALP* and *OC* was significantly upregulated in OA SBOs compared to normal SBOs. *OPN* expression was upregulated in OA SBOs, however, failed to reach the significance. *: $p \leq 0.05$.

Figure 3: Effects of normal and OA ACCs on SBOs matrix deposition in

the direct and indirect co-culture system. (A) Phenotypic characterization of normal and OA ACCs was determined by the mRNA expression of COL2 and AGG after 14 days in the pellet culture. A representative of three normal and three OA ACC pellets are shown. **(B)** Normal SBOs were cultured with normal and OA ACCs in both direct and indirect co-culture systems in osteogenic differentiation medium. Matrix mineralization was determined by alizarin red staining. Total staining density of each well was quantified by using Image J in both direct **(C)** and indirect co-cultures **(D)**, and results are shown as mean ± SEM, n=5. *: $p<0.05$.

Figure 4: ALP activity and osteogenic gene expression of normal SBOs cultured with normal or OA ACC CM. (A) ALP activity determined by a colorimetric assay in SBOs after culture with CM from normal or OA ACCs for 7 days as indicated in figures. The bars are mean ± SEM, n=5. *: $p<0.05$. Expression levels of *CBFA1***(B)**, *ALP***(C)**, *OPN*, **(D)** *OC* **(E)** were determined by quantitative PCR after culturing SBOs with normal or OA ACCs CM , at 3, 7, and 14 days. SBOs cultured in non-conditioned medium were used as control. Relative mRNA levels were normalized to 18srRNA. Bar equal mean ± SEM; n=5. *: $p<0.05$.

Figure 5: Phosphorylated ERK1/2, p38 and JNK expression in normal SBOs cultured with OA or normal CM. (A) Total cell protein was isolated from SBOs cultured with normal or OA ACCs CM and Western blot was performed to determine phosphorylation changes of ERK1/2, p38, and JNK pathways at day 7 and day 14. Tubulin was used as a loading control. **(B-D)** Quantification of band density was performed from pERK1/2 (B), p38 (C), and pJNK (D) bands obtained on day 7 using Image J. Each value represents protein bands from three different experiments. *: $p<0.05$.

Figure 6: The basal levels of phosphorylated ERK1/2 were measured by immunoblot methods in normal and OA SBOs. A significantly higher level of phosphorylated ERK1/2 was detected in OA SBOs compared to the level in normal SBOs.

Figure 7: Inhibition of ERK1/2 phosphorylation reversed OA ACCs CM induced SBOs differentiation. Normal SBOs were cultured alone or with OA ACCs CM for 14 days in the presence (+) or absence (-) of SB203580 (5μM) to

inhibit p38 phosphorylation, PD98059 (10μM) to inhibit ERK 1/2 phosphorylation or SP600125 (10μM) to inhibit the JNK phosphorylation in osteogenic conditions, respectively. **(A)** Western blot analysis revealed that the addition of the respective inhibitors reduced OA ACCs CM induced p38, ERK1/2 and JNK phosphorylation up to 80 % in SBOs **(B)** Response of SBOs after culturing with or without OA ACCs CM on the extra cellular matrix deposition in the presence or absence of different inhibitors. **(C)** Response of SBOs after culturing with or without OA ACCs CM on the gene expression of *ALP*, *OC*, *OPN* and *CBFA1* in the presence or absence of different inhibitors. mRNA levels were normalized to *18s* and the relative expressions were given. *: $p<0.05$.

Figure 8: Effect of U0126, an ERK1/2 inhibitor in the SBOs co-cultured with normal and OA ACCs. (A) pERK1/2 was significantly increased in SBOs co-cultured with OA ACCs. (B) OA ACCs also increased the expression of OCN and ALP compared to normal ACCs and SBOs alone. (C) U0126 at 15 uM concentration significantly reduced the ERK1/2 phosphorylation in SBOs induced by OA ACCs. (D) Presence of U0126 reversed the expression of OC and ALP in SBOs induced by OA ACCs. Results are mean ±SEM, where $P\geq0.05$ was considered significant.

Figure 9: Diagrammatic representation of co-culture models. (A) Direct co-cultures: ACC pellets were prepared as described in methodology and were placed directly on the SBOs monolayer. **(B)** Indirect co-cultures (conditioned media (CM)): SBOs CM was prepared as described in methodology section and cultured with ACCs micromasses.

Figure 10: Characterization of micromass culture of normal and OA ACCs. (A) The mRNA expression of chondrogenic and hypertrophic marker genes was compared between normal and OA ACC 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 up regulated in OA SBOs compared to normal SBOs. However, the expression of *COL2* an *AGG* was significantly decreased in OA ACCs. Results are shown as mean ± SD. *: $p\leq0.05$.

(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 11: 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 in the co-culture with OA SBOs. ACC pellets grown alone acted as controls. **(B-C)** Chondrogenic markers of *COL2* and *AGG* were downregulated in the co-culture with OA SBOs, but hypertrophic markers of *CBFA1, COL10,* and *ALP* were significantly upregulated in the co-culturing ACC pellets. mRNA levels were normalized against *GAPDH* and *18s* and the relative expression is presented. Results are shown as mean ± SD. * represents a significant difference ($p<0.05$).

Figure 12: Effects of normal and OA SBOs CM on ACCs matrix deposition and gene expression in indirect co-culture. (A) ACCs were cultured with conditioned media from normal or OA SBOs and control ACC micromasses were cultured in non-conditioned media. GAG deposition decreased and matrix mineralization increased in the co-culture with OA SBOs after 7 days. **(B&C)** Expression levels of *COL2* and *AGG* decreased and *CBFA1, COL10,* and *ALP* levels increased by quantitative PCR after culturing ACC micromasses for 7 days in conditioned media. Results are shown as mean ± SD. * represents a significant difference ($p<0.05$).

Figure 13: MAPK-signalling 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 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

phopho-ERK1/2 (**C**) and phospho-p38 (**D**). Each value represents protein bands from three separate experiments. The mean ± SD is shown. * represents a significant difference ($p<0.05$).

Figure 14: Inhibition of ERK1/2 phosphorylation reversed OA SBOs conditioned media induced ACC phenotypic changes. (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 in 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. * represents a significant difference ($p<0.05$).

Figure 15: Effects of p38 inhibition on ACCs cultured with normal SBOs CM. (A) ACCs micromasses were cultured in conditioned media 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. SB203580 inhibited p38 phosphorylation, but enhanced pERK1/2 activity. **(B-C)** p38 inhibition decreased the expression of *COL2* and *AGG*, but increased *CBFA1*, *COL10*, and *ALP* mRNA level in ACCs micomasses after co-culturing with normal SBOs. mRNA levels were normalized against *GAPDH* and *18s* and the relative expression is shown. *represents a significant difference ($p<0.05$).

Figure 16: Effect of U0126, an ERK1/2 inhibitor in the ACCs co-cultured with normal and OA SBOs. (A) pERK1/2 was significantly increased in ACCs co-cultured with OA SBOs. (B) OA SBOs also increased the expression of CBFA1 and COL10 compared to normal SBOs and ACCs alone. (C) U0126 at 15 uM concentration significantly reduced the ERK1/2 phosphorylation. (D) Presence of U0126 reversed the expression of CBFA1 and COL10 in ACCs induced by OA SBOs. Results are mean ± SEM, where $P\geq0.05$ was considered significant.

Figure 17: pERK-1/2 pathway inhibitor U0126 has partial protective effects against OA cartilage degradation: (A-D) Gross morphology findings at the cartilage surface in sham, menisectomy (MSX), anterior cruciate ligament (ACLT) and mono-ido-acetate OA models in U0126 treated vs untreated animals. No cartilage changes were detected in the sham group either in inhibitor treated or untreated animals. Cartilage surface roughness, fibrillation, small osteophytes or areas of peripheral fibrous tissue proliferation were present both in tibia and femur at 8 weeks and 4 weeks where the meniscus was removed; however the damage was less in MSX models when treated with U0126 every 3 days at a concentration of 0.5 mM in 50 µl/ joint. The ACLT model demonstrated cartilage surface damage around the margins of the tibia and center of femoral condyles, although this damage was much less in U0126 treated animals. One single intra articular injection of MIA resulted in severe cartilage degeneration. Due to the severity of the MIA treatment, the damage was less apparent in U0126 treated animals (in circle).

Figure 18: Large prominent osteophyte formation, a characteristic feature of OA knee joints were observed in the MSX OA models. It was found that the size and incedent of osteophytes was significantly decreased when the animals were treated with U0126 compared to untreated animals (arrow).

Figure 19: Histological findings demonstrating the efficacy of U0126 in the rat OA models. Animals were sacrificed after 8 weeks of experimental period and stained with saffronin-o and OA was scored based on the Mankin scale. **(A)** Saffronin-o staining demonstrated proteoglycan depletion in the OA animal models compared to sham. However, U0126 treated animals showed less depletion compared to untreated OA animal models. **(B)** Saffronin-o sections were further quantified using Mankin score (14 scale). Bar graphs showing the total Mankin score from the histological evaluation of joint cartilage at the weight bearing area of the tibial chondyle in the different experimental groups. U0126 treated animal models showed significantly reduced Mankin score compared to untreated and sham animals. Data is mean ± SD, where $P < 0.05$ indicates statistical significance.

Figure 20: Microstructure parameters in subchondral plate analysed by micro-CT. (A) MSX OA models showed an increase in BV (bone volume) and

BV/TV (bone volume/tissue volume) compared to shams at week 8. The increased BV and BV/TV were partially reversed when the animals were treated with the U0126. In the ACLT model, BV and BV/TV were decreased with respect to that in sham knees. This phenomenon was in part reversed upon treatment with U0126 ($P < 0.05$). Similarly, in the MIA model BV and BV/TV showed a downward trend with respect to healthy controls, however, treatment with U0126 failed to show any effect due to the severity of the MIA model. **(B)** 3D image of MSX-OA showing uneven rough texture on the surface of the subchondral bone. U0126 treatment partially lessened this surface damage. Similar results (*i.e.* a partial reduction in subchondral bone surface damage) were obtained following treatment of the ACLT-OA and MIA-OA models with the U0126 inhibitor.

Figure 21: U0126 treatment reduces OA cartilage degradation markers. Cartilage tissue protein was isolated from all the animal groups and subjected to Western blot analysis. pERK-1/2 was significantly increased in the OA models compared to sham indicating the pathological relevance of this signalling pathway in relation to OA cartilage. U0126 downregulated the pERK-1/2 expression in the treatment groups indicating that the pathway was indeed down regulated. Furthermore, U0126 also decreased the expression of ADAMTS5 (gene reported to be responsible for OA cartilage degradation (Majumdar *et al.*, 2007)), VEGF and COL10 (markers of cartilage hypertrophy, typical of OA (Pullig *et al.*, 2000)) in ACLT and MSX models compared to sham and untreated OA models. However in the MIA model, although an increase in ADAMTS5 and VEGF was identified, no difference was observed in response to inhibitor treatment, again due to the severity of the MIA model. COL10 was not expressed in any group in the MIA model. Tubulin was used as a loading control. Representative images from three different animals are shown.

Figure 22: U0126 treatment enhanced the OA cartilage anabolic activity. Chondrocytes were cultured in serum-free chondrogenic medium for 1 wk from all the experimental groups. After 1 wk culture, RNA was extracted and Col2a1 mRNA expression was assessed by real time RT-PCR. Cartilage anabolic marker Col2a1 expression was decreased in all OA animal models compared to shams. Treatment

with U0126 partially restored the Col2a1 loss. The bar graphs represent the mean ± SD of three independent experiments (n=3).

Figure 23: U0126 treatment reduces OA subchondral bone changes. Bone tissue protein was isolated from all the animal groups and subjected to Western blot analysis. pERK-1/2 was significantly increased in the OA models compared to sham indicating the pathological relevance of this signalling pathway in relation to OA subchondral bone changes. U0126 downregulated the pERK-1/2 in treatment groups indicating that the pathway intended to be inhibited was indeed down regulated. Furthermore, ALP (Alkaline phosphatase), a marker of osteogenesis that has been reported to be increased in the human OA bone compared to normal patients relating to abnormal bone remodelling typical of OA patients (Sanchez *et al.*, 2008). Similar to these findings it was found that ALP expression was also increased in ACLT and MSX models compared to sham. U0126 decreased the expression of ALP induced in ACLT and MSX models compared to sham and untreated OA models. However in the MIA OA model, a decrease in ALP expression was identified possibly because of the bone degradation induced by MIA. No changes were shown in U0126 treated MIA OA model. Tubulin was used as a loading control. Representative images from three different animals areshown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is predicated, at least in part, by the present inventors' unexpected discovery that pathological interactions between chondrocytes and bone osteoblasts during osteoarticular diseases, including osteoarthritis, induces MAPK (ERK1/2) signalling and suppresses MAPK (p38) signalling.

More particularly, the inventors surprisingly found that MAPK-signalling pathway modulators may facilitate the development of prophylactic and/or therapeutic treatment regimes of a range of bone and joint disorders that are associated with increased cell differentiation and matrix mineralization, such as osteoarthritis.

In one aspect, the invention provides a method of prophylactic and/or therapeutic treatment of an osteoarticular disease in a mammal, said method including the step of modulating a MAPK-signalling pathway of an osteoblast and/or

a chondrocyte in said mammal, whereby (i) modulation of the MAPK-signalling pathway, associated with an interaction between osteoarthritic osteoblasts and normal chondrocytes, prevents or inhibits differentiation of a normal chondrocyte to an osteoarthritic phenotype; and/or (ii) modulation of the MAPK-signalling pathway, associated with an interaction between osteoarthritic chondrocytes with normal osteoblasts, prevents or inhibits differentiation of a normal osteoblast to an osteoarthritic phenotype; to thereby treat or prevent said osteoarticular disease.

As used herein, the term "*prophylactic treatment*" means that the treatment described herein may be used to prevent an osteoarticular disease from occurring before the onset of any noticeable symptoms. Thus, in some embodiments, the prophylactic treatment may be suitable for treating a mammal that has had, and/or is susceptible to developing, an osteoarticular disease (*e.g.* a human that is genetically predisposed to developing osteoarthritis and/or a human that has suffered a joint injury).

By "*osteoarticular disease*" is meant a disease in which both cartilage and bone tissue are damaged. An osteoarticular disease can be inflammatory rheumatism, metabolic arthropathy, degenerative rheumatism, rheumatoid arthritis, osteoarthritis, spondylarthritis, gout, chondrocalcinosis or arthrosis, although without limitation thereto.

One particularly preferred form of an osteoarticular disease is osteoarthritis.

As used herein, "*osteoarthritis*", or "*OA*", refers to a group of diseases characterized by the degradation of joints including articular cartilage and the subchondral bone next to it. The term osteoarthritis may refer to primary and/or secondary osteoarthritis.

The term "*MAPK-signalling pathway*" refers to a signalling pathway of one or more molecules comprising, or regulated by, mitogen-activated protein kinases (MAPKs), including but not limited to ERKs and p38 MAP kinases. This pathway suitably includes phosphatases and/or protein kinases acting directly on, upstream, and/or downstream of a MAPK. A MAPK may, for example, be activated by an upstream protein kinase (*e.g.* a MAPK kinase such as MEK1/2 in the case of ERK1/2 or MEK3/6 in the case of p38 MAPK) or inhibited by the activity of a phosphatase

(*e.g.* Protein Phosphatase 2A, PP2A). A downstream transcription factor of a MAPK-signalling pathway (*e.g.* CFBA1) may be activated by ERK1/2 and inhibited by p38.

The term "*MAPK-signalling pathway modulator*", or "*modulator*", refers to a molecule that at least partly decreases or increases an activity and/or expression of a component of the MAPK-signalling pathway as hereinbefore defined. Accordingly, administration of a modulator may either decrease or increase MAPK-signalling.

In some embodiments, the term modulator may also refer to agonists, antagonists, analogs and/or mimetics, although without limitation thereto.

In certain embodiments, the modulator is an inhibitor.

As referred to herein, an "*inhibitor*" may be a nucleic acid construct, a compound, an antibody and/or a nucleic acid that silences, knocks-down, blocks, inhibits, reduces, suppresses or otherwise lowers the expression and/or activity a target molecule (*e.g.* ERK1/2, MEK1/2 and/or CFBA1) in a cell.

In one embodiment, the modulator inhibits a protein kinase that activates a MAPK-signalling pathway by phosphorylating a target molecule (*e.g.* p38).

In another embodiment, the modulator activates a phosphatase that inhibits a MAPK-signalling pathway by dephosphorylating a target molecule (*e.g.* ERK1/2).

In yet another embodiment, the modulator is an inhibitor of the catalytic activity of a member of MAPK-signalling pathway (*e.g.* ERK1/2), for example by binding to its active site.

In still another embodiment, the modulator is a nucleic acid molecule (*e.g* an RNAi molecule) that silences the expression and/or activity of a target molecule (*e.g.* CFBA1, ERK1/2 and/or MEK1/2).

Such a modulator will typically be administered to modulate MAPK-signalling in cartilage and/or bone tissue. It will be appreciated that the modulator may be administered to modulate MAPK-signalling in specific cells such as articular cartilage chondrocytes (ACCs) and/or subchondral bone osteoblasts (SBOs).

In one particular embodiment, said modulator is administered to modulate MAPK-signalling in articular cartilage chondrocytes (ACCs).

Typically, according to this particular embodiment, said modulator modulates the expression and/or activity of one or more hypertrophic markers selected from the

group consisting of Collagen-10 (COL10), Alkaline Phosphatase (ALP), CFBA1 and MMP13.

In another particular embodiment, said MAPK-signalling pathway modulator is administered to modulate MAPK-signalling in subchondral bone osteoblasts (SBOs).

Typically, according to this particular embodiment, said modulator modulates the expression and/or activity of one or more osteogenic molecules selected from the group consisting of CBFA1, Alkaline Phosphatase (ALP), Osteopontin (OPN) and Osteocalcin (OC).

The skilled addressee will also appreciate that a reduction in activity and/or expression of these osteogenic molecules may improve one or more of the symptoms that are associated with an osteoarticular disease, such as osteoarthritis, by at least partly reducing bone metabolism, abnormal bone remodelling, matrix mineralization and/or bone sclerosis, which are characteristic features of osteoarthritis.

Preferably, said modulator is capable of modulating the expression and/or activity of one or more molecules selected from the group consisting of ERK1/2, MEK1/2, p38, and CFBA1.

Suitably, said modulator is an inhibitor that lowers, suppresses or otherwise reduces the expression of ERK1/2 and/or CFBA1.

Suitably, said modulator increases, induces or otherwise up-regulates the expression and/or activity of p38 in cells such as ACCs.

As used herein, the administration of an ERK1/2 inhibitor will typically at least partly suppress, alleviate, prevent and/or eliminate the symptoms encountered by a mammal, such as a human, that suffers from an osteoarticular disease including osteoarthritis.

In some embodiments, the ERK12 inhibitor may be administered to a mammal that has had, or is susceptible to, an osteoarticular disease before the onset of any noticeable symptoms (*e.g.* a human with a genetic predisposition to osteoarthritis and/or a human that has suffered a joint injury). Suitably, this administration at least partly prevents the symptoms from appearing.

In certain embodiments, administration of the ERK1/2 inhibitor to a mammal (*e.g.* a human) that suffers from an osteoarticular disease (*e.g.* osteoarthritis) at least partly: reduces osteoarthritic cartilage degeneration, decreases osteophyte size and incidence, reduces proteoglycan depletion, reduces subchondral bone surface damage, decreases the expression and/or activity of one or more osteoarthritic cartilage degradation markers, reduces alkaline phosphatase (ALP) expression and/or activity, and abnormal bone remodeling associated therewith, and/or enhance osteoarthritic cartilage anabolic activity, although without limitation thereto.

A CFBA1 inhibitor may, for example, be used to lower, reduce or suppress the expression and/or activity of CFBA1 to reduce and/or delay osteogenic differentiation and/or mineralization *in vivo* and/or *in vitro*.

In one embodiment, a CFBA1 inhibitor may be administered to specifically reduce, suppress and/or silence the expression and/or activity of CFBA1 in matrix-depositing cells.

In yet another embodiment, said MAPK-signalling modulator is a p38 activator.

Suitably, a p38 activator suppresses, reduces and/or inhibits the expression and/or activity of ERK1/2 and/or CFBA1.

It will be appreciated from the foregoing that the invention provides nucleic acid inhibitors of the MAPK-signalling pathway.

Thus, in some embodiments of the invention, it may be desirable to at least partly inhibit the expression of ERK1/2, MEK1/2, and/or CFBA1 in one or more chondrocytes and/or osteoblasts of a mammal (*e.g.* a human) suffering from an osteoarthritic disease and/or condition. It may also be beneficial to inhibit a phosphatase that specifically dephosphorylates p38 in cells such as ACCs.

In particular embodiments, nucleic acid inhibitors are RNA inhibitors, such as a short-interfering RNA (siRNA), a short/small hairpin RNA (shRNA), a micro RNA (miRNA), or other RNA molecule, that at least partly reduce the expression and/or stability of a target nucleic acid. A target sequence can include a DNA sequence, such as a gene or the promoter region of a gene, or an RNA sequence, such as an mRNA encoding a protein of the MAPK-signalling pathway.

Recent developments in RNA-interference based therapeutics are summarized in Castanotto and Ross, 2009, and examples of novel synthetic materials suitable for encapsulation and intracellular delivery of nucleic acids may be found in Whitehead *et al.*, 2009.

Non-limiting examples of RNAi inhibition of an ERK1 and/or ERK2 kinase. may be found in Zeng *et al.*, 2005, Di Benedetto *et al.*, 2009, Frémin *et al.*, 2007, Bessard *et al.*, 2008, and Lefloch *et al.*, 2008. Commercially available ERK1/2 RNAi constructs (*e.g.* siRNAs) can be found at www.cellsignal.com/products/6560.html. Systems that may be suitable for silencing ERK1/2 include the pSUPER RNAi system (www.bio-protech.com.tw/databank/pSUPER_Protocol.pdf). RNAi inhibition of MEK1/2 is described in Chatterjee *et al.*, 2004. Commercially available MEK1 RNAi constructs (*e.g.* siRNAs and shRNAs) can be found at www.scbt.com/datasheet-35899-mek-kinase-1-siRNA-m.html although without limitation thereto.

In yet another embodiment, said RNAi at least partly inhibits CFBA1. This can for example be achieved by placing an anti-CFBA1 siRNA into a vector system which can be regulated to yield the desired reduction in CFBA1 expression and/or activity depending on the inflammatory state of the tissue and/or cells.

In still another particular embodiment, said RNAi inhibits a phosphatase (see for example Montalibet and Kennedy, 2005). Suitably, said phosphatase dephosphorylates p38 in cell such as ACCs.

In another aspect, the invention provides a method of screening for, designing, engineering or otherwise producing a modulator for use in treatment of an osteoarticular disease, said method including the steps of determining whether a modulator can modulate a MAPK-signalling pathway in one or more cells and/or tissues that are associated with an orteoarticular disease.

In one embodiment, said modulator is an ERK1/2 inhibitor.

In another embodiment, said modulator is a CFBA1 inhibitor.

In yet another embodiment, said modulator is a p38 activator.

The modulators of this aspect may be identified by way of screening libraries of molecules such as synthetic chemical libraries, including combinatorial libraries,

by methods such as described in Nestler & Liu, 1998, Comb. Chem. High Throughput Screen. **1** 113 and Kirkpatrick *et al.*, 1999, Comb. Chem. High Throughput Screen **2** 211 and in United States Patent No. 7,291,456.

It is also contemplated that libraries of naturally-occurring molecules may be screened by methodologies such as the ones reviewed in Kolb, 1998, Prog. Drug. Res. **51** 185.

More rational approaches to designing modulators may employ X-ray crystallography, NMR spectroscopy, computer assisted screening of structural databases, computer-assisted modelling, or more traditional biophysical techniques which detect molecular binding interactions, as are well known in the art.

A review of structural bioinformatics approaches to drug discovery is provided in Fauman *et al*, 2003, Meth. Biochem. Anal. **44**: 477. More recent reviews on emerging drug discovery approaches (*e.g.* chemogenomics) may be found in Pirard, 2009, Vintonyak *et al.*, 2009, Schreyer and Blundell, 2009, and Strömbergsson and Kleywegt, 2009.

Computer-assisted structural database searching and bioinformatic approaches are becoming increasingly utilized as a procedure for identifying and/or engineering agonists and antagonist molecules. Examples of database searching methods may be found in United States Patent No. 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics) and United States Patents 7,158,891 and 5,680,331, which are directed to more general computational approaches to protein modelling and structural mimicry of protein activity.

Virtual screening methods that may be employed to design modulators are provided in Perola, 2006, Brewerton, 2008, and Kolb *et al.*, 2009.

A Computer Aided Drug Design (CADD) method that facilitates high throughout screening of millions of compounds, suitable for the development of signal-regulated kinase inhibitors, is described in Burkhard *et al.*, 2009.

Recent advances in lead molecule design are reviewed in Shaikh *et al.*, 2007.

Generally, other applicable methods include any of a variety of biophysical techniques which identify molecular interactions. Methods applicable to potentially useful techniques such as competitive radioligand binding assays, electrophysiology,

analytical ultracentrifugation, microcalorimetry, surface plasmon resonance and optical biosensor-based methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

A person skilled in the art will appreciate that modulating agents may be in the form of a binding partner and as such, identified by interaction assays such as yeast two-hybrid approaches and the like, but without limitation thereto. Two-hybrid screening methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

In another aspect, the invention provides a pharmaceutical composition comprising one or more MAPK-signalling modulators selected from the group consisting of: (i) a modulator produced according to the method of the aforementioned aspect, (ii) an ERK1/2 inhibitor; (iii) a CFBA inhibitor; and (iv) a p38 activator; and a pharmaceutically acceptable carrier, diluent or excipient, for use in the treatment of an osteoarticular disease or condition in a mammal.

Preferably said osteoarticular disease is selected from the group consisting of inflammatory rheumatism, metabolic arthropathy, degenerative rheumatism, rheumatoid arthritis, osteoarthritis, spondylarthritis, gout, chondrocalcinosis and arthrosis.

More preferably, said osteoarticular disease is osteoarthritis.

In general terms, by "*carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in administration to a mammal such as a human. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulphate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, isotonic saline, pyrogen-free water, tricaine, wetting or emulsifying agents, bulking agents, coating binders, fillers, disintegrants, diluents, lubricants, pH buffering agents.

In one preferred embodiment, the pharmaceutical composition is

administered via local delivery to a joint.

The skilled addressee will appreciate that since cartilage can absorb nutrients, as well as drugs from synovial fluid during joint movement, the pharmaceutical composition may be injected into the diseased joint cavity. For example by puncturing the joint cavity using a needle and subsequently injecting the pharmaceutical composition into the intra-articular fluid, (*i.e.* via intra-articular injection). It will also be appreciated that the pharmaceutical composition may be control released depending on the delivery agent(s) and the condition of the patient.

In other embodiments, the pharmaceutical composition is administered via topical and/or oral delivery systems. Preferably, the pharmaceutical composition targets the modulator to a site of, or containing, osteoarthritic cells or tissues so that non-specific inhibition or activation of a MAPK-signalling pathway is at least partly avoided or minimized. It will be appreciated that the methods of the invention may be broadly directed to any mammal that suffers from an osteoarticular disease or condition, such as osteoarthritis or rheumatoid arthritis, in which both bone and cartilage tissue are damaged. It will also be appreciated that a mammal that is being treated for said osteoarticular disease or condition may be administered a "*therapeutically effective amount*" of the modulator or a pharmaceutical composition comprising the same.

As referred to herein, a "*therapeutically effective amount*" is an amount sufficient to achieve a desired biological effect, for example an amount that is effective to prophylactically or therapeutically treat an osteoarticular disease or condition in a mammal such as a human.

In one particular embodiment, it is an amount of a modulator that is effective to reduce, alleviate, suppress, eliminate and/or prevent symptoms associated with osteoarticular diseases such as, but not limited to, joint pain, stiffness, tenderness, inflammation, creaking and locking of joints.

In another particular embodiment, it is an amount of a modulator effective to at least partly repair and remodel bone and/or cartilage tissue in a mammal that suffers from an osteoarticular disease.

The skilled addressee will appreciate that the therapeutically effective amount

includes a quantity of modulator sufficient to achieve a desired effect in a mammal that is being treated for osteoarticular disease or condition.

This may, for instance, be an amount that is sufficient to at least partly improve the signs and/or symptoms associated with diseases of the joints and bone such as osteoarthritis and gout.

The skilled addressee will also appreciate that an effective amount of the modulator can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount of the modulator, or a pharmaceutical composition comprising the same, will be dependent on the subject being treated, the severity and type of the osteoarticular disease or condition being treated, and the manner of administration.

The exact amount of modulator is readily determined by one of skill in the art based on the age, weight, sex, and physiological condition of the subject. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Administration of the modulator can be utilized whenever repair and regeneration of bone or cartilage tissue is desired, for example, at the first sign of symptoms of an osteoarticular disease, such as but not limited to inflammatory rheumatism, metabolic arthropathy, degenerative rheumatism, rheumatoid arthritis, osteoarthritis, spondylarthritis, gout, chondrocalcinosis or arthrosis, and following injury. The modulator may also be administered as a preventative measure in the absence of any symptoms (*e.g.* to a genetically predisposed human and/or a human that has suffered a joint injury).

Therapeutically effective amounts of modulators, or compositions comprising the same, may be administered by a number of routes, including parenteral administration, for example, intravenous, intraperitoneal, intramuscular, intrasternal, or intraarticular injection, or infusion.

The therapeutically effective amount of modulators to reduce mineralization, bone sclerosis, and for use in repair and/or regeneration is that amount that achieves a desired effect in a mammal (*e.g.* a human) that is being treated.

In one embodiment, this may be the amount of modulator that may be

necessary to inhibit advancement, or to cause regression of an osteoarticular disease or condition.

The modulator can be administered in a pharmaceutically acceptable carrier, such as buffered saline or another medium suitable for administration to a subject.

In some embodiments, pharmaceutical compositions containing one or more modulators may also contain one or more additional pharmaceutical agents, such as one or more anti-microbial agents (for example, antibiotics, anti-viral agents and anti-fungal agents), anti-inflammatory agents (for example, glucocorticoids such as hydrocortisone, dexamethasone or prednisone, or non-steroidal anti-inflammatory agents such as acetylsalicylic acid, ibuprofen or naproxen sodium).

The skilled addressee will appreciate that such additional pharmaceutical agents can be administered before, during, or after administration of the modulator, depending on the desired effect. This administration can be by the same route or by different routes, and either at the same site or at a different site.

Preferably, the mammal is a human.

So that the invention may be fully understood and put into practical effect, the skilled reader is directed to the following non-limiting detailed Examples.

EXAMPLES

Introduction

The role of the mitogen-activated protein kinase (MAPK) signalling network in osteogenesis has been demonstrated in several studies (Hipskind and Bilbe, 1998; Wang *et al.*, 2007; Ge *et al.*, 2007). MAPKs are proline-directed kinases that include the mitogen-regulated extracellular signal-regulated kinase (ERK), the stress-activated protein kinases/c-Jun NH_2 terminal kinases (JNK) and the p38 kinases (p38). MAPKs have important functions as mediators of cellular responses to a variety of extracellular stimuli (Cobb, 2001) and typically, the MAPK kinase (MEK)/ERK family is stimulated by growth factors, while the JNK/p38 pathways are activated by cellular stresses, cytokines, and hypoxia (Pearson *et al.*, 2001; Xiao *et al.*, 2002). While the MAPK-signalling pathways have been shown to be involved in various aspects of cartilage biology, such as cartilage matrix synthesis and homeostasis (Zhen *et al.*, 2001; Watanabe *et al.*, 2001), their potential roles during

the development and progression of degenerative joint diseases, such as osteoarthritis, have remained unknown. Given that cell-cell interactions between subchondral bone osteoblasts (SBOs) and articular cartilage chondrocytes (ACCs) can occur both distally and in close proximity, direct and indirect co-culture systems were designed to study the cross-talk between osteoarthritic and normal SBOs and ACCs and the involvement of the MAPK-signalling pathway during these processes. Three different *in vivo* OA rat models were subsequently used to determine whether manipulation of the signals that take place between cartilage and the underlying bone during osteoarthritis using MAPK signalling pathway modulators would alleviate symptoms and slow down disease progression.

EXAMPLE 1

OSTEOARTHRITIC CARTILAGE CHONDROCYTES ALTER SUBCHONDRAL OSTEOBLAST DIFFERENTIATION VIA A MAPK-SIGNALLING PATHWAY INVOLVING ERK1/2

Materials and Methods

Reagents: *Cell culture:* Dulbecco's Modified Eagle's Medium (DMEM) and antibiotics (penicillinG and streptomycin) were purchased from GIBCO (Invitrogen, Mt Waverley, VIC, Australia); fetal bovine serum (FBS) was obtained from Thermo (In Vitro Technologies, Nobel Park, VIC, Australia); osteogenic supplements - dexamethasone, β-glycerophosphate and L-ascorbic acid were from Sigma (Castle Hill, NSW, Australia); chondrogenic supplements – TGF β3 were from R&D systems (Bio Scientific, Gymea, NSW, Australia), and proline and ITS+ from Sigma.

Inhibitors: MAP kinase pathway specific inhibitors for p38 (SB203580), for ERK1/2 (PD98059), and for JNK (SP600125) were purchased from Calbiochem (Novabiochem, Alexandria, NSW, Australia).

Assays: ALP assay kit was from Bioassay Systems (BioCore Pty Ltd, Alexandria, NSW, Astralia).

Antibodies: phospho p38, phospho ERK1/2 and phospho JNK antibodies were purchased from Cell Signalling Technology (Genesearch, Arundel, QLD, Australia).

Articular cartilage sample collection and phenotypic determination: OA ACCs (n=5) were sourced from the main defective area of the medial compartment

cartilage showing degenerative changes undergoing knee replacement surgery. The average age of the OA patients participating in this study was 65.20 ± 5.94 (range 60-75 years old). **Normal ACCs** (n=5) were obtained from trauma patients, where knee tissue was available. Normal patients were healthy adults aged between 41-53 years old, with no clinical signs or symptoms of joint, metabolic or hormonal diseases (osteoporosis), none of whom were taking medications which might affect cartilage or bone metabolism. In order to eliminate early osteoarthritis, patient samples showing any evidence of cartilage changes were excluded. Such changes included softening of the hyaline articular cartilage, thining and fibrous dislocation, ulcerations of the cartilage, and light sclerosis of the subchondral bone; normal cartilage was graded as 0. All radiographs were reviewed by experienced clinicians. The cartilage was classified according to the Mankin score of the cartilage, where score 0 indicated normal cartilage and score over 3 indicated advanced degenerative OA cartilage (van der Sluijs *et al.*, 1992; Brismar et al., 2002; Kellgren and Lawrence, 1957). Chondrocytes from the cartilage tissues were isolated following a method described previously (Patti *et al.*, 1999) and only first and second passage chondrocytes were used for subsequent experiments. The patients selected for this study had all ceased taking anti-inflammatory medication at least one month prior to surgery.

Subchondral bone sample and phenotypic determination: Bone specimens were taken within 5mm of the subchondral bone plate. **OA SBOs** (n=5) were sourced from the weight bearing sites from the patients suffering advanced OA, where the cartilage was degraded and showing prominent subchondral bone erosion and density. **Normal SBOs** (n=5) were collected from the patients mentioned above undergoing fracture repair surgery, with no evidence of subchondral bone erosion or cartilage degeneration according to the criteria (Altman *et al.*, 1986). SBOs were isolated according to the method described by Beresford (Beresford *et al.*, 1983 and 1984) The bone cell phenotype was confirmed by determining the production of early bone markers alkaline phosphatase (ALP) and osteocalcin (OC). All bone cell populations tested negative, by flow cytometry, for the hemopoietic cell marker CD45, and were also negative for oil red-O staining and fast red staining, indicating

the absence of adipogenic and fibroblastic lineage specific cells.

Co-culture models: There is an absence of any well established co-culture models available to study the dynamic interactions between ACCs and SBOs. Therefore, a co-culture system that allowed for the maintenance of the chondrocyte phenotype, as well as studying both direct and indirect cell interactions was designed.

Direct co-culture: $2x10^5$ cells of normal ACCs and OA ACCs were resuspended in a serum free chondrogenic media (high glucose DMEM supplemented with 10 ng/mL TGF-β3, 10nM dexamethasone, 50 mg/mL L-ascorbic acid, 10 mg/mL sodium pyruvate, 10 mg/mL proline, and ITS+ (final concentration: 6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 mg/mL selenious acid, 5.33 mg/mL linoleic acid, and 1.25 mg/mL bovine serum albumin)) and centrifuged at 6,000 × *g* for 20 min to form a pellet. The pellets were grown in the 2 mL of chondrogenic differentiation media for 2 weeks under 3D conditions before being placed directly on a monolayer of normal SBOs ($2x10^4$) in 24 well plates. The co-cultures were performed in DMEM medium containing osteogenic supplements (10nM dexamethasone, 10mM β-glycero-phosphate, 50μg/mL ascorbic acid). After 7 days of co-culture the cells were fixed in 4% paraformaldehyde for 10 min and stained with 1% alizarin red to assess the effect of ACCs on SBOs matrix deposition. Alizarin red staining intensity was measured with the Image J image processing software (http://rsb.info.nih.gov/ij/index.html). The direct co-culture model is shown in ***Figure 1A***.

Indirect co-culture: Two types of indirect co-cultures systems were used.

Method 1: A Cell Culture Insert system (BD Sciences, North Ryde, NSW, Australia) with a pore size 0.4 μm was used to separate ACCs and SBOs, thus ensuring that the cells only communicated via soluble factors. Normal SBOs (75,000 cells/well) were seeded in 24 well plates in complete DMEM medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) and allowed to settle for 24 hr to avoid accidental mixing of cells. High density micromass droplets were prepared as described previously (Stanton *et al.*, 2004). Briefly, trypsinized ACCs were resuspended in growth media at final cell density of 2.5×10^6 cells/ml and spotted as 10 μL/well droplets on cell culture inserts, incubated for 2 hr at 37°C, after which the inserts were placed above the normal SBOs monolayer and the co-cultures were

incubated in DMEM medium containing osteogenic supplements (10nM dexamethasone, 10mM β-glycero-phosphate, 50μg/mL ascorbic acid).

Method 2: Preparation of ACCs conditioned medium (CM): Normal or OA ACC pellets were prepared as described above and differentiated for 2 weeks in 2 mL of chondrogenic medium. During this period, media was replenished for every 3 days. After 2 weeks of differentiation, ACC pellets were incubated with 2 mL complete DMEM media (DMEM supplemented with 10%FCS and 1% penicillin/streptomycin) for 48 hr. Conditioned medium (CM) from normal or OA ACCs was collected and centrifuged at low speed of 1,000 × *g* for 15 min and the supernatant transferred to a fresh tube and stored at -80^{0}C to be used in subsequent experiments. In co-culture experiments normal SBOs were incubated with 300 μL of conditioned media mixed with an equal volume fresh media supplemented with osteogenic supplements at different time points. SBOs were replenished with fresh CM every 2 to 3 days. Control SBOs were incubated in complete medium (DMEM supplemented with 10%FCS and 1% penicillin/streptomycin) following the same process as CM at 37 ^{0}C and frozen. The SBOs were harvested for total cell lysate, RNA and protein on days 3, 7, and 14. Real time quantitative PCR (RT-qPCR) and western blotting was performed to determine the relative changes of SBOs cultured with CM from normal or OA ACCs *vs.* SBOs cultured in control media. Extracellular matrix deposition was determined by fixing the cells in 4% paraformaldehyde and staining with a 1% alizarin red solution (***Figure 1B***). All experiments were performed in triplicates for each of the matched cell populations.

Detection of MAP kinase activation: Western blot analysis was performed to determine the degree of MAPK signal activation by the expression of phospho-p38, phospho-ERK1/2 and phospho-JNK in SBOs cultured with CM from normal or OA ACC, as well as SBOs cultured in control media, on days 7 and 14. The MAP kinase mediated cellular interactions were also evaluated in the indirect co-cultures by the use of MAPK specific inhibitors (p38: SB203580; ERK1/2: PD98059 and U0126; JNK: SP600175). Briefly, SBOs were incubated with or without the MAPK inhibitors in conditioned media prepared as described above after dissolving the concentrated stock solutions of each inhibitor in DMSO. The final concentration of

DMSO never exceeded 0.1% (v/v) and the same amount of DMSO vehicle was added to the control medium. The medium was replenished every 2 to 3 days. Pilot experiments showed that the optimum concentration to be 10 μM for ERK1/2 inhibition; 5 μM for p38 inhibition, and 10 μM for JNK inhibition. At these concentrations there was no observable change in the proliferation rates between control cells and inhibitor treated cells, nor was there any evidence of cytotoxicity, as assessed by LDH (lactose dehydrogenase) assays. All experiments were performed in triplicate.

Cell proliferation assay: The rate of cell proliferation was determined by DNA content using a CyQuant Cell Proliferation Assay kit (Molecular Probes, Invitrogen) following the manufacturer's instructions. Total cellular DNA was estimated by fluorometry at 490/520 nm and comparison with a DNA standard curve, expressed in nanograms of DNA/culture well.

ALP activity measurements: Intracellular ALP activity was determined with a Quantichrom^{tm} Alkaline Phosphatase Assay Kit, a *p*-nitrophenyl phosphate (pNP-PO_4) based assay. SBOs cells, after 7 days in conditioned media from normal or OA ACCs, were rinsed twice with PBS, and lysed in 0.5 mL 0.2% Triton X-100 in MilliQ water, followed by 20 min agitation at room temperature. Fifty microliter of sample was mixed with 100 μL working solution and absorbance measured after 5 min at 405 nm in a microplate reader.

RNA Extraction and real time quantitative -PCR (RT-qPCR): 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 1 μg 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 template 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 the *18s* and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) housekeeping genes by comparative cycle of threshold (Ct) value method (ABI user bulletin # 2). The difference between the mean C_t values of the gene of interest and the housekeeping gene was labelled ΔC_t, and the difference between ΔC_t and the C_t value of the calibrator sample was labelled $\Delta\Delta C_t$. The $\log_2$ ($\Delta\Delta C_t$) gave the relative value of gene expression.

Western blot: Total protein was harvested by lysing the cells in a 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, Dee Why, NSW, Australia). The cell lysate was clarified by centrifugation and the protein concentration determined by a bicinchoninic acid protein assay (Sigma-Aldrich). 10 μg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel. The protein was transferred to a nitrocellulose membrane, and blocked in a Tris-Tween buffer containing 5% non-fat milk. The membranes were incubated with primary antibodies against phospho-p38 (1:1,000), phospho-ERK1/2 (1:2,000) and phospho-JNK (1: 1,000) overnight at 4°C. The membranes were washed three times in TBS-Tween buffer, and then incubated with an anti-rabbit secondary antibody at 1:2,000 dilutions for 1 hr. The protein bands were visualized using the ECL Plus™ Western Blotting Detection Reagents (GE Healthcare, Rydalmere, NSW, Australia) and exposed on X-ray film (Fujifilm, Stafford, QLD, Australia). Immunoblot negatives were analyzed by densitometry using Image J software.

Statistical analysis: Results are presented as a mean ± SEM of an average of five samples of three distinct experiments and are representative of the experiments. Repeated measures ANOVA with post hoc tests were used to ascertain statistical significance; $p \leq 0.05$ was considered to be a significant difference.

Results

Expression of osteogenic genes in SBOs: The mRNA expression of osteogenic marker genes was compared between normal and OA SBOs after 14 days in differentiation media. The expression of *CBFA1* ($p \leq 0.05$), *ALP* ($p \leq 0.05$) and *OC* ($p \leq 0.05$) was significantly upregulated in OA SBOs compared to normal SBOs (***Figure 2***). *OPN* expression ($p \geq 0.05$) was upregulated in OA SBOs, but failed to reach the significance threshold. These results indicated that OA SBOs had greater osteogenic potential than normal SBOs.

Cell proliferation: The cell proliferation of SBOs was assessed in the co-cultured vs. non co-cultured SBOs and no significant differences were observed in either direct or indirect co-culture systems at any of the time points (3, 7, and 14 days).

OA ACCs enhanced SBOs differentiation in both direct and indirect cell co-cultures: Chondrogenic characterization of normal and OA ACCs was evaluated by the expression of chondrogenic markers of type II collagen (COL2) and aggrecan (AGG) in the pellet cultures after two weeks. It was observed that COL2 and AGG were expressed in all three cell lines collected from both normal and OA patients (***Figure 3A***). The expression of *CBFA1*, *COL10* and *ALP* was all significantly upregulated in OA ACCs compared to normal ACCs, whereas the expression of *COL2* and *AGG* was significantly down regulated in OA ACCs compared to normal ACCs in quatitative PCR studies (results not shown). After 7 days of direct co-culture in osteogenic growth medium, the ability of normal SBOs to undergo differentiation, in the presence or absence of normal or OA ACCs, was investigated. The matrix deposition was assessed and revealed that SBOs co-cultured with OA ACCs enhanced SBOs matrix deposition, as was evidenced by a more intense alizarin red stain compared to SBOs cultured alone. By contrast, matrix deposition was significantly reduced in SBOs when co-cultured with normal ACCs, compared with SBOs co-cultured with OA ACCs (***Figure 3B&C***). Controls grown in the absence of osteogenic supplements did not stain for alizarin red (data not shown). Similarly, in the indirect co-culture system, SBOs differentiated in the presence of CM from normal ACCs had significantly delayed bone nodule formation, and conversely, co-culturing SBOs in the presence of CM from OA ACCs resulted in

increased SBOs matrix mineralization (***Figure 3B&D)***. Similar results were also obtained from the co-cultures performed using the cell culture inserts (data not shown).

Co-culture of SBOs with OA ACCs CM enhanced osteoblast specific gene expression: Next it was assessed whether the staining pattern was reflected at the cellular level. In order to avoid possible mixing of cells, all further experiments were only carried out with SBOs cultured in conditioned media from normal or OA ACCs. As shown in the ***Figure 4A***, at day 7, there was a significant increase in the ALP activity in SBOs cultured in OA ACCs CM, whereas the ALP activity of SBOs exposed to normal ACCs CM was reduced, compared to SBOs cultured in control media or CM from OA ACCs. RT-qPCR revealed that expression of the osteoblast transcription factor *CBFA1* was significantly upregulated in the SBOs cultured in CM from OA ACCs from day 3 until day 14. The expression level of *CBFA1* was significantly lower, across all time points, in SBOs exposed to CM from normal ACCs CM compared to those exposed to CM from OA ACCs (***Figure 4B)***. *ALP* expression was determined at each time point, given it is an early marker of osteoblast differentiation. *ALP* expression was upregulated almost immediately, in SBOs cultured in control media and the SBOs that were cultured in the presence of CM in a time dependent fashion. However, the degree of expression of *ALP* was much greater when SBOs were cultured in CM from OA ACCs ***(Figure 4C)***. Expression level of *OPN* was significantly upregulated in SBOs exposed to CM from OA ACCs, compared to the SBOs exposed to CM from normal ACCs, in a time dependent manner ***(Figure 4D)***.

Osteocalcin (OC) is a late marker of osteoblast differentiation and its expression was assessed at all the time points. At days 3 and 7 there was no significant differences in *OC* expression observed in any of the groups. However, at day 14 the transcription levels of *OC* were several fold increased in both SBOs cultured in control media, as well as those cultured in CM from normal and OA ACC, where the culture group containing CM from OA ACCs showed the strongest response ***(Figure 4E).*** Together these results suggest that normal ACCs secrete factors that are responsible for the

delay of SBOs differentiation; whereas OA ACCs appear to lose the ability to initiate these factors, and instead lead to increased SBOs differentiation.

MAP kinase signalling pattern in the SBOs cultured with normal or OA ACCs CM: The temporal characteristics of MAP kinase activation was investigated by Western blot analysis to determine if signalling in SBOs was altered in response to culturing in CM from normal or OA ACCs on days 7 and 14. When SBOs were cultured with CM from OA ACCs, phospho-ERK1/2 increased significantly ($p \leq 0.05$) and in a time dependent fashion, compared to cultures performed with CM from normal ACCs or control media. Phosphorylation of p38, on the other hand, was not altered significantly, whereas JNK phosphorylation was significantly upregulated in SBOs cultured in CM from either normal or OA ACCs. There was no difference in phospho-JNK expression between cells grown in CM from normal and OA ACCs ($p \geq 0.05$) culture groups ***(Figure 5A-D)***.

These results suggested that the OA ACCs significantly increased the ERK1/2 phosphorylation in normal SBOs upon co-culture. To confirm that this phenomenon was clinically relevant, the basal levels of phosphorylated ERK1/2 were measured by immunoblot method mentioned above in fresh isolated normal and OA SBOs after 7 days in DMEM culture media. The phenotype of normal and OA SBOs was characterized subsequently (***Figure 2***). The results revealed significantly higher levels of phosphorylated ERK1/2 in OA SBOs compared to that in normal SBOs (***Figure 6***).

PD98059 reversed OA ACCs induced SBOs differentiation: It became clear that ERK1/2 phosphorylation increased in SBOs cultured with CM from OA ACCs, compared to cultures grown in CM from normal ACCs or control media. The ERK1/2 specific inhibitor PD98059 was used to determine if this pathway was involved in the increased SBOs differentiation induced by CM from OA ACCs. It was determined that SB203580 (p38 inhibitor), PD98059 (ERK1/2 inhibitor), and SP600125 (JNK inhibitor) decreased significantly the phosphorylation of p38, ERK1/2, and JNK, respectively (***Figure 7A***). PD98059, at a final concentration of 10 μM, robustly reduced mineralization in SBOs caused by CM from OA ACCs by up to 80%, but only 10-20% decrease in the control of SBOs without CM ***(Figure 7B)***.

The reduction of ERK1/2 phosphorylation strongly inhibited the effects of OA ACCs CM induced SBOs phenotype changes ***(Figure 7B&C)***, evident by a decrease of matrix deposition and downregulation of osteogenic gene expression, such as *ALP* ($p \leq 0.05$), *OC* ($p \leq 0.05$) and *CBFA1* ($p \leq 0.05$) compared with SBOs culture alone and the application of SB203580, and SP600125 in the CM of OA ACCs. This is an indication that ERK1/2 activation by OA ACCs CM may be responsible for the abnormal SBOs phenotype. Similarly, inhibition of the p38 and JNK pathways, with the inhibitors SB203580 and SP600125 respectively, inhibited their respective phosphorylation by up to 90% ***(Figure 7A)***. It is interesting however; that inhibition of p38 and JNK did not seem to affect OA ACCs CM induced SBOs differentiation ***(Figure 7B-C)***. These results therefore provide evidence that the interaction between OA ACCs and SBOs, leading to increased differentiation of the latter, may be mediated through the activation of the ERK1/2 pathway.

Effect of U0126, an ERK1/2 inhibitor in the SBOs co-cultured with normal and OA ACCs.

pERK1/2 activation was investigated by Western blot analysis to determine if signalling in SBOs was altered in response to co-culturing with normal or OA ACCs. When SBOs were cultured with OA ACCs, phospho-ERK1/2, OC and ALP increased significantly compared to cultures performed with normal ACCs or SBOs alone (***Figure 8A&B***). These results suggested that the OA ACCs significantly increased the ERK1/2 signal phosphorylation and SBOs differentiation. The ERK1/2 specific inhibitor U0126 was used to determine if this pathway was directly involved in the increased SBOs differentiation induced by OA ACCs. It was detected that U0126 decreased significantly the phosphorylation of ERK1/2 at 15uM concentration (***Figure 8C***). Also, U0126 at a final concentration of 15μM, robustly reduced ALP and OCN gene expression (***Figure 8D***). This is an indication that ERK1/2 activation by OA ACCs may be responsible for the abnormal SBOs phenotype.

EXAMPLE 2

MITOGEN ACTIVATED PROTEIN KINASE ERK1/2 AND P38 IN THE REGULATION OF HYPERTROPHIC CHANGES OF NORMAL ARTICULAR CARTILAGE CHONDROCYTES INDUCED BY

OSTEOARTHRITIC SUBCHONDRAL OSTEOBLASTS

Materials & Methods

Articular cartilage sample collection and phenotypic determination: 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 based on saffranin-O and H&E histology staining (Mankin *et al.*, 1971). Chondrocytes from the cartilage tissues were isolated following a method described previously (Patti *et al.*, 1999). 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 (Altman *et al.*, 1986). 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 et al. (1983, 1984).

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. $2x10^5$ 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 6,000 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 (Stanton *et al.*, 2004). Briefly, following trypsinization the ACCs 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 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 (Muraglia *et al.*, 2003).

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 1 week. 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. 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), PD98059, (Novabiochem p38 and ERK1/2 pathway inhibition and U0126 (Promega). 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 (Invitrogen), 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: Results were presented as a mean ± SD and are representative of at least three distinct experiments. Repeated measures ANOVA with post hoc tests were used to assess statistical significance, where $p \leq 0.05$ was considered significant.

Results

Expression of chondrogenic and hypertrophic genes in normal and OA ACCs: The mRNA expression of chondrogenic and hypertrophic marker genes was compared between normal and OA ACCs. The expression of *CBFA1* ($p \leq 0.05$), *COL10* ($p \leq 0.05$) and *ALP* ($p \leq 0.05$) was all significantly upregulated in OA ACCs compared to normal ACCs, whereas the expression of *COL2* and *AGG* expression ($p \leq 0.05$) was significantly down regulated in OA ACCs compared to normal ACCs (***Fig 10A***). 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 10B***). On the other hand ERK1/2 phosphorylation was significantly up regulated in OA ACCs compared with normal ACCs (***Fig 10C***).

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

Direct co-culture: 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 11A, 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 11A, 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 11A, 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 11B***). In contrast, co-culture of ACC pellets with OA SBOs lead to increased matrix mineralization which was evident by a more intense alizarin red stain compared to co-culture groups containing normal SBOs and non co-cultured ACCs. 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 11C***).

Indirect co-culture: When ACC micromasses were cultured with normal or OA SBO CM, alcian blue staining reveled 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 12A, 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 12A, 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 12B***). By contrast, the expression of cartilage hypertrophy markers *CBFA1, COL10* and *ALP*, were significantly upregulated in the presence of OA SBOs CM (***Fig 12C***) compared to both normal SBOs CM and control groups.

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

The effect of normal and OA SBOs on the ACCs was assessed with respect to alteration of MAPK-signalling 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 lead 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 13A-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 increasing the expression of phospho-p38 in a concentration dependent manner (***Fig 14A***). 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. These findings indicated that the inhibition of ERK1/2 phosphorylation lead to a positive feed back of p38 phosphorylation in the ACCs stimulated by OA SBOs. RT-qPCR showed that ERK1/2 inhibition by PD98059 reversed the expression of hypertrophic gene expression of *CBFA1, COL10* and *ALP* in ACCs micromasses in the present of OA SBOs CM (***Fig 14B***). By contrast, the expression of *COL2* and *AGG* was upregulated by ERK1/2 inhibition (***Fig 14C***).

Inhibitor of p38 augmented ERK1/2 phosphorylation leading to decreased chondrogenesis and increased hypertrophic changes of ACCs

Inhibition of the p38 phosphorylation by SB203580 activated ERK1/2 phosphorylation in ACCs cultured in normal SBO CM, in a concentration dependent manner, evidence that a reduction in p38 activity is accompanied by a co-contaminant increase of ERK1/2 phosphorylation (***Fig 15A***). 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 present of normal SBOs CM (***Fig 15C&D***). These results indicate there was a significant shift of ACCs towards a more hypertrophic phenotype in the absence of phosphorylated p38 signalling. Similar results were also obtained in normal ACCs culture alone (data not shown).

Effect of U0126, an ERK1/2 inhibitor in the ACCs co-cultured with normal and OA SBOs.

The effect of normal and OA SBOs on the ACCs was assessed with respect to alteration of ERK1/2 signalling cascade in co-cultures. The results showed that the signal phosphorylation of ERK1/2 and hypertrophic gene expression of CBFA1 and COL10 was significantly augmented when ACCs were co-cultured with OA SBOs compared to ACCs alone and ACCs co-cultured with normal SBOs (***Fig 16A&B***). These results suggest that upregulation of ERK1/2 was involved in the pathological interaction of OA SBOs with ACCs. Incubation with ERK1/2 inhibitor, U0126 in ACCs cultured with OA SBOs had the effect of decreasing the expression of phospho-ERK1/2 at 15 uM concentration (***Fig 16C***). qRT-PCR showed that ERK1/2 pathway inhibition by U0126 reversed the expression of hypertrophic gene such as *CBFAF1 and COL10* in ACCs induced by OA SBOs (***Fig 16D***).

EXAMPLE 3

IN VIVO STUDY OF THE ABILITY OF MAPK-ERK1/2 INHIBITORS TO ALLEVIATE SYMPTOMS OF OSTEOARTHRITIS (OA) AND REDUCE DISEASE PROGRESSION USING RAT OA MODELS

Materials and Methods

Animals. Wistar rats (11-12 weeks old) weighing about 300-350 grams were used for this experiment.

Rat OA models: Animal models have been extensively used to study the pathogenesis of OA and to evaluate potential anti-arthritic drugs for clinical use. In general, these models fall into 3 categories, spontaneous models, surgically induced models and chemically induced models (Bendele, 2002). Spontaneous OA occurs in the knee joints of various strains of mice and guinea pigs with an increased incidence and severity as they age. However, the disadvantage of this model is that they take a long time (> 2 years) to develop OA related changes (Bendele, 2002). Therefore, for it was determined to develop OA in rats using a surgically induced method and a chemically induced method, which develop OA changes in less than 4 weeks. Surgery induced OA can be developed via two methods: By removing the medial

compartment meniscus disk (MSX) or by transecting anterior cruciate ligament (ACLT) or chemically induced by giving single intra-articular injection of Mono-ido-acetate (MIA). Briefly, for MSX models, after giving anaesthesia (combination of zoletil + xylazil, intra-peritoneal) the medial collateral ligament is transacted just below its attachment to the meniscus, so that when the joint space opens, the meniscus is reflected toward the femur. The meniscus was then cut at its narrowest point without damaging the tibial surface resulting in complete medial meniscus transaction. The surgical wound was then closed by suturing in two layers. A sham group on the left knee was subjected to the same surgical procedure, without the excision of the ligament or any meniscus manipulation. For ACLT model, after giving anaesthesia to the rats, right knee joint was exposed through a medial parapatellar approach. The patella was dislocated laterally and the knee placed in full flexion followed by ACL transection with micro-scissors. The joint capsule and subcutaneous layer were sutured separately and the skin was closed by vicryl 3.0. Sham animals underwent the same surgical procedure with the omission of ACL transaction. After the surgery, both MSX and ACLT rats OA models received pain killer (Buprenorphine 0.05 mg/kg) and antibiotic (Gentamycin (5 mg/kg). For MIA model, rats were anaesthetized and MIA was injected 1 mg in 50 microliter volume in 0.9% saline) into the right joint cavity through the patellar ligament. The control animals were injected with 0.9% saline only (sham control).

Reagents: ERK inhibitor U0126 was purchased from Promega.

Experimental design: The rats were divided randomly into the following groups. Each group comprised of 6 animals (3 males + 3 females) and two time points (4 weeks and 8 weeks).

Group	Treatment (right knee)	Group	Control (left knee)
1	MSX + Saline	1	MSX sham + Saline
2	MSX + ERK inhibitor (U0126)	2	MSX sham + ERK inhibitor (U0126)
3	ACLT + Saline	3	ACLT sham + Saline
4	ACLT + ERK inhibitor (U0126)	4	ACLT sham+ ERK inhibitor (U0126)
5	MIA + Saline	5	MIA sham + Saline

6	MIA + ERK inhibitor (U0126)	6	MIA sham+ ERK inhibitor (U0126)

Inhibitor treatment: From day 7 post-surgery all animals as indicated in the above table were given an intra articular injection of ERK1/2 inhibitor (U0126) every 3 days at a concentration of 0.5 mM in 50 µl using 30G needle. The control group received same volume of vehicle alone without administration inhibitor. At the end of each time point, rats were euthanized.

Assessment of the severity of OA cartilage in U0126 treated vs untreated animals: Whole knee joints were removed by dissection, fixed in 4% paraformaldehyde, and decalcified in 10% EDTA. After dehydration and paraffin embedding, serial 5 µm sagittal sections from the lateral and medial compartment of the joint were cut. Two sections obtained at 100 µm intervals from the non weight-bearing region and weight-bearing region of each knee joint were stained with Safranin o–fast green. OA severity in the tibial plateau was evaluated according to Mankin's histologic grading system (Mankin's score: 0 to 14) (Mankin *et al.*, 1971; Mankin & Buckwalter, 2002), and a cartilage destruction score was assigned for each knee sample (n=6).

Assessment of the severity of OA subchondral bone changes in U0126 treated vs untreated animals: Morphological changes of subchondral bone in inhibitor treated vs untreated OA animal models were determined using Micro-CT. Femurs and tibia were scanned with micro CT (Scanco 40, Switzerland) with isotropic voxel size of 18 µm. The x-ray tube voltage was 55 kV and the current was 145 µA, with a 0.5 mm aluminium filter. The exposure time was 1180 ms. The data set was segmented with an inbuilt software. In both the medial and the lateral part of each femoral, a circler (area 0.004 cm^2) was selected. Similarly, in the tibial scan, circles were selected with area of 0.004 cm^2. The circles were located in the middle of the load-bearing subchondral bone areas. Selected areas contained subchondral plate, but did not contain subchondral trabecular bone or growth-plate tissue. A total of 25 consecutive tomographic slices were analysed. The meaningful measurements such as bone volume (mg HA/ccm) and BV/TV (%) were analysed in the subchondral bone plate of U0126 treated OA animals and untreated OA models.

Western blotting: Total protein lysates were harvested by lysing the bone and cartilage tissue samples 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). 5 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 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).

Cell Culture: Articular chondrocytes were isolated from the knee joints of rats by cutting the cartilage tissue into small pieces and digested with 0.2% Type II collagenase for 3 h. The released cells were cultured in high density micromasses as described previously in high glucose medium supplemented with 10% FBS, penicillin and streptomycin as described previously. After 2 days the media was changed to chondrogenic media (serum-free medium-high glucose DMEM supplemented with 10 ng/mL transforming growth factor-β3, 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). After differentiation for 1 week in chondrogenic media total RNA was isolated from micromasses and subjected to qPCR analysis. Osteoblasts were isolated by mincing the bone into small pieces with sterile bone cutter, and then washed several times with 1X PBS and placed in T25 flasks with a sterile forceps and air dried for 10 min in a laminar flow hood. High glucose DMEM supplemented with 10% FBS and 50u/ml penicillin and 50µg/ml streptomycin was added to the bone pieces and incubated in a standard humidified incubator at 37°C containing 5% CO_2 / 95% atmospheric air. Cells started to emerge from bone pieces approximately

after 1 week. After confluence the cells were passaged and cultured under osteogenic conditions (10nM dexamethasone, 10mM β-glycero-phosphate, 50μg/mL ascorbic acid) for a week and RNA was isolated for further analysis.

RNA Extraction and Quantitative RT-PCR (qRT-PCR): Total RNA was isolated with TRIZOL reagent from articular cartilage chondrocytes (ACCs) and subchondral bone osteoblasts (SBOs) to test the gene expression differences in sham, OA and OA + U0126 groups. Complementary DNA was synthesized using Superscript III from 1μg total RNA. PCR primers were designed based on cDNA sequences from the NCBI Sequence database using the Primer Express® software and the primer specificity was confirmed by BLASTN searches. RT-qPCR was performed on an ABI Prism 7300 Thermal Cycler with SYBR Green detection reagent for Type 2 collagen (Col2a1: forward: 5′ TCCTAAGGGTGCCAATGGTGA 3′, reverse: 5′ AGGACCAACTTTGCCTTGAGGAC 3’). The relative expression of the genes of interest was normalized against housekeeping beta-actin (forward: 5′ GGAGATTACTGCCCTGGCTCCTA 3′, reverse: 5′ GACTCATCGTACTCCTGCTTGCTG 3′).

Behavioural studies: Some of the most common signs indicating the health status of a rat include observation of behaviour, assessment of physical appearance, and measurement of body weight. Behavioural parameters include observation of unprovoked behaviour and responses to external stimuli. Classic changes in physical appearance include exophthalmia or enophthalmia (bulging or sunken eyes, respectively), nasal or ocular discharge, rough coat, and hunched back. These clinical indicators can be scored as degree-of-deviation-from-normal, thereby allowing an animal to be monitored over time to asses any side effects that U0126 may have. However, none of the animals showed above symptomatic signs during the course of experimental period.

Statistical analysis: The data from animal work was analysed by repeated measures ANOVA and post-hoc testing, with significance at $p \leq 0.05$, using SPSS statistical software.

DISCUSSION

In this study, it was observed initially that SBOs isolated from OA patients

produced significantly greater levels of the *CBFA1, ALP*, and *OC* mRNA when compared to SBOs isolated from the healthy patients. This indicates that the OA SBOs possess greater osteogenic potential than normal SBOs. However, the question as to how these changes occur at the cellular level, and therefore the nature of molecular mechanisms involved, has remained unanswered.

Using both direct and indirect co-culture models, it was demonstrated that ACCs, isolated from a healthy joint, decreased the differentiation potential of SBOs. This inhibitory regulation of SBOs differentiation by the normal ACCs may therefore be one of the factors responsible for regulating subchondral bone remodelling, thereby maintaining the normal joint homeostasis. It was shown that when OA ACCs were co-cultured with normal SBOs, there was a demonstrable increase in SBOs differentiation, characterized by a significant upregulation of osteogenic markers such as *CBFA1*, *ALP*, *OPN*, *OC*, as well as bone matrix deposition. These findings are of clinical significance, since they show that dysregulation of SBOs, by OA ACCs, results in increased bone metabolism, leading to changes to subchondral bone architecture and subsequent bone sclerosis, a characteristic feature of OA.

In mammals, once growth formation is complete and has ceased, tissues, such as articular cartilage tend, under normal conditions, not to undergo hypertrophic differentiation. However, cartilage hypertrophy has been reported to be reinitiated in the course of pathological conditions. Thus, the observations presented here, that OA, but not normal ACCs, increased SBOs differentiation is therefore most probably a result of the phenotypic changes in the OA cartilage. Indeed, the ACCs from the OA patients the inventors used showed strong mRNA expression for hypertrophic specific genes such as *COL10*, *ALP*, *CBFA1* and *MMP-13*.

The mechanism by which bi-directional interaction between bone and cartilage cells are regulated in SBOs, when co-cultured with normal ACCs or OA ACCs, was also investigated in this study, by elucidating the signalling pathways that are modulated by cellular interactions. Here it has been demonstrated that co-culturing SBOs with OA ACCs, leads to a significant activation of the ERK1/2 signalling pathway, whereas normal ACCs failed to activate the same pathway, which indicates that OA induced SBOs phenotype is transduced via the ERK1/2

dependent pathway. The direct involvement of this pathway was confirmed by co-culturing SBOs with OA ACCs in the presence of ERK1/2 pathway specific inhibitors. The ERK1/2 inhibition significantly reduced the OA ACCs induced SBOs differentiation. Importantly, the inventors demonstrated that the basal levels of ERK1/2 phosphorylation increased in OA SBOs compared to normal SBOs, an indication of the clinical relevance of this pathway in OA pathogenesis. As one of the major components of the MAPK family, ERK is associated with osteoblast differentiation and it was therefore a significant result that the expression of *CBFA1*, a key transcriptional activator of osteoblast differentiation, was significantly suppressed when cells were treated with the ERK1/2 inhibitors PD98059 and U0126. In another study, the importance of MAPK-signalling pathways was demonstrated as the means by which OA SBOs induce altered ACCs phenotypic changes, and provided some insight into the cross-talk that take place between the p38 and ERK1/2 signalling pathways during this pathological interaction process.

ACCs isolated from OA patients were found to produce 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.

Using both direct and indirect co-culture methods, the inventors 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 symptom of OA is an upregulation of hypertrophy and mineralization related markers and a complete breakdown of the native chondrocyte phenotype (Pullig *et al.*, 2000 (1 & 2)). These observations suggest that the interaction of OA SBOs lead to these typical clinical symptoms of OA changes in ACCs. The transition of ACCs to hypertrophic changes may contribute to damage to the extracellular matrix by triggering localized inflammation which precedes cartilage degeneration, indicating that the phenotypic conversion of ACCs to hypertrophy is pathological for the health and integrity of articular cartilage leading to its degeneration.

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, it has been demonstrated that the basal levels of ERK1/2 phosphorylation increased and p38 decreased in OA ACCs compared to normal ACCs, an indication of the clinical relevance of this 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. In the absence of ERK1/2, ACCs hypertrophy was reversed and there was a return to the chondrogenic phenotype of ACCs. This 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 is further supported by results showing that when p38 is neutralized by an inhibitor in ACCs co-cultured with normal SBOs, a weakening of chondrogenic phenotype and increase of hypertrophic differentiation is observed, thus negating the effects of normal SBOs had on ACCs. Together this data indicates that OA SBOs decrease p38 phosphorylation, resulting in an upregulation of ERK1/2 activity, with a resulting reduced chondrogenic phenotype and increased hypertrophic differentiation.

The inventors showed that PD98059 and U0126 (anti ERK1/2) significantly reduced the expression of the transcription factor *CBFA1*, whereas SB203580 (anti p38) activated this transcription factor. This suggests that the two pathways both regulate *CBFA1*, one by turning it on (ERK1/2) and the other by turning it off (p38), thus effecting the changes induced by SBOs on the ACCs.

The data presented in Examples 1 and 2 showed that normal ACCs inhibited SBO differentiation, whereas OA ACCs enhanced SBO differentiation. The inventors have also discovered a novel feedback/feedforward mechanism between the ERK1/2 and p38 pathways in OA SBOs and ACCs interactions, which appears to play a role in the hypertrophic changes of normal chondrocytes. These data provide an insight into MAPK-signalling pathways involved in the molecular mechanisms of osteoarthritis pathogenesis, which have significant clinical implications. Specific targeting of these molecular events by ERK1/2 inhibitors, p38 activators as well as with RNA interference will help control both ACC and SBO phenotypic changes

both during and perhaps prior to the disease progression of osteoarticular diseases, particularly osteoarthritis, and provide a rationale for novel treatment strategies.

The inventors subsequently used an *in vivo* rat OA model to investigate whether MAPK-ERK 1/2 modulators may be used to manipulate the signals that take place between cartilage and the underlying bone and slow down disease progression. As described in detail in Example 3, and Figures 17-23, the inventors found that specific targeting of the MAPK-signalling pathway using an ERK 1/2 inhibitor significantly reduced the progression of OA and the symptoms associated therewith. For example, ***Fig. 17*** shows that the ERK 1/2 inhibitor U0126 confers protection against OA cartilage degradation, while ***Fig. 18*** illustrates that U0126 is capable of decreasing the size and incidence of abnormally large osteophytes, a characteristic feature of OA knee joints. The inventors also discovered that treatment with U0126 resulted in reduced proteoglycan depletion compared to untreated animals, further emphasizing the benefits of treating subjects suffering from OA with ERK1/2 inhibitors (***Fig. 19***). Interestingly, as shown in ***Fig. 20***, OA knees treated with U0126 showed less subchondral bone surface damage compared to untreated OA control knees indicating that U0126 is capable of at least partially reducing surface damage to the subchondral bone. The inventors also found that treatment with the ERK 1/2 inhibitor led to reduced expression of cartilage degradation markers including ADAMTS5, VEGF and COL10 when compared to sham and untreated OA models (***Fig. 21***). As shown in ***Fig. 22***, U0126 not only reduced cartilage degradation but also promoted cartilage anabolic activity which is advantageous for an OA sufferer as a net loss of proteoglycan content (extracellular cartilage matrix) is one of the common features of all stages of osteoarthritic cartilage degeneration. Treatment with U0126 also resulted in reduced levels of Alkaline Phosphatase (ALP), a marker of osteogenesis known to induce abnormal bone remodelling in OA patients, compared to untreated OA controls (***Fig. 23***).

In conclusion, the inventors have shown that treatment of a subject suffering from OA with an ERK1/2 modulator (*e.g.* U0126) alleviates a number of undesirable OA symptoms thereby improving the wellbeing and prognosis of patients suffering from osteoarthritis. These novel treatment methods and compositions, which target

the MAPK-ERK1/2 signalling pathway, advantageously provide relief without the need for surgical intervention. The enhanced treatment options described herein are also likely to reduce an individual's dependence on strong anti-inflammatory medication, which is associated with inconvenient side-effects (*e.g.* stomach ulcers and liver damage) and, at most, only provides temporary relief.

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CLAIMS

1. A method of prophylactic and/or therapeutic treatment of an osteoarticular disease in a mammal, said method including the step of modulating a MAPK-signalling pathway of an osteoblast and/or a chondrocyte in said mammal, whereby (i) modulation of the MAPK-signalling pathway, associated with an interaction between osteoarthritic osteoblasts and normal chondrocytes, prevents or inhibits differentiation of a normal chondrocyte to an osteoarthritic phenotype; and/or (ii) modulation of the MAPK-signalling pathway, associated with an interaction between osteoarthritic chondrocytes with normal osteoblasts, prevents or inhibits differentiation of a normal osteoblast to an osteoarthritic phenotype, to thereby treat or prevent said osteoarticular disease.

2. The method of Claim 1, wherein modulation of the MAPK-signalling pathway of the osteoarthritic osteoblast and/or the normal chondrocyte prevents or inhibits differentiation of the normal chondrocyte to the osteoarthritic phenotype.

3. The method of Claim 2, wherein the MAPK-signalling pathway of the osteoarthritic osteoblast is modulated.

4. The method of Claim 2, wherein the MAPK-signalling pathway of the normal chondrocyte is modulated.

5. The method of Claim 1, wherein modulation of the MAPK-signalling pathway of the osteoarthritic chondrocyte and/or the normal osteoblast prevents or inhibits differentiation of the normal osteoblast to the osteoarthritic phenotype.

6. The method of Claim 5, wherein the MAPK-signalling pathway of the osteoarthritic chondrocyte is modulated.

7. The method of Claim 5, wherein the MAPK-signalling pathway of the normal osteoblast is modulated.

8. The method of any one of Claims 1-7, wherein said osteoarticular disease is selected from the group consisting of inflammatory rheumatism, metabolic

arthropathy, degenerative rheumatism, rheumatoid arthritis, osteoarthritis, spondylarthritis, gout, chondrocalcinosis and arthrosis.

9. The method of Claim 8, wherein said osteoarticular disease is osteoarthritis.

10. The method of any one of Claims 1-9, wherein said chondrocytes are, or comprise, articular cartilage chondrocytes (ACCs).

11. The method of any one of Claims 1-9, wherein said osteoblasts are, or comprise, subchondral bone osteoblasts (SBOs).

12. The method of any one of Claims 1-11, wherein said MAPK-signalling pathway modulation reduces matrix mineralization.

13. The method of any one of Claims 1-12, wherein said MAPK-signalling pathway modulation reduces bone sclerosis.

14. The method of any one of Claims 1-13, wherein said MAPK-signalling pathway modulation modulates the expression and/or activity of one or more molecules selected from the group consisting of ERK1/2, p38, and CFBA1.

15. The method of any one of Claims 1-14, wherein said MAPK-signalling pathway modulation reduces the expression and/or activity of ERK1/2 and/or CFBA1.

16. The method of any one of Claims 1-15, wherein said MAPK-signalling pathway modulation increases the expression and/or activity of p38.

17. The method of any one of Claims 1-16, wherein said MAPK-signalling pathway is modulated by an ERK1/2 inhibitor.

18. The method of any one of Claims 1-17, wherein said MAPK-signalling pathway is modulated by a CFBA1 inhibitor.

19. The method of any one of Claims 1-18, wherein said MAPK-signalling pathway is modulated by a p38 activator.

20. The method of any one of Claims 1-19, wherein said mammal is a human.

21. A method of screening for, designing, engineering or otherwise producing a MAPK-signalling pathway modulator for use in treatment of an osteoarticular disease, said method including the steps of determining whether a MAPK-signalling pathway modulator can modulate a MAPK-signalling pathway in

one or more cells and/or tissues that are associated with an osteoarticular disease.

22. The method of Claim 21, wherein said osteoarticular disease is osteoarthritis.

23. The method of Claim 21 or Claim 22, wherein said MAPK-signalling pathway modulator is an ERK1/2 inhibitor.

24. The method of Claim 21 or Claim 22, wherein said MAPK-signalling pathway modulator is a CFBA1 inhibitor.

25. The method of Claim 21 or Claim 22, wherein said MAPK-signalling pathway modulator is a p38 activator.

26. The method of any one of Claims 21-25, wherein said mammal is a human.

27. A pharmaceutical composition comprising one or more MAPK-signalling modulators selected from the group consisting of: (i) a MAPK-signalling pathway modulator produced according to the method of any one of Claims 21-26, (ii) an ERK1/2 inhibitor; (iii) a CFBA1 inhibitor; and (iv) a p38 activator, and a pharmaceutically acceptable carrier, diluent or excipient, for use in the treatment of an osteoarticular disease or condition in a mammal.

28. The pharmaceutical composition of Claim 27, wherein said osteoarticular disease is selected from the group consisting of inflammatory rheumatism, metabolic arthropathy, degenerative rheumatism, rheumatoid arthritis, osteoarthritis, spondylarthritis, gout, chondrocalcinosis and arthrosis.

29. The pharmaceutical composition of Claim 28, wherein said osteoarticular disease is osteoarthritis.

30. The pharmaceutical composition of any one of Claims 27-29, wherein said mammal is a human.

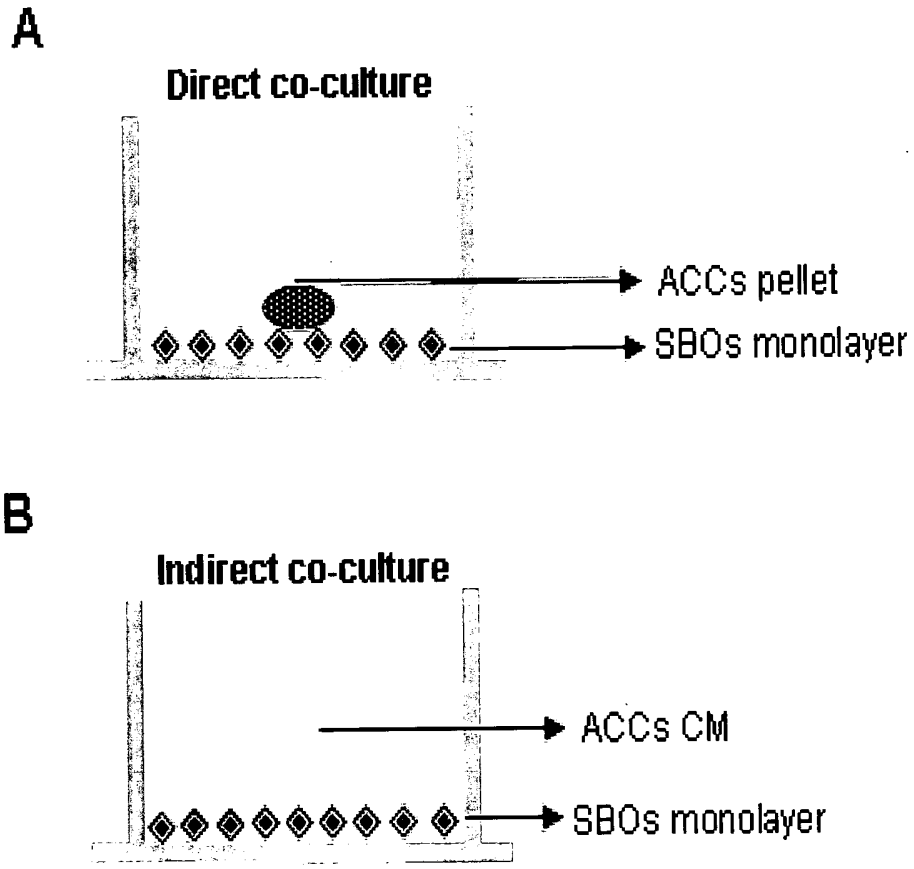

A
Direct co-culture
ACCs pellet
SBOs monolayer
B
Indirect co-culture
ACCs CM
SBOs monolayer

FIG. 1

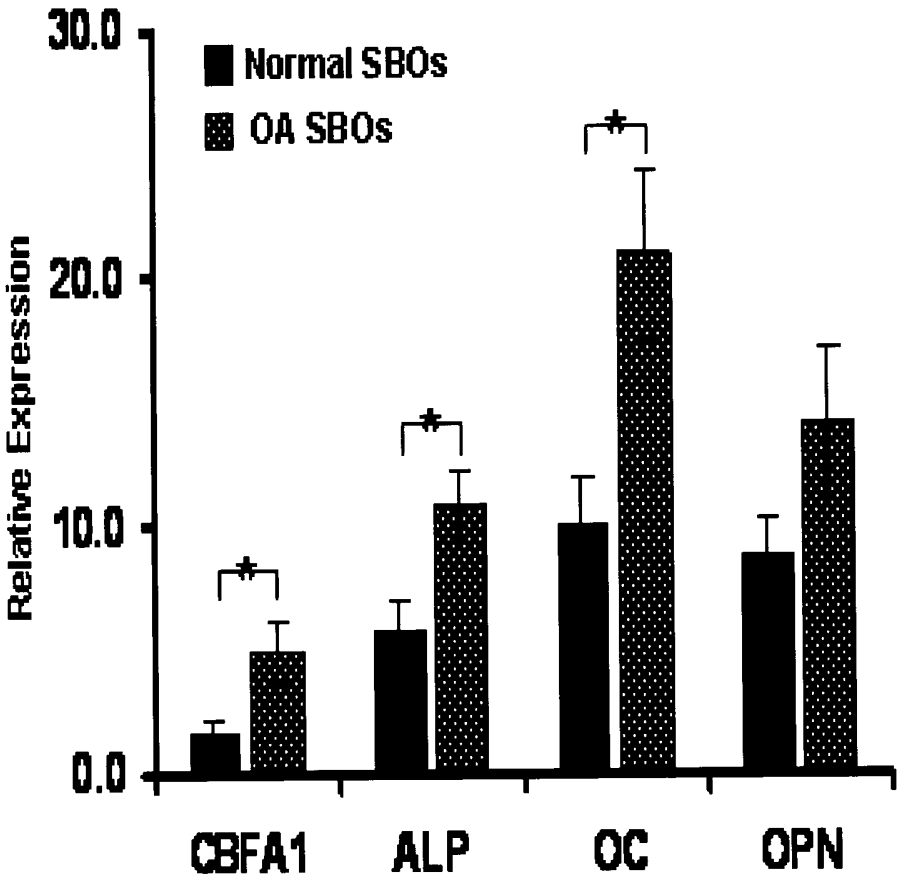

30.0
20.0
10.0
0.0
Relative Expression
Normal SBOs
OA SBOs
*
*
*
CBFA1
ALP
OC
OPN

FIG. 2

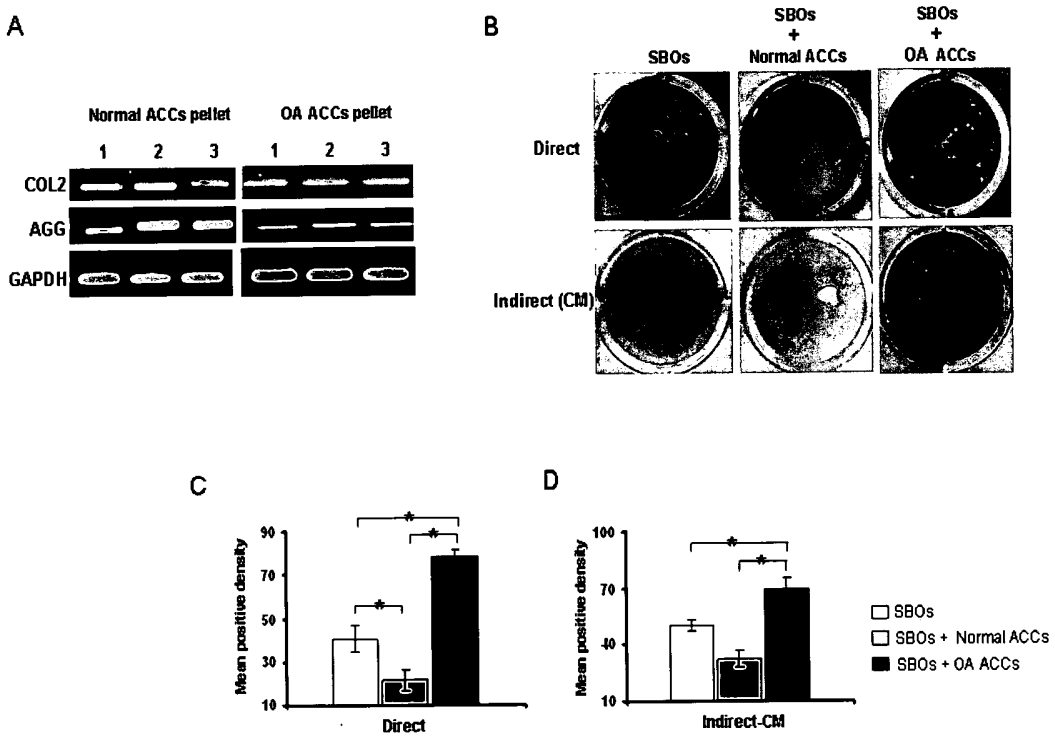

A
Normal ACCs pellet
OA ACCs pellet
1 2 3 1 2 3
COL2
AGG
GAPDH
B
SBOs
SBOs + Normal ACCs
SBOs + OA ACCs
Direct
Indirect (CM)
C
Mean positive density
90
70
50
30
10
Direct
D
Mean positive density
100
70
40
10
Indirect-CM
SBOs
SBOs + Normal ACCs
SBOs + OA ACCs

FIG. 3

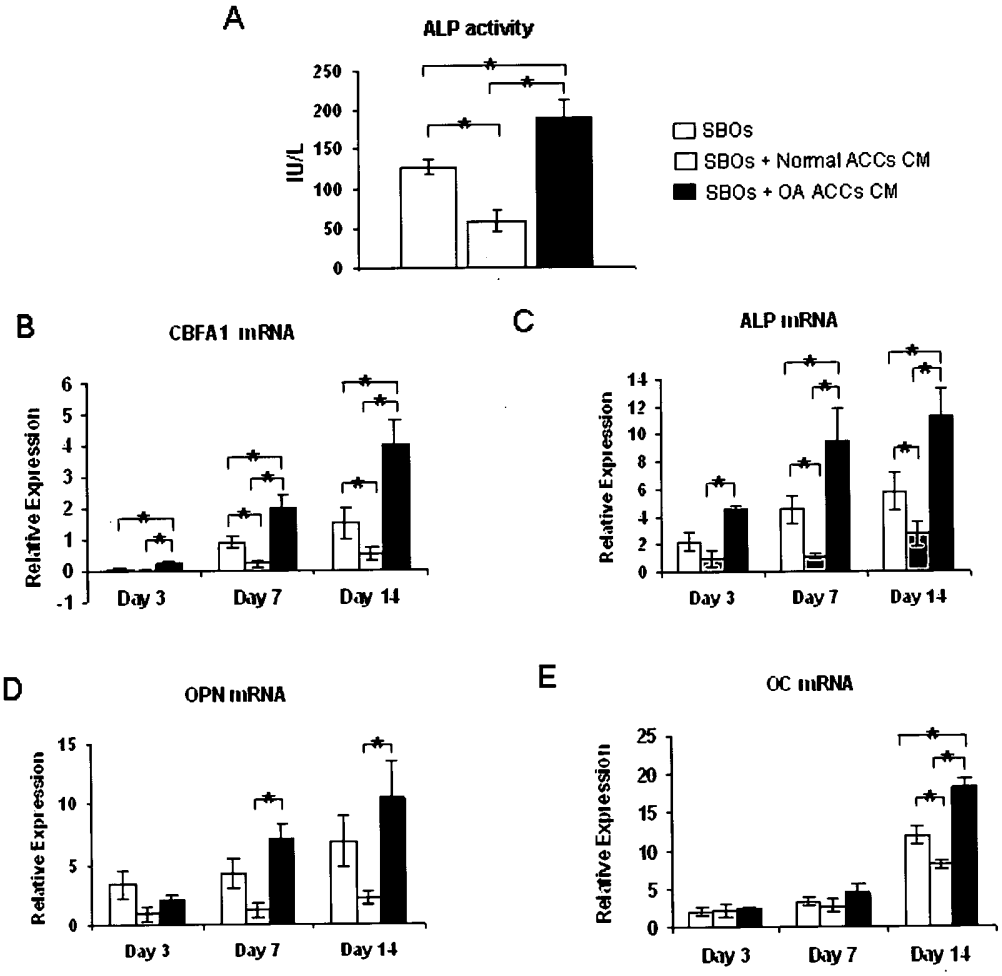

A
ALP activity
IU/L
250
200
150
100
50
0
SBOs
SBOs + Normal ACCs CM
SBOs + OA ACCs CM
B
CBFA1 mRNA
Relative Expression
Day 3
Day 7
Day 14
C
ALP mRNA
Relative Expression
Day 3
Day 7
Day 14
D
OPN mRNA
Relative Expression
Day 3
Day 7
Day 14
E
OC mRNA
Relative Expression
Day 3
Day 7
Day 14

FIG. 4

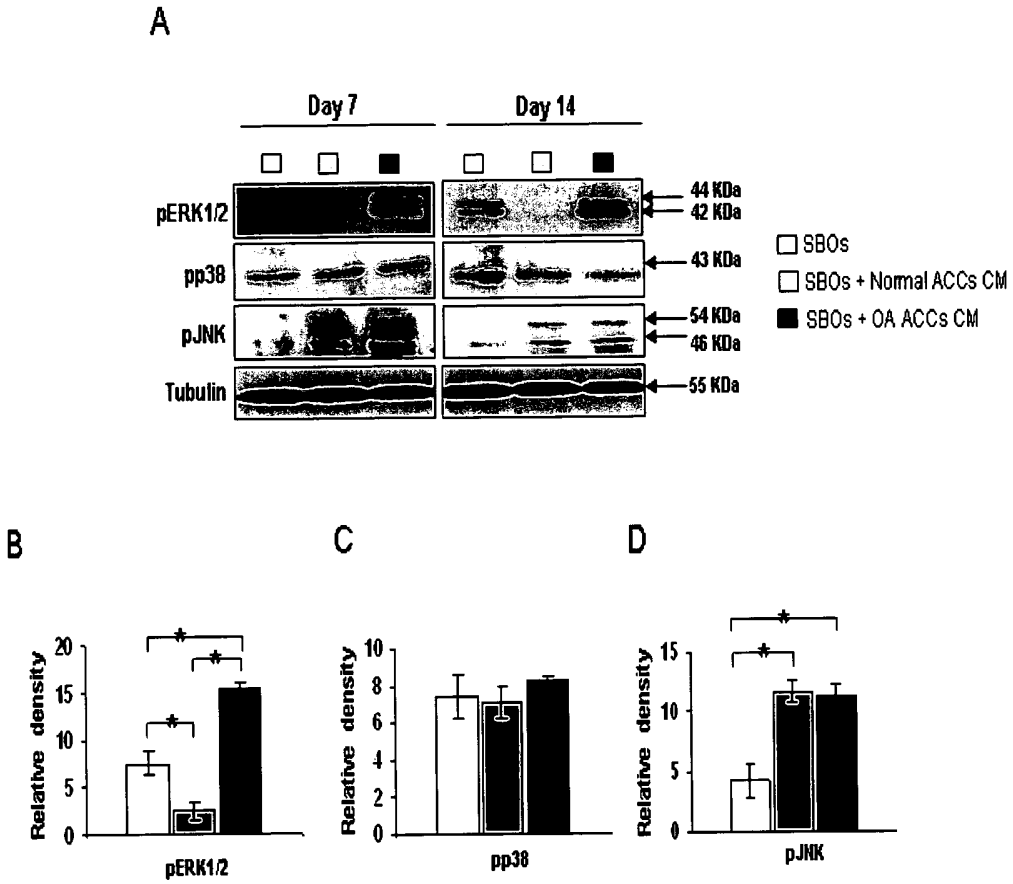

A
Day 7
Day 14
pERK1/2
pp38
pJNK
Tubulin
44 KDa
42 KDa
43 KDa
54 KDa
46 KDa
55 KDa
SBOs
SBOs + Normal ACCs CM
SBOs + OA ACCs CM
B
C
D
Relative density
pERK1/2
pp38
pJNK

FIG. 5

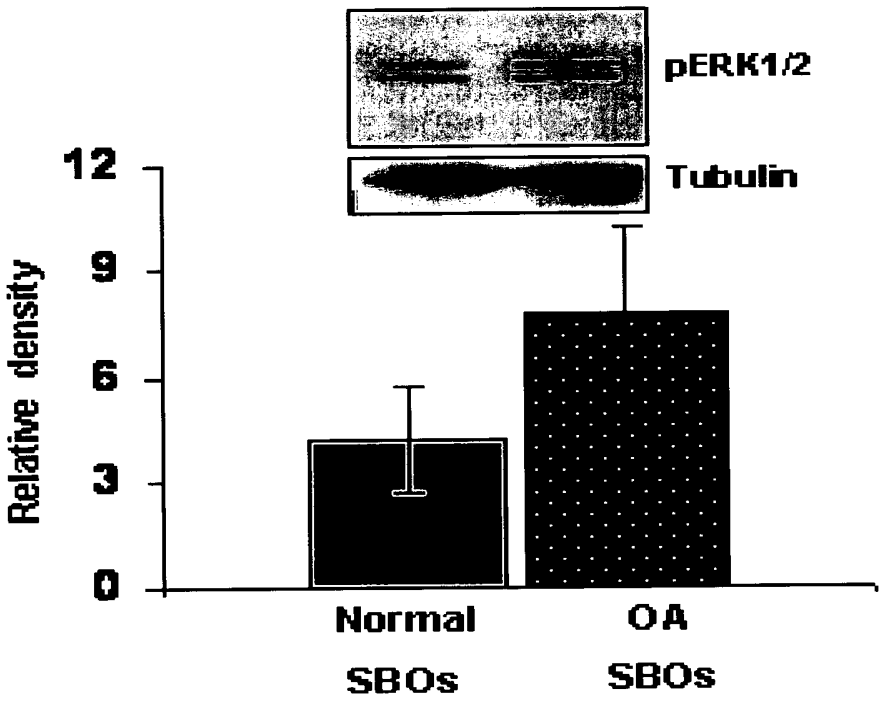

pERK1/2
Tubulin
Relative density
12
9
6
3
0
Normal SBOs
OA SBOs

FIG. 6

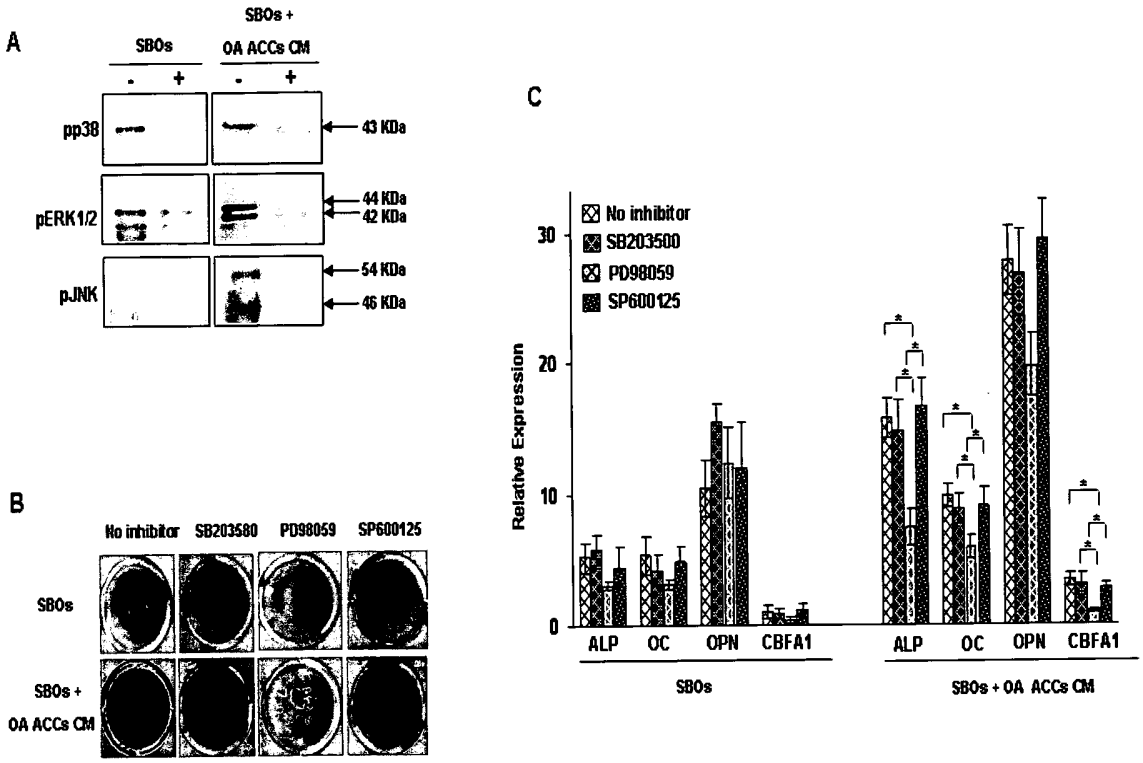

A
SBOs
SBOs + OA ACCs CM
- + - +
pp38
pERK1/2
pJNK
43 KDa
44 KDa
42 KDa
54 KDa
46 KDa
B
No inhibitor
SB203580
PD98059
SP600125
SBOs
SBOs + OA ACCs CM
C
No inhibitor
SB203500
PD98059
SP600125
Relative Expression
0
10
20
30
ALP
OC
OPN
CBFA1
SBOs
SBOs + OA ACCs CM

FIG. 7

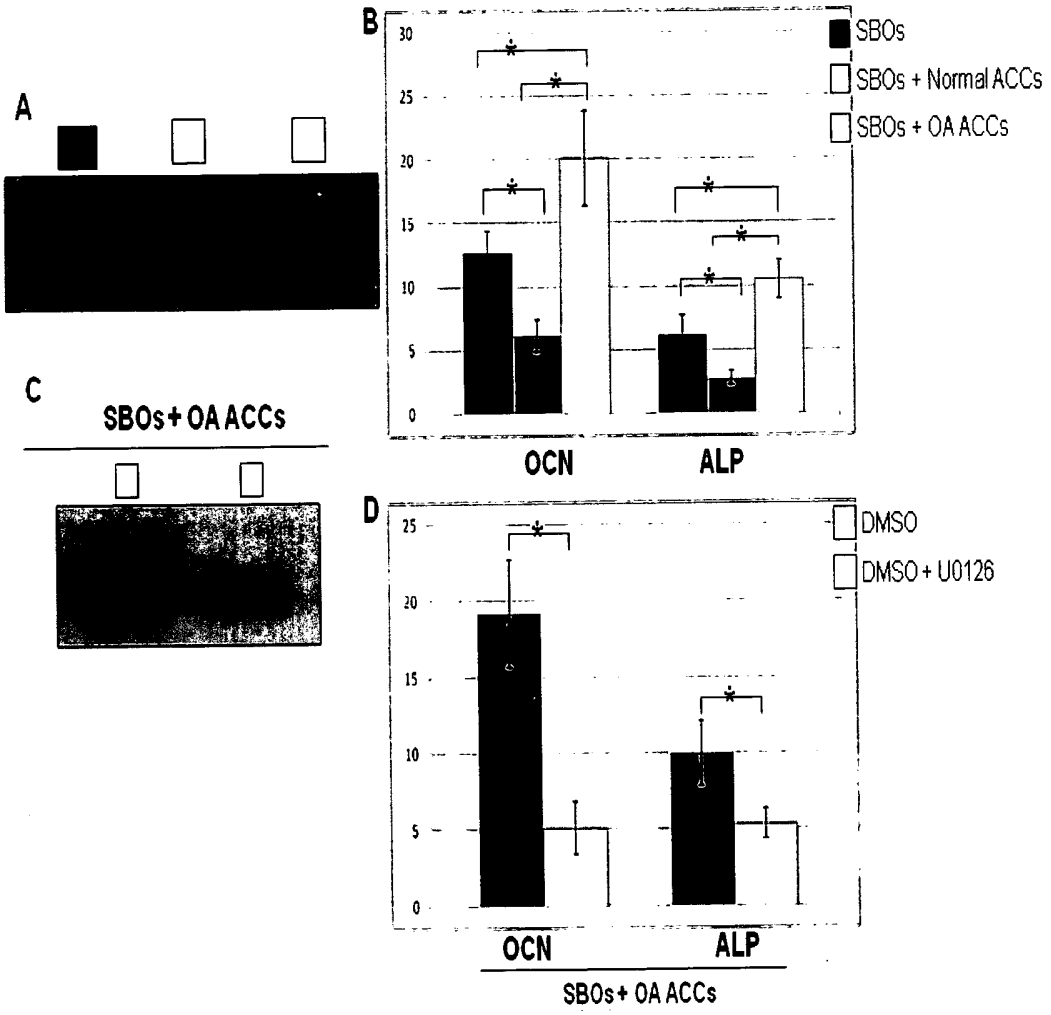

A
B
SBOs
SBOs + Normal ACCs
SBOs + OA ACCs
30
25
20
15
10
5
0
OCN
ALP
C
SBOs + OA ACCs
D
DMSO
DMSO + U0126
25
20
15
10
5
0
OCN
ALP
SBOs + OA ACCs

FIG. 8

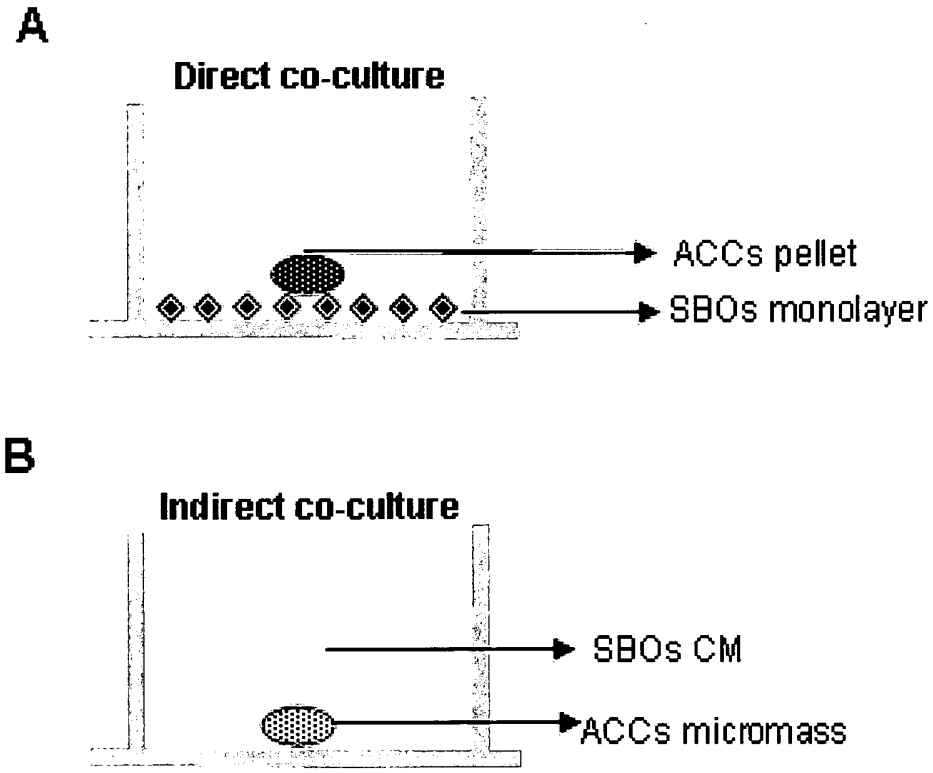

A
Direct co-culture
ACCs pellet
SBOs monolayer
B
Indirect co-culture
SBOs CM
ACCs micromass

FIG. 9

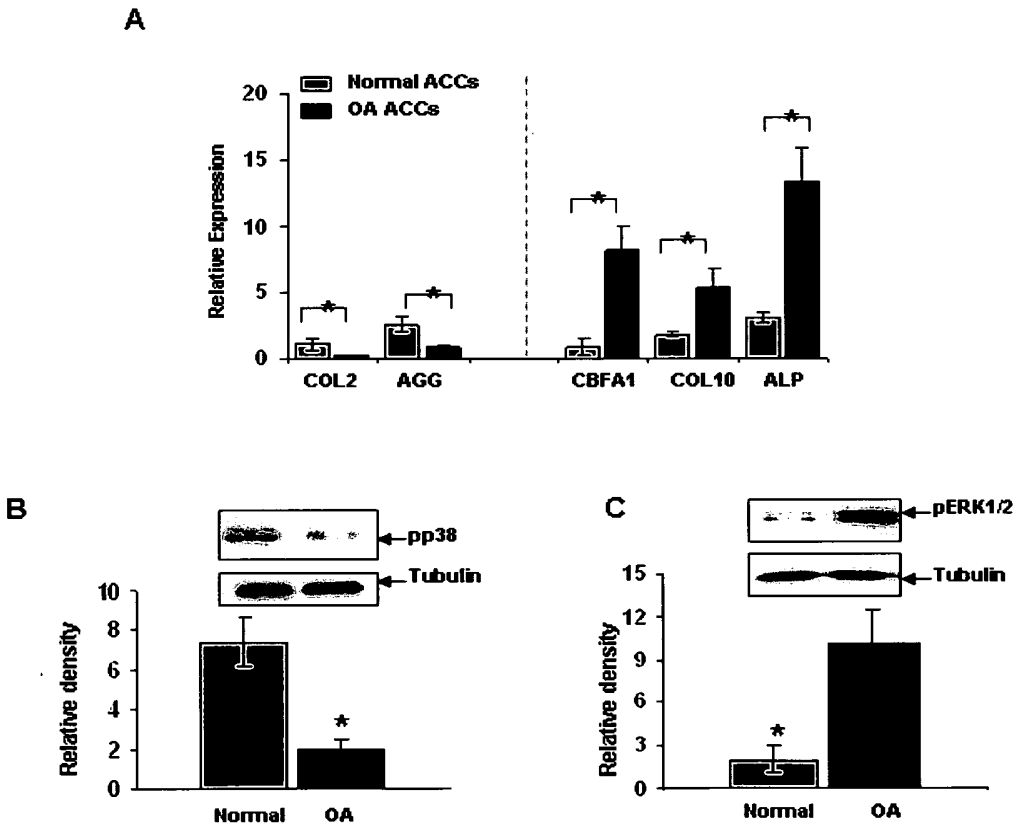

A
Normal ACCs
OA ACCs
Relative Expression
20
15
10
5
0
COL2
AGG
CBFA1
COL10
ALP
B
pp38
Tubulin
Relative density
10
8
6
4
2
0
Normal
OA
C
pERK1/2
Tubulin
Relative density
15
12
9
6
3
0
Normal
OA

FIG. 10

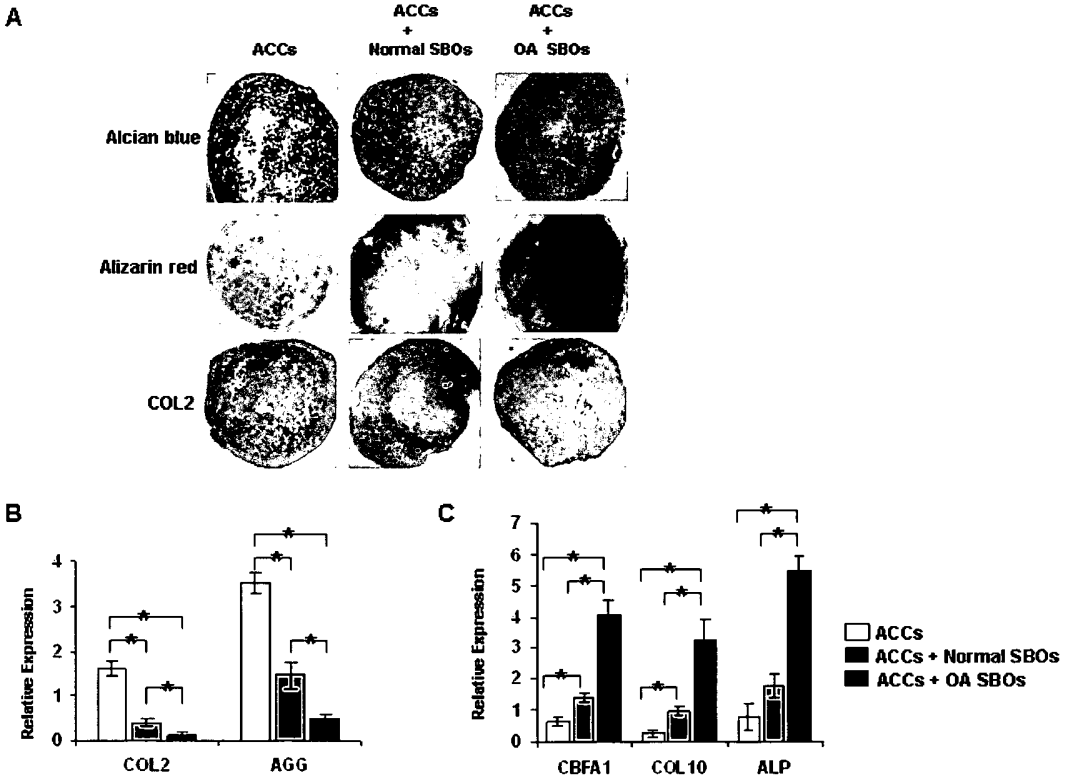

A
ACCs
ACCs + Normal SBOs
ACCs + OA SBOs
Alcian blue
Alizarin red
COL2
B
Relative Expression
COL2
AGG
C
Relative Expression
CBFA1
COL10
ALP
ACCs
ACCs + Normal SBOs
ACCs + OA SBOs

FIG. 11

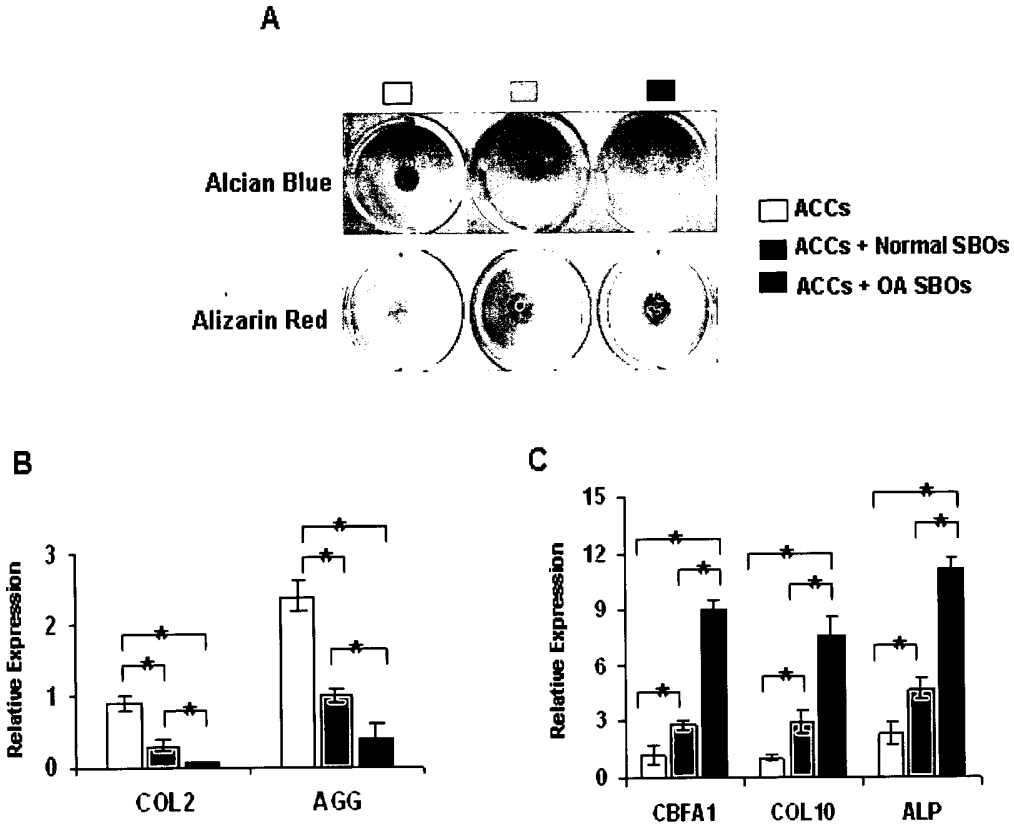

A
Alcian Blue
Alizarin Red
ACCs
ACCs + Normal SBOs
ACCs + OA SBOs
B
Relative Expression
0
1
2
3
COL2
AGG
C
Relative Expression
0
3
6
9
12
15
CBFA1
COL10
ALP

FIG. 12

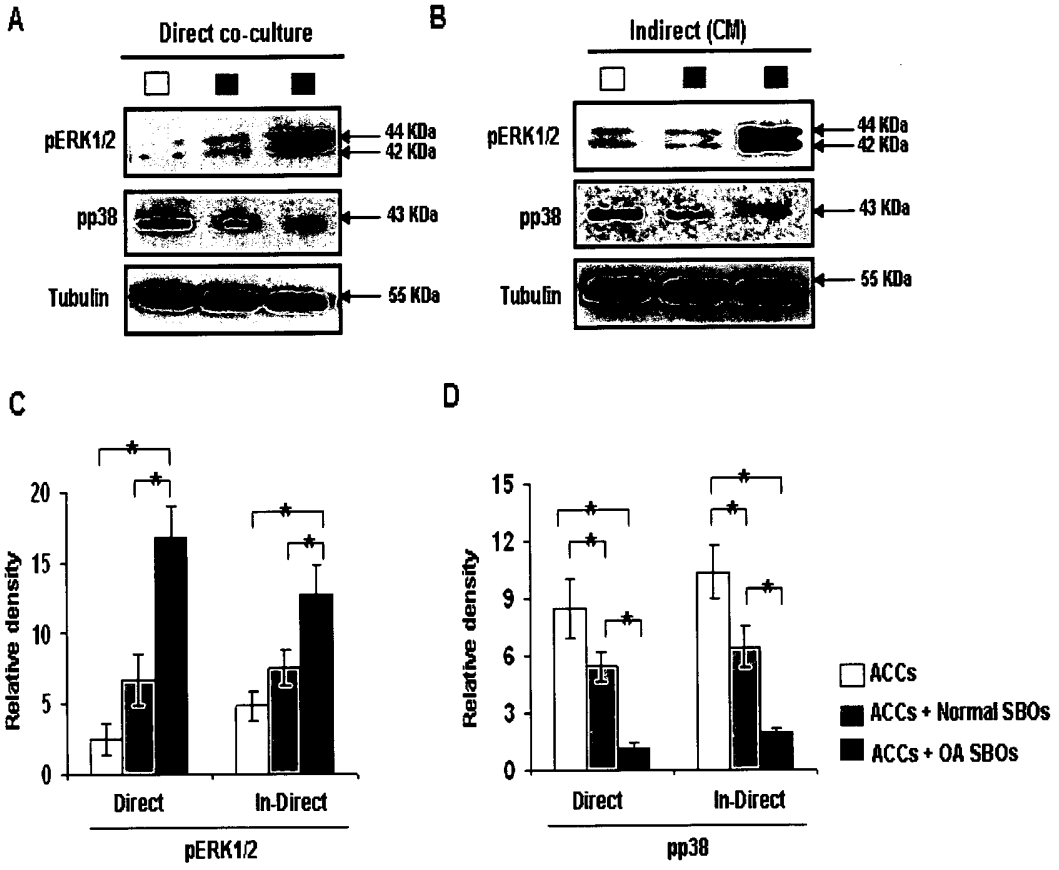

A
Direct co-culture
pERK1/2
pp38
Tubulin
44 KDa
42 KDa
43 KDa
55 KDa
B
Indirect (CM)
pERK1/2
pp38
Tubulin
44 KDa
42 KDa
43 KDa
55 KDa
C
Relative density
20
15
10
5
0
Direct
In-Direct
pERK1/2
D
Relative density
15
12
9
6
3
0
Direct
In-Direct
pp38
ACCs
ACCs + Normal SBOs
ACCs + OA SBOs

FIG. 13

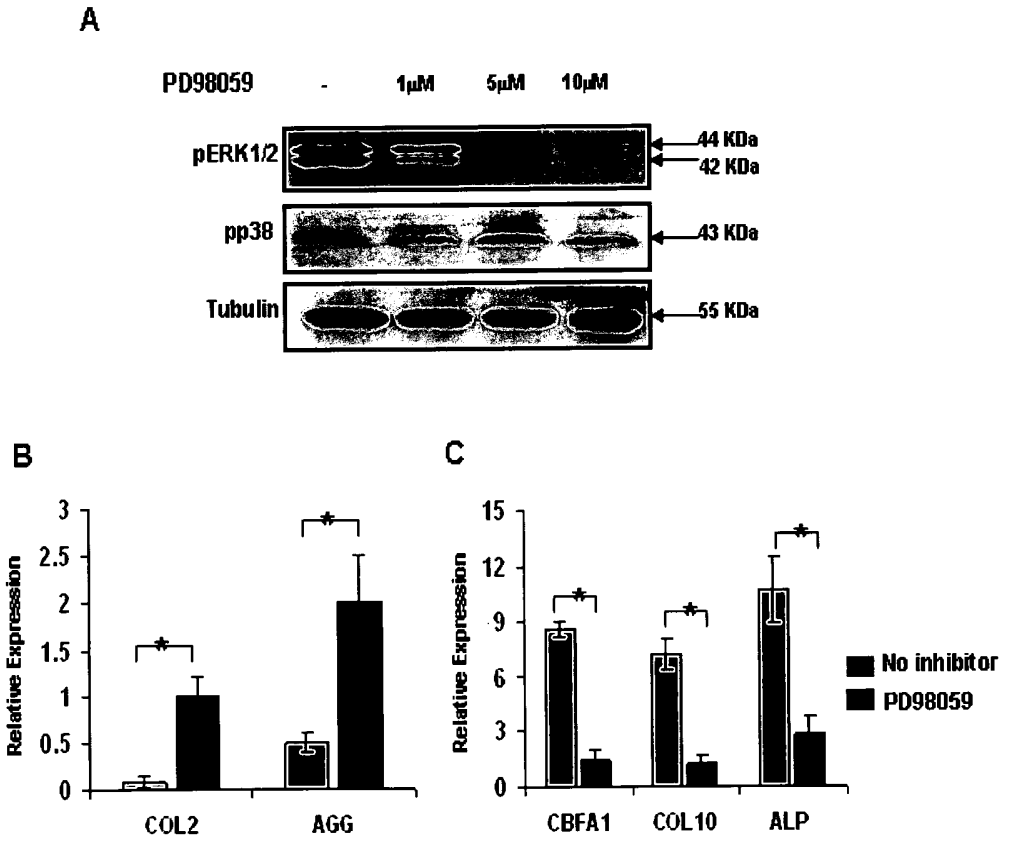

A
PD98059
-
1μM
5μM
10μM
pERK1/2
44 KDa
42 KDa
pp38
43 KDa
Tubulin
55 KDa
B
Relative Expression
3
2.5
2
1.5
1
0.5
0
COL2
AGG
C
Relative Expression
15
12
9
6
3
0
CBFA1
COL10
ALP
No inhibitor
PD98059

FIG. 14

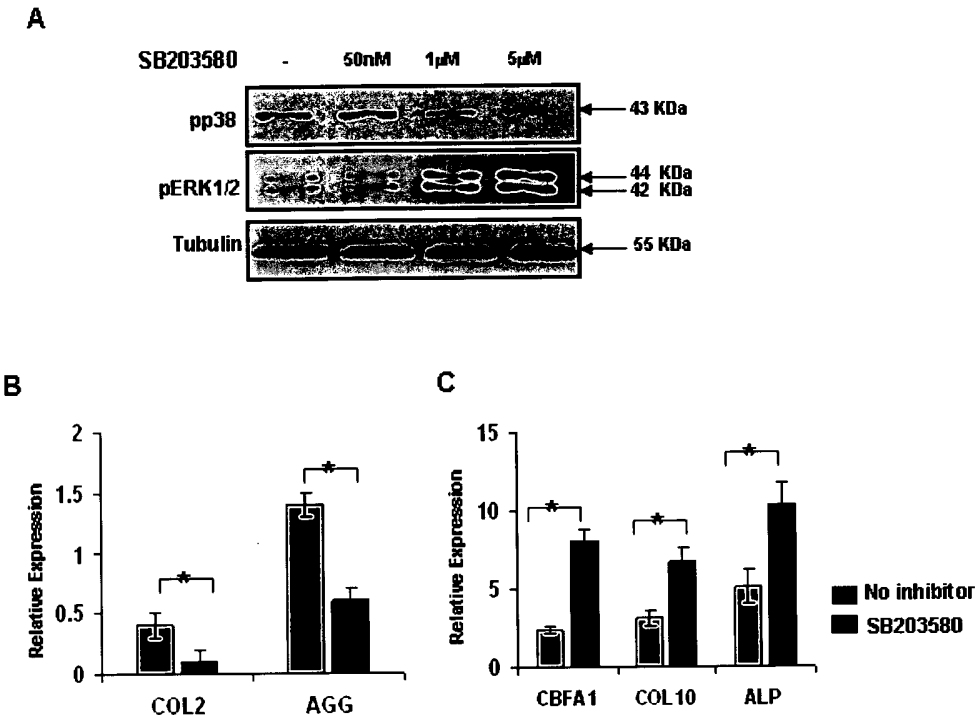

A
SB203580
-
50nM
1μM
5μM
pp38
pERK1/2
Tubulin
43 KDa
44 KDa
42 KDa
55 KDa
B
Relative Expression
2
1.5
1
0.5
0
COL2
AGG
C
Relative Expression
15
10
5
0
CBFA1
COL10
ALP
No inhibitor
SB203580

FIG. 15

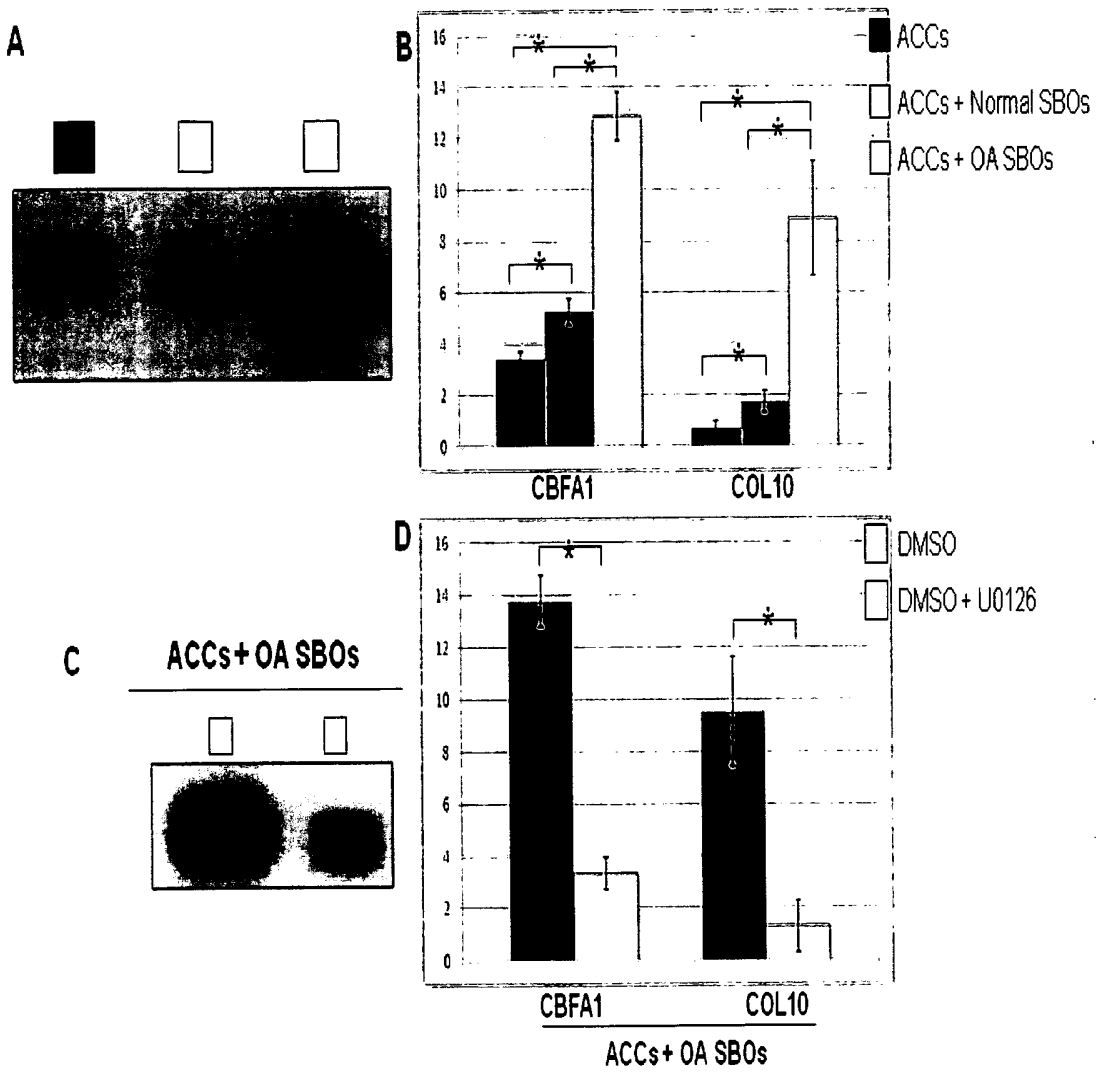

A
B
ACCs
ACCs + Normal SBOs
ACCs + OA SBOs
CBFA1
COL10
C
ACCs + OA SBOs
D
DMSO
DMSO + U0126
CBFA1
COL10
ACCs + OA SBOs

FIG. 16

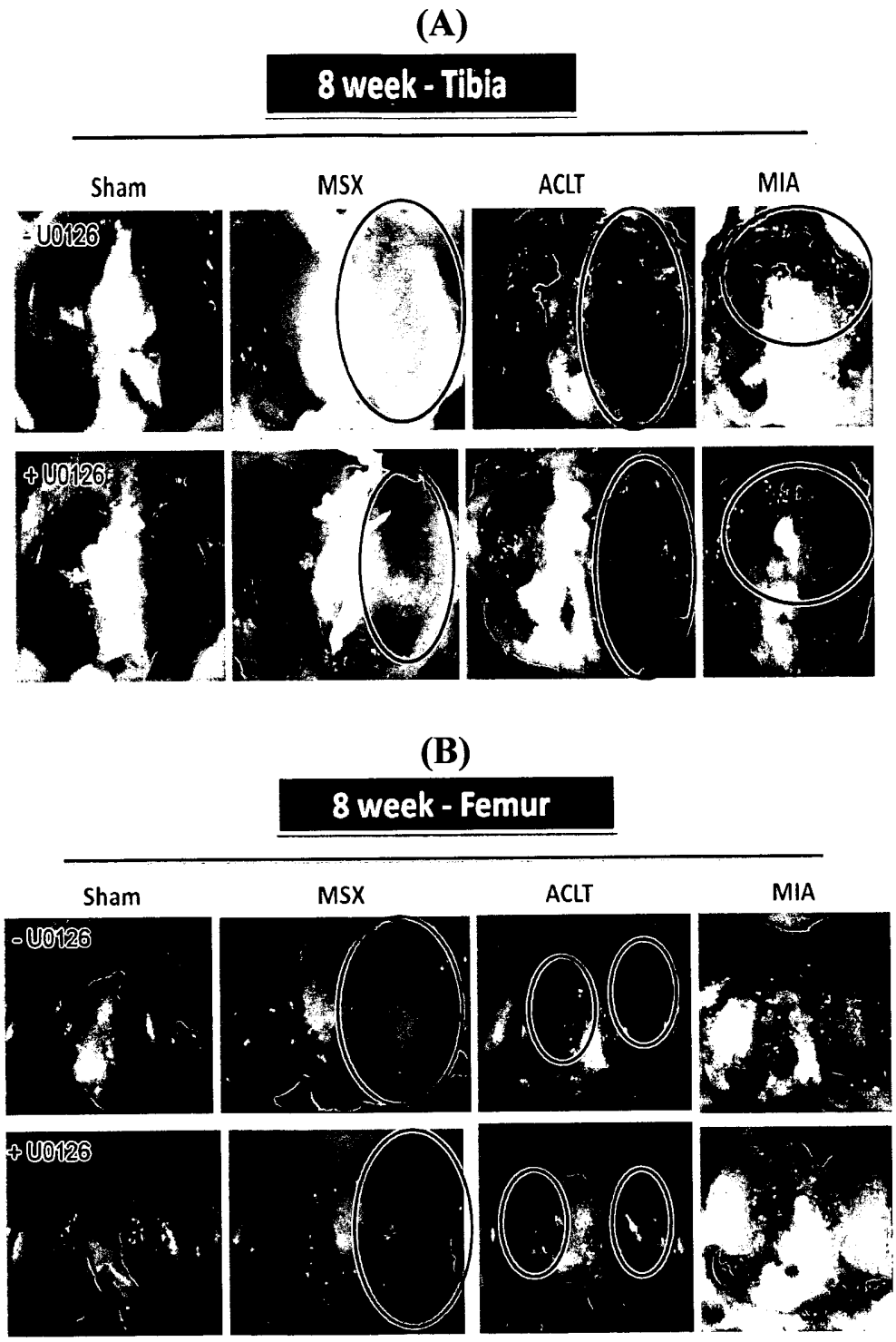
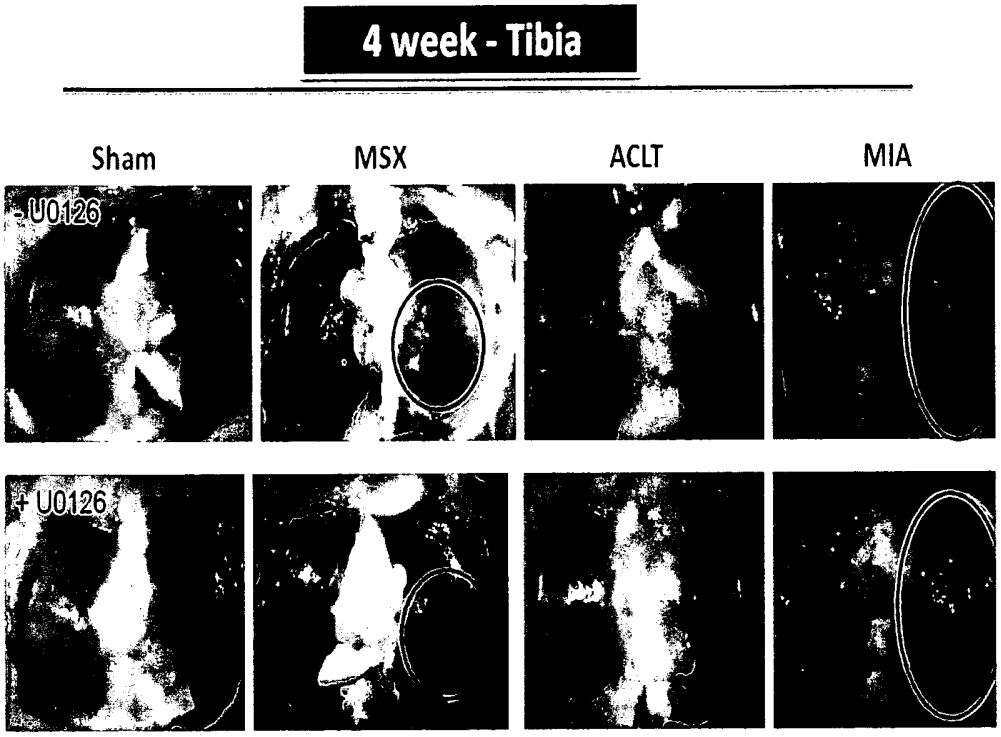

(A)
8 week - Tibia
Sham
MSX
ACLT
MIA
- U0126
+ U0126
(B)
8 week - Femur
Sham
MSX
ACLT
MIA
- U0126
+ U0126

FIG. 17

(C)



(D)

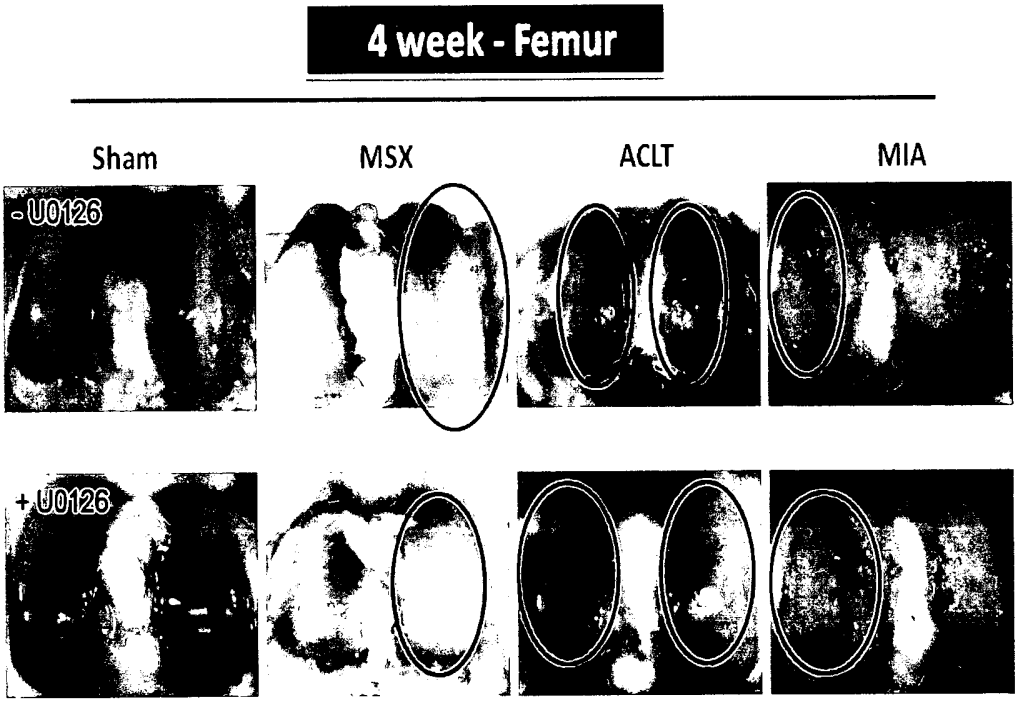


FIG. 17 (CONT'D)

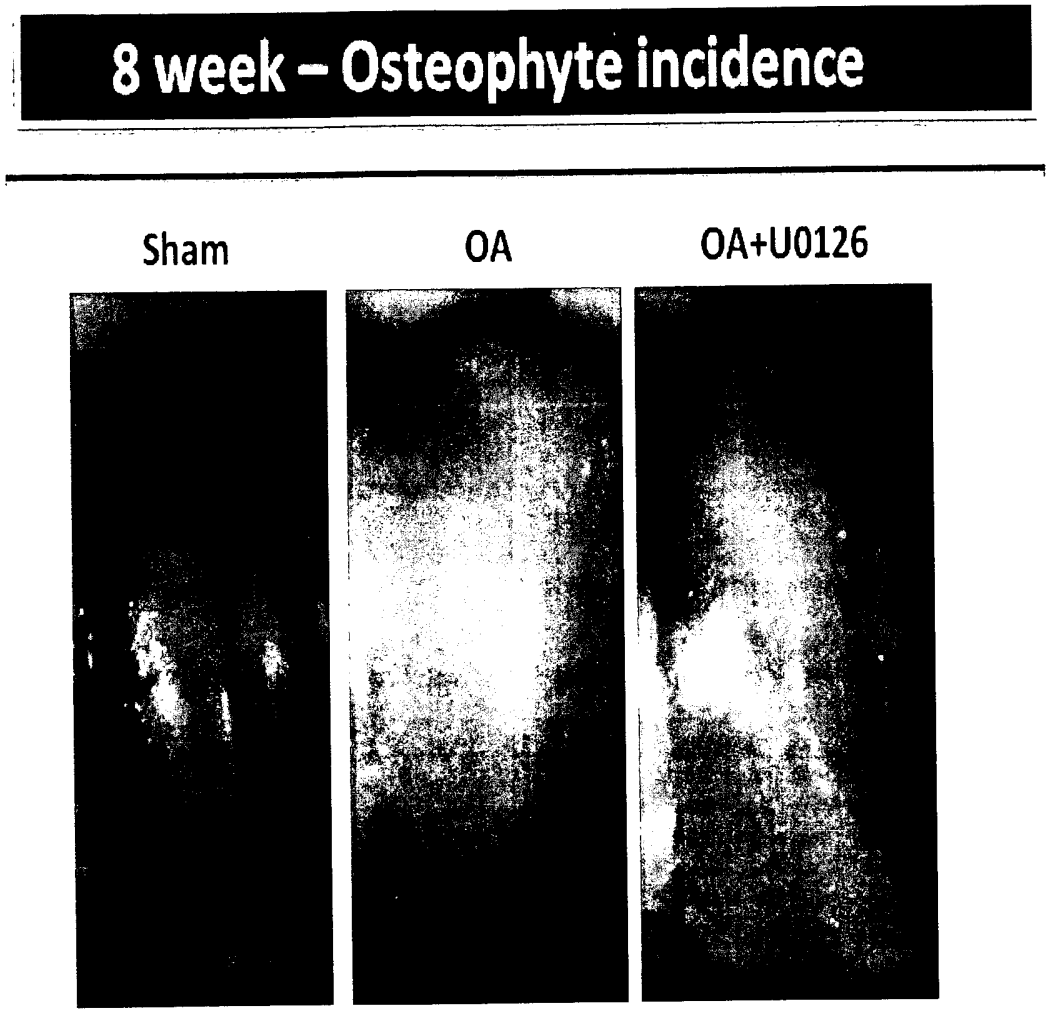
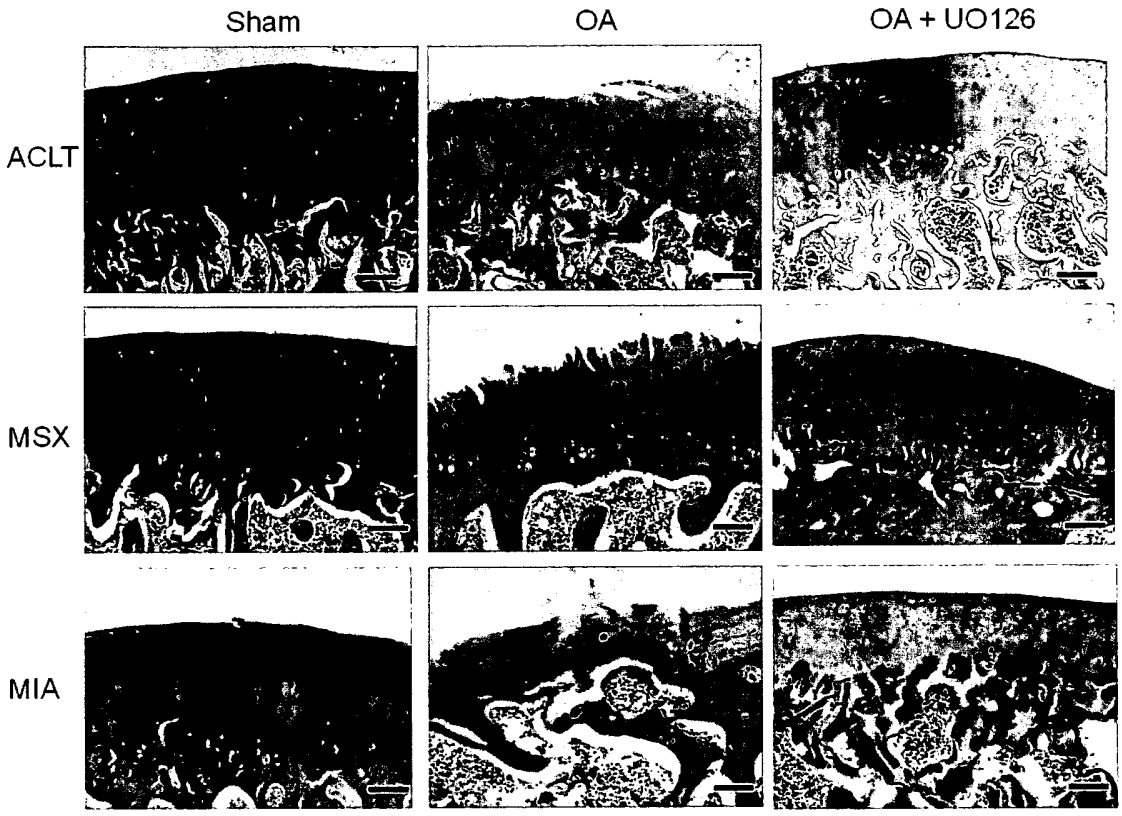


FIG. 18

(A)


Sham
OA
OA + UO126
ACLT
MSX
MIA

(B)

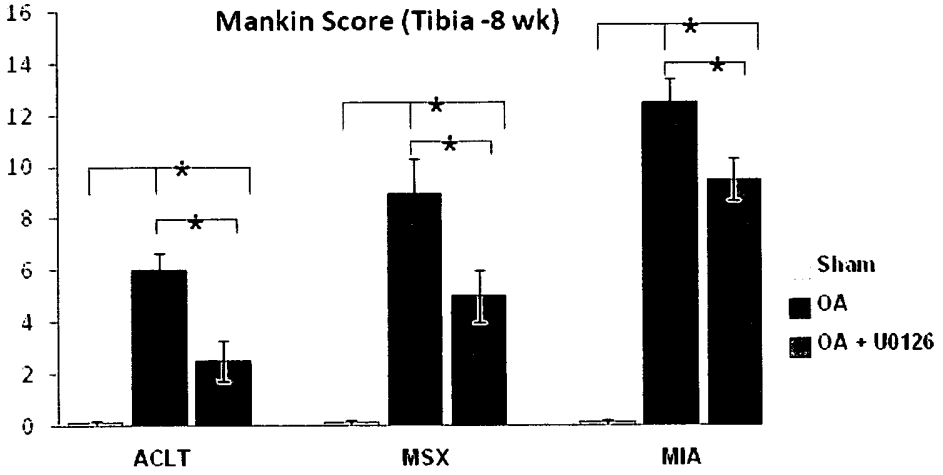
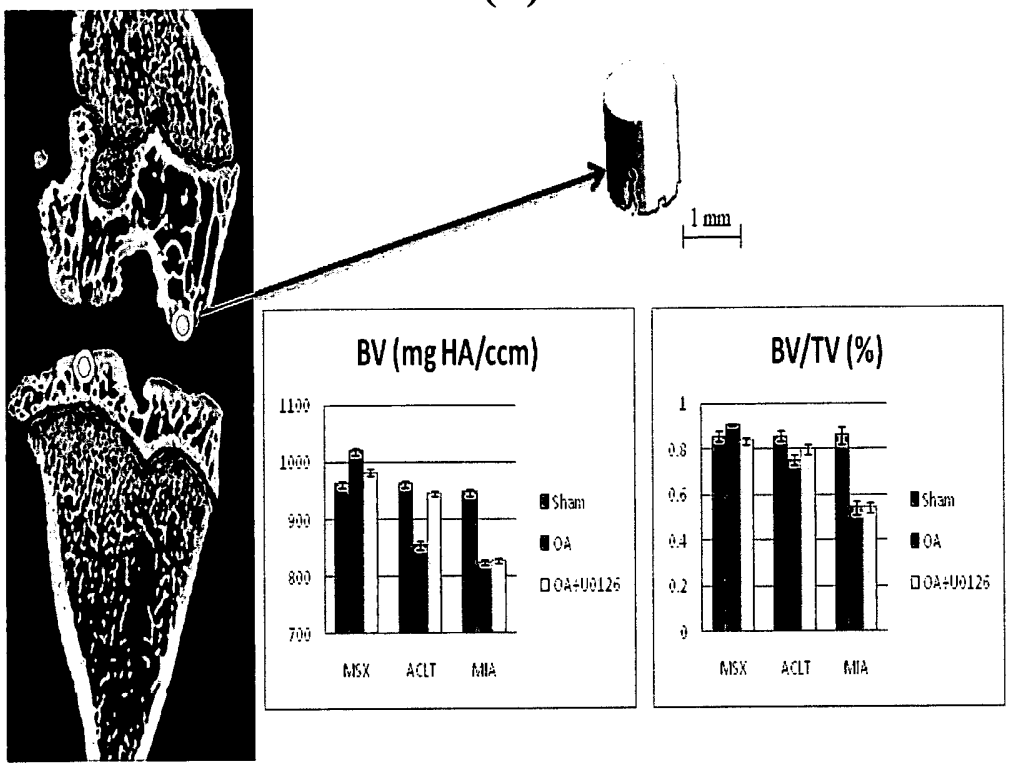

Mankin Score (Tibia -8 wk)
16
14
12
10
8
6
4
2
0
ACLT
MSX
MIA
Sham
OA
OA + U0126

FIG. 19

(A)


1 mm
BV (mg HA/ccm)
1100
1000
900
800
700
MSX
ACLT
MIA
Sham
OA
OA+U0126
BV/TV (%)
1
0.8
0.6
0.4
0.2
0
MSX
ACLT
MIA
Sham
OA
OA+U0126

(B)

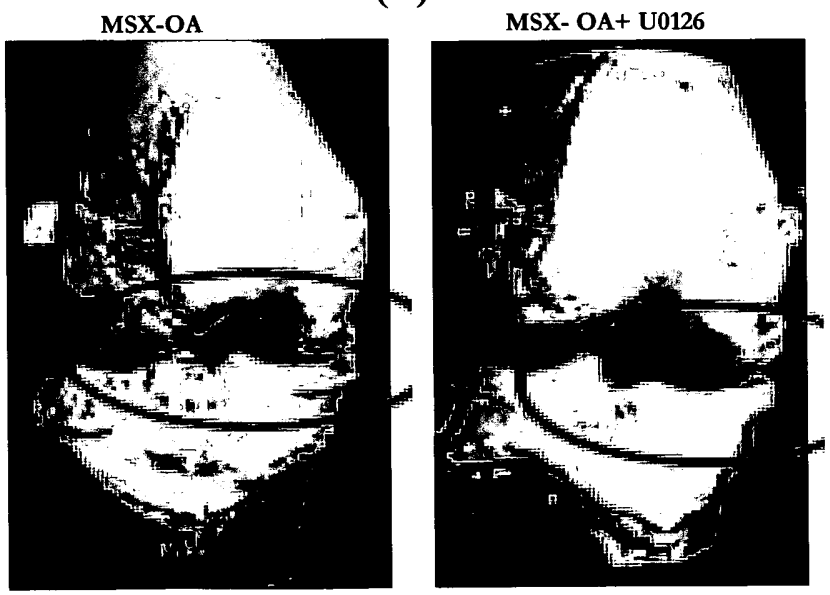

MSX-OA
MSX- OA+ U0126

FIG. 20

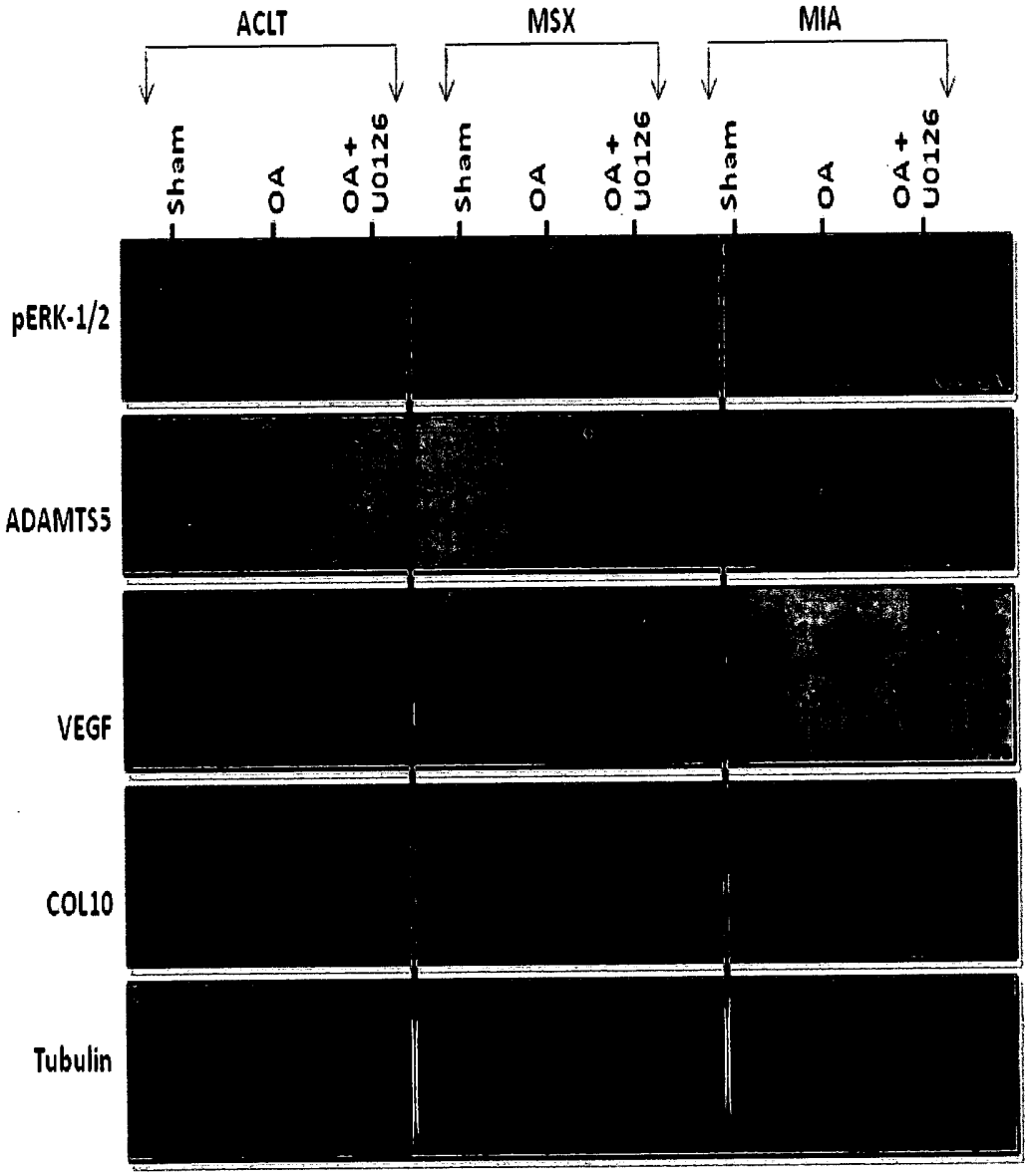

ACLT
MSX
MIA
Sham
OA
OA + U0126
Sham
OA
OA + U0126
Sham
OA
OA + U0126
pERK-1/2
ADAMTS5
VEGF
COL10
Tubulin

FIG. 21

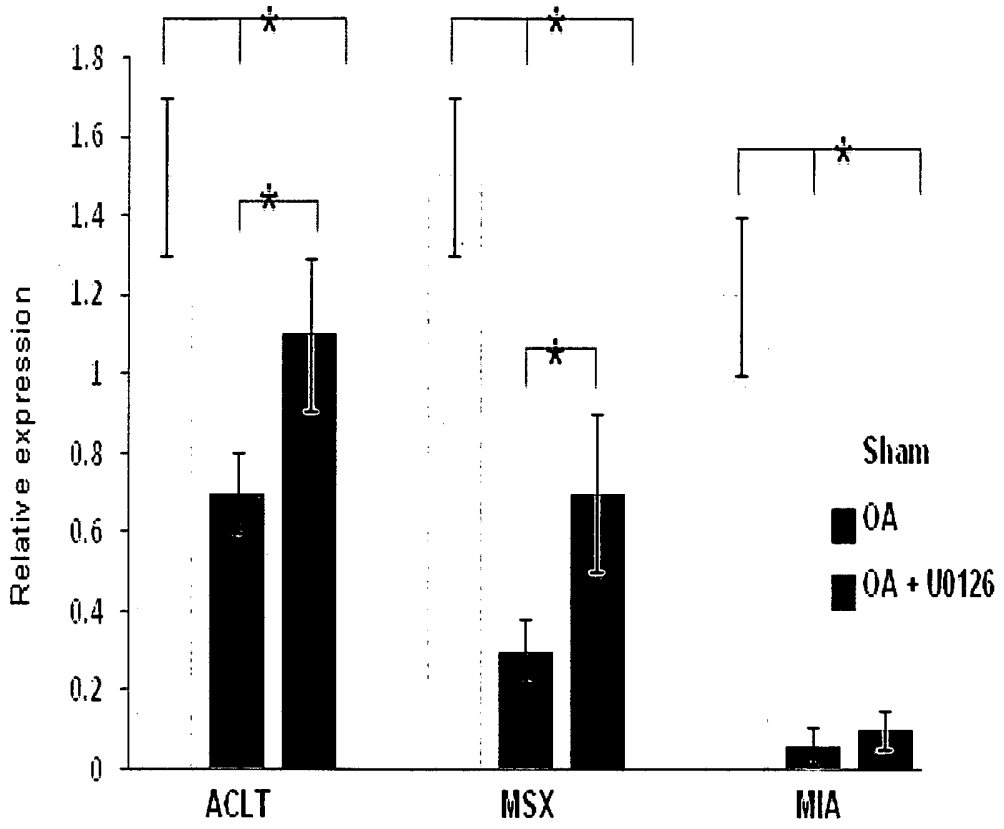

Relative expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ACLT
MSX
MIA
Sham
OA
OA + U0126

FIG. 22

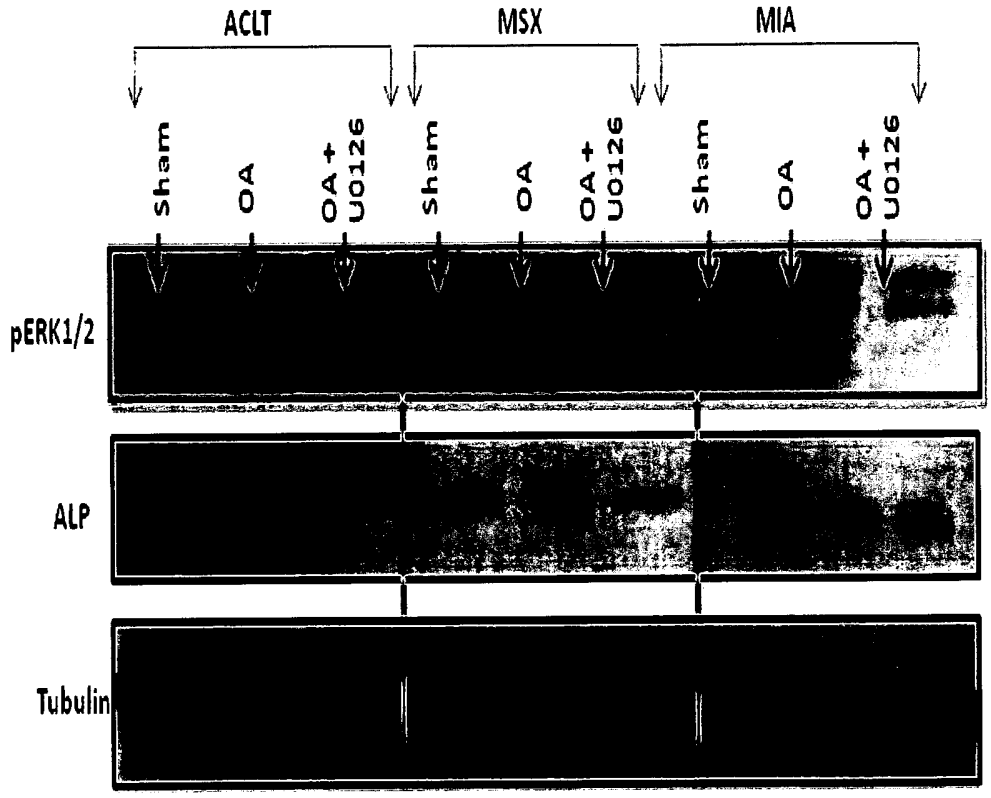

ACLT
MSX
MIA
Sham
OA
OA + U0126
Sham
OA
OA + U0126
Sham
OA
OA + U0126
pERK1/2
ALP
Tubulin

FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/001400

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 31/352 (2006.01) *A61K 31/4439* (2006.01)
A61K 31/416 (2006.01) *A61P 19/00* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, WPI, EPODOC (Keywords; MAPK, ERK 1, ERK 2, P38, CFBA1, Osteoarthritis, Osteoarthritic Disease, Degenerative Joint Disease, Rheumatism, Gout, Modulate, Inhibit, Activate, and like terms)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PRASADAM I et al. Osteoarthritic cartilage chondrocytes alter subchondral bone osteoblast differentiation via MAPK signaling pathway involving ERK1/2. Bone. 2010. 46: 226-235. 22 October 2009 See Abstract, Materials and Methods and Conclusions	1-30
X	SAKLATVALA, JEREMY. Inflammatory Signaling in Cartilage: MAPK and NF-KB Pathways in Chondrocytes and the Use of Inhibitors for Research into Pathogenesis and Therapy of Osteoarthritis. Current Drug Targets. 2007. 8:305-313. See Fig 3 and pages 309-310.	1-30
X	BROWN, KIMBERLY K et al. P38 MAP kinase inhibitors as potential therapeutics for the treatment of joint degeneration and pain associated with osteoarthritis. Journal of Inflammation. 2008. 5:22. See Abstract	21, 22 and 26-30

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2010	25 NOV 2010
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **JAMES SUNG** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2747

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/001400

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PELLETIER, JEAN-PIERRE et al. In vivo selective inhibition of mitogen-activated protein kinase kinase 1/2 in rabbit experimental osteoarthritis is associated with a reduction in the development of structural changes. Arthritis & Rheumatism. 2003. 48(6): 1582-1593. See Abstract, pages 1585-1589, 1591-1592	1-30
X	KIMURA, H. et al. The Chondroprotective Agent ITZ-1 Inhibits Interleukin-1β–Induced Matrix Metalloproteinase–13 Production and Suppresses Nitric Oxide–Induced Chondrocyte Death. J. Pharmacol. Sci. 23 April 2009. 110, pages 201-211. See abstract, Material and Methods and Discussion	1-30