Cross-talk of subchondral bone osteoblasts and articular cartilage chondrocytes: a new insight in understanding osteoarthritis pathogenesis

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Abstract

Osteoarthritis (OA) is the most common musculoskeletal disorder and represents a major health burden to society. In the course of the pathological development of OA, articular cartilage chondrocytes (ACCs) undergo atypical phenotype changes characterized by the expression of hypertrophic differentiation markers. Also, the adjacent subchondral bone shows signs of abnormal mineral density and enhanced production of bone turnover markers, indicative of osteoblast dysfunction. Collectively these findings indicate that the pathological changes typical of OA, involve alterations of the phenotypic properties of cells in both the subchondral bone and articular cartilage. However, the mechanism(s) by which these changes occur during OA development are not completely understood. The purpose of this project was to address the question of how subchondral bone osteoblasts (SBOs) and ACCs interact with each other with respect to regulation of respective cells' phenotypic properties and in particular the involvement of mitogen activated protein kinase (MAPK) signalling pathways under normal and OA joint condition. We also endeavoured to test the influence of cross-talk between SBOs and ACCs isolated from normal and OA joint on matrix metalloproteinase (MMP) expression.

For this purpose tissues from the knees of OA patients and normal controls were collected to isolate SBOs and ACCs. The cellular cross-talk of SBOs and ACCs were studied by means of both direct and indirect co-culture systems, which made it possible to identify the role of both membrane bound and soluble factors. Histology, immunohistochemistry, qRT-PCR, zymography, ELISA and western blotting were some of the techniques applied to distinguish the changes in the co-cultured *vs.* non co-cultured cells. The MAPK signalling pathways were probed by using targeted MAPK inhibitors, and their activity monitored by western blot analysis using phospho MAPK specific antibodies.

Our co-culture studies demonstrated that OA ACCs enhanced the SBOs differentiation compared to normal ACCs. We demonstrated that OA ACCs induced these phenotypic changes in the SBOs *via* activating an ERK1/2 signalling pathway. The findings from this study thus provided clear evidence that OA ACCs play an integral role in altering the SBO phenotype.

In the second study, we tested the influence of normal SBOs and OA SBOs on ACCs phenotype changes. The results showed that OA SBOs increased the hypertrophic gene expression in co-cultured ACCs compared to normal SBOs, a phenotype which is considered as pathological to the health and integrity of articular cartilage. It was demonstrated that these phenotype changes occurred via de-activation of p38 and activation of ERK1/2 signaling pathways. These findings suggest that the pathological interaction of OA SBOs with ACCs is mediated by cross-talking between ERK1/2 and p38 pathways, resulting in ACCs undergoing hypertrophic differentiation.

Subsequent experiments to determine the effect on MMP regulation, of SBOs and ACCs cross-talk, revealed that co-culturing OA SBOs with ACCs significantly enhanced the proteolytic activity and expression of MMP-2 and MMP-9. In turn, co-culture of OA ACCs with SBOs led to abundant MMP-2 expression in SBOs. Furthermore, we showed that the addition of ERK1/2 and JNK inhibitors reversed the elevated MMP-2 and MMP-9 production which otherwise resulted from the interactions of OA SBOs-ACCs. Thus, this study has demonstrated that the altered interactions between OA SBOs-ACCs are capable of triggering the pathological pathways leading to degenerative changes seen in the osteoarthritic joint.

In conclusion, the body of work presented in this dissertation has given clear *in vitro* evidence that the altered bi-directional communication of SBOs and ACCs may play a role in OA development and that this process was mediated by MAPK signalling pathways. Targeting these altered interactions by the use of MAPK inhibitors may provide the scientific rationale for the development of novel therapeutic strategies in the treatment and management of OA.

Keywords: Osteoarthritis, Cell interactions, Cross-talk, Subchondral bone osteoblasts, Articular cartilage chondrocytes, Mitogen activated protein kinase, Matrix Metalloproteinases, Co-culture.

List of publications

The following is a list of publications, submitted manuscripts that have been derived from the work performed for this thesis.

1. **Prasadam I**, Friis T, Shi W, Gennip S, Crawford R, Xiao Y. Osteoarthritic cartilage chondrocytes alter subchondral bone osteoblast differentiation via MAPK signalling pathway involving ERK1/2. **Bone, 2009, In press (IF 4.5).**

2. **Prasadam I**, Gennip S, Friis T, Shi W, Crawford R, Xiao Y. Mitogen-activated Protein Kinase ERK1/2 and p38 in the regulation of hypertrophic changes of normal articular cartilage chondrocytes induced by osteoarthritic subchondral osteoblasts. **Arthritis & Rheumatism, 2009, In press (IF 6.8).**

3. **Prasadam I**, Crawford R, Xiao Y. Cell interactions of osteoarthritic subchondral bone osteoblasts and articular chondrocytes aggravate MMP-2 and MMP-9 production through the mediation of ERK1/2 and JNK phosphorylation. **Osteoarthritis and Cartilage, 2009, Submitted (IF 4.0)**.

4. **Prasadam I**, Forsythe M, Friis T, Shi W, Crawford R, Xiao Y. Post-natal changes in osteoarthritis development: Histology and Immunohistochemistry. **In preparation.**

Publications during PhD candidature, but not related to the work contained in this thesis:

Xiao Y, Fu H, **Prasadam I**, Yaw-Ching Y, Jeffrey H. Gene Expression Profiling of Bone Marrow Stromal Cells from Juvenile, Adult, Aged and Osteoporotic Rats: With an Emphasis on Osteoporosis. **Bone, 2007, 40(3):pp. 700-715 (IF 4.5).**

List of conferences and abstracts

1) Cell interactions of osteoarthritic subchondral bone osteoblasts and articular chondrocytes aggravate MMP-2 and MMP-9 production through the mediation of ERK1/2 and JNK phosphorylation. Institute of Health and Biomedical Innovation post graduate student conference. 16-11-2009, Brisbane, Australia - **Oral presentation.**

2) The cross talk of subchondral bone osteoblasts and articular chondrocytes in osteoarthritis development. Australian and New Zealand Orthopaedic Research Society **(**ANZORS), 18-11-2008, Brisbane, Australia - **Oral presentation.**

3) Subchondral bone osteoblasts can induce chondrocyte mineralization during osteoarthritis development and this process is relevant to cartilage degradation. 2nd Joint meeting of the International Bone & Mineral Society, 9-03-2008, Davos, Switzerland- **Poster presentation**

Patents

Methods and compositions for the treatment of osteoarticular cartilage disease. US provisional patent application - 61/255,259.

Declaration

The work contained within this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due references were made.

Signed…….. (Indira Prasadam)

Date…….....

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Last but not least, thanks to my spiritual mentor Shirdi Sai baba. You have made my life more bountiful. May your name be exalted, honoured, and glorified, Om Sri Sai Ram. This thesis is dedicated on his name.

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Cross-talk of subchondral bone osteoblasts and articular cartilage chondrocytes: a new insight in understanding osteoarthritis pathogenesis

List of Abbreviations

ACCs	Articular cartilage chondrocytes
ALP	Alkaline phosphatase
AGG	Aggrecan
BSA	Bovine serum albumin
COLI	Type I collagen
COLII	Type II collagen
COLX	Type X collagen
CPI	C-terminal type I procollagen propeptide
CC	Calcified cartilage
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucliec acid
ECM	Extracellular matrix
ERK1/2	Extracellular signal-regulated kinases 1 and 2
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
IL-6	Interleukin 6
hMSC	human mesenchymal stem cells
IGF	Insulin-like growth factor
JNK	c-Jun amino-terminal kinases
KDa	Kilodaltons
MAPKs	Mitogen activated protein kinases

MMP	Matrix metalloproteinase
nm	Nanometer
NCC	Non calcified cartilage
OA	Osteoarthritis
OC	Osteocalcin
OPN	Osteopontin
p38	Protein kinase 38
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SBOs	Subchondral bone osteoblasts
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOX9	Sex determining region Y - box 9
TGF-β1	Transforming growth factor beta 1
TGases	Transglutaminases
T25	25 cm^2 tissue culture flask
T75	75 cm^2 tissue culture flask
TM	Tide mark
TBST	Tris buffered saline with Tween
uPA	Urokinase plasminogen activator

Chapter 1

Preface and Aims

RESEARCH PROBLEM

Osteoarthritis (OA), is a common cause of joint dysfunction, pain and long-term disability, and can have a profound negative impact on the quality of life of its sufferers. The ever growing prevalence and incidence of OA makes this condition a major healthcare problem (Flores and Hochberg 2003; Moskowitz *et al.* 2004). For decades the articular cartilage itself has been the central focus of research aimed to understand the OA patho-physiology. OA articular cartilage is characterized by an initial loss of proteoglycans from the upper zone followed by the degradation of the collagen network (Mankin *et al.* 1971). Several studies have provided evidence that synthesis of cartilage specific molecules such as type II collagen (COLII) and aggrecan (AGG) are significantly increased immediately after onset of the disease, suggesting that the articular chondrocytes try to repair the damaged matrix. However, this repair process eventually appears to fail, leading to irreversible cartilage degeneration (Eyre *et al.* 1980; Floman *et al.* 1980). In addition, the expression of hypertrophy and mineralization related markers has been reported in OA articular cartilage, which would be indicative of atypical phenotype changes in the cartilage during the course of disease development (Pullig *et al.* 2000; Pullig *et al.* 2000; Iannone and Lapadula 2003). The long-term molecular events responsible for these phenotypic transitions in OA articular cartilage are not well understood. However, OA changes are not just limited to cartilage. Recent studies suggest changes in juxta-articular subchondral bone as an important clinical manifestation. Bone turnover and composition, and the behaviour of osteoblasts are factors that are reported to be abnormal in OA patients (Lajeunesse 2004; Botter *et al.* 2008; Raynauld *et al.* 2008).

Substantial changes to both the subchondral bone and cartilage are common in most of the patients suffering from OA. Much knowledge about the cartilage and bone structural changes is already known, but as yet researchers have had little success in elucidating the pathogenesis of OA. Therefore, a new overall working hypothesis is necessary to better understand the stages involved in the disease progression in order to improve treatment options. *In vivo* OA pathology (both bone and cartilage) is more prominent in medial compartment compared to lateral compartment in femoro-tibial joint, and this is a clear indication that changes in the bone and cartilage during the

development of OA are associated with each other. This notion is further supported by the fact that certain OA animal models showed synchronized changes to both the subchondral bone and the cartilage during disease development (Botter *et al.* 2009). Furthermore, it has been proposed that subchondral bone sclerosis precedes and contributes to cartilage damage (Anderson-MacKenzie *et al.* 2005), but also that the cartilage degeneration itself can affect the abnormal bone remodelling (Bobinac *et al.* 2003). These observations suggest that both subchondral bone and cartilage cells can influence each other, hinting to a possibility of "altered bi-directional communication" during the development of OA. However, a significant knowledge gap currently exists in the understanding as to what extent cartilage cells and subchondral bone cells interact with each other with respect to regulation of differentation under normal and OA diseased conditions.

Matrix metalloproteinases (MMP) -2 and -9 are reported to be major culprits responsible for cartilage degeneration and abnormal bone remodelling during OA development (Burrage *et al.* 2006; Hulejova *et al.* 2007). It is clear that cell differentiation status and MMP expression levels are inter-related events (Murphy and Nagase 2008) and that an abnormal cell differentiation status can stimulate the production of MMPs. Given that interaction of subchondral bone osteoblasts (SBOs) and articular cartilage chondrocytes (ACCs) regulate each others differentiation, it would be of great interest to test whether altered cross-talk of OA SBOs and ACCs stimulate the levels of proteases further than it was required, thereby accelerating the degenerative changes in the joint.

The exact molecular mechanism(s) involved during the cross-talk of SBOs and ACCs in the normal and OA joint has yet to be investigated. Fully differentiated cells maintain their specialized character through signalling pathways, and amongst these signalling factors, the mitogen activated protein kinases (MAPKs) are reported to be responsible for the conversion of a large number of extracellular stimuli into specific cellular responses that range from positive and negative roles on cell proliferation and differentiation (Cobb 1999; Pearson *et al.* 2001). These kinases act as regulators of cell function by the addition of negatively charged phosphate groups to proteins, which activates the catalytic potential of the proteins (Nordle *et al.* 2007). The

requirement of MAPK signalling pathways, in particular protein kinase 38 (p38), extracellular signal regulated kinase 1 and 2 (ERK1/2), and c-Jun amino-terminal kinases (JNK) during various phases of cartilage and bone biology is well established (Hu *et al.* 2003; Stanton *et al.* 2003). Given the important role of MAPK signalling pathways in cartilage and bone biology, we considered whether these pathways influence the bi-directional communication of SBOs and ACCs.

Motivated by these issues, the overall aim of the thesis was to address the question of how SBOs and ACCs interact with respect to regulation of the other cell's phenotype and MMP expression under normal and OA joint conditions. A further aim was also to investigate the potential role of MAPK signalling pathways during this cellular communication process. In order to accomplish this, various *in vitro* co-culture models, which resembled the *in vivo* conditions, were developed. Molecular interactions can take place between cells at a distance as well as between cells in direct contact, therefore both direct and indirect co-culture models were designed, for this study purpose. Particular care was taken to maintain the chondrocyte phenotype in the co-culture models, since it is apparent that chondrocytes lose their phenotype in long-term monolayer cultures.

HYPOTHESES

Altered bi-directional communication between subchondral bone and articular cartilage cells lead to OA related phenotypic changes.

The specific aims of this thesis are summarized below:

1. To characterize the molecular changes associated with cartilage (Chapter 3&5) and subchondral bone (Chapter 4) in OA joint.
2. To investigate what effects ACCs, isolated from normal and OA joints, have on changes to SBOs differentiation (Chapter 4).
3. To investigate what effects SBOs, isolated from normal and OA joints, have on ACCs phenotype. In particular, we investigated the role of SBOs in inducing hypertrophic differentiation in ACCs (Chapter 5).

4. To examine whether altered bi-directional cell communication between OA SBOs and OA ACCs is a critical event which orchestrates the abnormal production of gelatinases MMP-2 and -9 (Chapter 6).
5. To test whether the bi-directional interactions of SBOs and ACCs is mediated *via* MAPK dependent signalling pathways (Chapter 4, 5, and 6).

THESIS STRUCTURE

This thesis consists of seven chapters. **Chapter 1** introduces the topics and **Chapter 2** provides an extended literature review on the topics investigated. *The methods and materials relating to each research paper is contained within the relevant chapters*. In **Chapter 3,** we characterized morphological and cellular localization differences of osteochondral sections graded according to OA disease severity, with an emphasis on putative chondrocyte hypertrophy markers and make the case of relating these events to post-natal changes. The main objective of this study is to build my basic knowledge on OA pathogenesis both at morphological and cellular level.

Chapters 4-6, specifically focus on the study of the bi-directional interactions between SBOs and ACCs. For this purpose co-culture models were developed and a series of molecular and cell biological methods were used to distinguish the changes in co-cultured *vs*. non co-cultured cells. **Chapter 4** is a recently published study, conducted to determine how ACCs isolated from normal and OA joints affect the SBO phenotype. In this study, we also investigated the involvement of MAPK signalling pathways during this interaction process. In **Chapter 5**, the reciprocal interactions of normal and OA SBOs were tested on ACCs with respect to hypertrophic differentiation and MAPK signalling pathways (Accepted for publication in Arthritis and Rheumatism). In **Chapter 6**, the regulation of MMP-2 and MMP-9 during the interaction of SBOs and ACCs isolated from normal and OA joints was investigated to unravel the pathological cascades that were triggered during the interactions of these cells. **Chapter 7** provides a summary and draws conclusions of the studies in the preceding chapters to demonstrate what new insights have been added to our understanding of the aetiology and biology of OA. Suggestions for future directions are discussed.

THESIS FORMAT

This thesis was written according to the guidelines of Queensland University of Technology's "Thesis by publication" format. References for Chapters 1, 2 and 7 are listed at the end of this thesis in the author-date format, and all figures and tables are listed in numerical order, within the individual chapters. Chapter 3-6 are presented in the required manuscript format of the journal to which they were submitted for publication, some variation in endnote style may therefore appear in these chapters. All abbreviations used in Chapters 1-7 are listed in front of the thesis in page number XII.

Chapter 2

Literature Review

BIOGRAPHY OF ARTICULAR CARTILAGE

Formation of articular cartilage: The sequences/phases involved during the development of articular joint have been described in many studies carried out on the limbs of mammalian and avian embryos (Fell 1925; Mitrovic 1978). The formation of an articular joint can be briefly divided into two phases. During the first phase, mesenchymal stem cells condense and undergo chondrogenic differentiation. In the second phase, a cartilaginous interzone first appears, specifying the joint structure (Pacifici *et al.* 2000; Koyama *et al.* 2007). As development continues the interzone becomes a clearly distinguishable, three zone structure. The middle zone is a layer of much looser mesenchyme tissue, separating the first and last zone and is responsible for generation of joint associated structures including synovium, ligaments and capsule (Pacifici *et al.* 2005). The first and last zone consists of chondrogenic like layers and plays an important role during initial oppositional longitudinal growth at the epiphysis (Pacifici *et al.* 2006).

It is fairly accepted now that the bulk of epiphysial chondrocytes will eventually undergo a secondary ossification process and subsequent endochondral ossification. However, the epiphysial chondrocytes that are in closer contact with the synovial cavity and tissues will survive and become "*permanent bona fide articular chondrocytes*"(Pacifici *et al.* 2000). The divergence in differentiation between articular cartilage and growth plate chondrocytes (transient chondrocytes) will occur after initial chondrocyte commitment, at the initial stage of joint specification, when the interzone first starts to appear (Onyekwelu *et al.* 2009). A brief account of stages involved during the articular joint development is shown in **figure *1***.

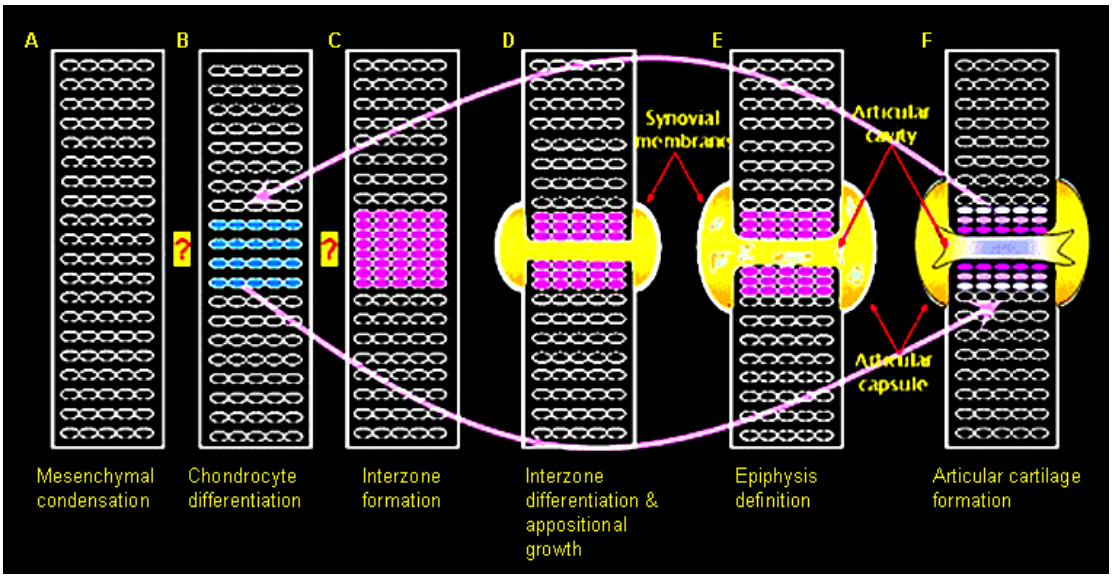

Figure 1: Diagram showing articular cartilage development. (A) Mesenchymal condensation with patterned site for joint development. **(B)** Mesenchymal cell differentiation into uninterrupted cartilaginous element. **(C)** Interzone formation **(D)** Interzone differentiation into an intermediate layer and two chondrogeneic layers, which contribute to longitudinal appositional growth **(E)** Epiphysis definition with concurrent decrease of appositional growth and formation of growth plates, which now become the main mechanism of longitudinal growth and **(F)** Development of articular chondrocytes and emergence of secondary ossification centres. (ww.kuleuven.be/rheumatology/images/develop1.gif and (Pacifici *et al.* 2000).

Structure of "permanent articular cartilage": Articular cartilage is a flexible load bearing tissue that covers the surface of diarthrodial joints. It is supported by bone and protects it by transferring equal amounts of load across the subchondral bone due to its exceptional elastic properties. There are three different types of cartilage in the body - *elastic cartilage* found in the epiglottis and eustachian tubes (passage ways that connect the ear to the throat**);** *fibrous cartilage*, found in the discs of the spine and knee; and *hyaline cartilage,* found in joints covering long bones (Freeman 1973). Whilst the structure of each type of cartilage differs slightly from one another, they all contain the same principal building components such as water, extra cellular matrix (ECM) components (Type II collagen (COLII) fibers, and proteoglycans (small leucine-rich proteoglycans, versican, perlecan, glycolproteins and aggrecan (AGG)).

Structure of cartilage comprises of 60-70% water (wet weight (WW)), 30-40 % (WW) or 70% dry weight (DW) of collagen and 5-10% (WW) or 10-20% (DW) of proteoglycans and 2-5% chondrocytes. The average thickness of the articular cartilage including the calcified cartilage layer is approximately 2.5 mm for adult human knee joint and it may differs between species (Mankin 2000). Approximately 10,000 chondrocyte cells are present per mm^3 of cartilage tissue (Mankin 2000). Chondrocytes attach to ECM components with the help of various matrix receptors and integrins (Loeser 1993). Cartilage is an avascular, fibrous connective tissue lacking nerve fibres.

The structure of articular cartilage is grossly classified as having four zones, the superficial zone, the middle zone, the deep zone, and the calcified zone (Poole *et al.* 2001). The majority of the superficial layer consists of collagen fibers and a few flattened chondrocytes arranged in a meshwork pattern. The middle layer contains chondrocytes that are larger, and the collagen fibres in this layer are randomly oriented. In the deep stratum, chondrocytes are arranged in vertical columns separated by collagenous fibrils. The last layer is called calcified cartilage since it contains partially mineralized matrix and hypertrophic chondrocytes and this zone shows many similarities with chondrocytes present in hypertrophic zone of growth plate cartilage (Sun and Kandel 1999; Mankin 2000). The calcified zone is separated from the deep zone of cartilage by the tidemark (***Figure 2***). The structure of the tidemark consists of 8-10 layers of collagen fibrils arranged as a thick bundle (Poole *et al.* 2001; Lyons *et al.* 2005).

Differentiation of normal articular cartilage: Healthy chondrocytes have a stable phenotype characterized by a round shape, a low rate of proliferation and by the production of extracellular matrix components such as proteoglycans and COLII. Articular cartilage chondrocytes under normal conditions are resistant to undergoing hypertrophy and express very low levels of matrix metalloproteinases (MMPs) (Onyekwelu *et al.* 2009). Typically, the differentiation process of chondrocytes, *in vitro* can be mimicked by addition of growth factors such as transforming growth factor- beta 3 (TGFβ3) and other essential supplements (Tang *et al.* 2009). Healthy chondrocytes remain in a post-mitotic inert state throughout life, with their declining

proliferative potential being attributed to replicative senescence and decrease of telomere length (Martin *et al.* 2004).

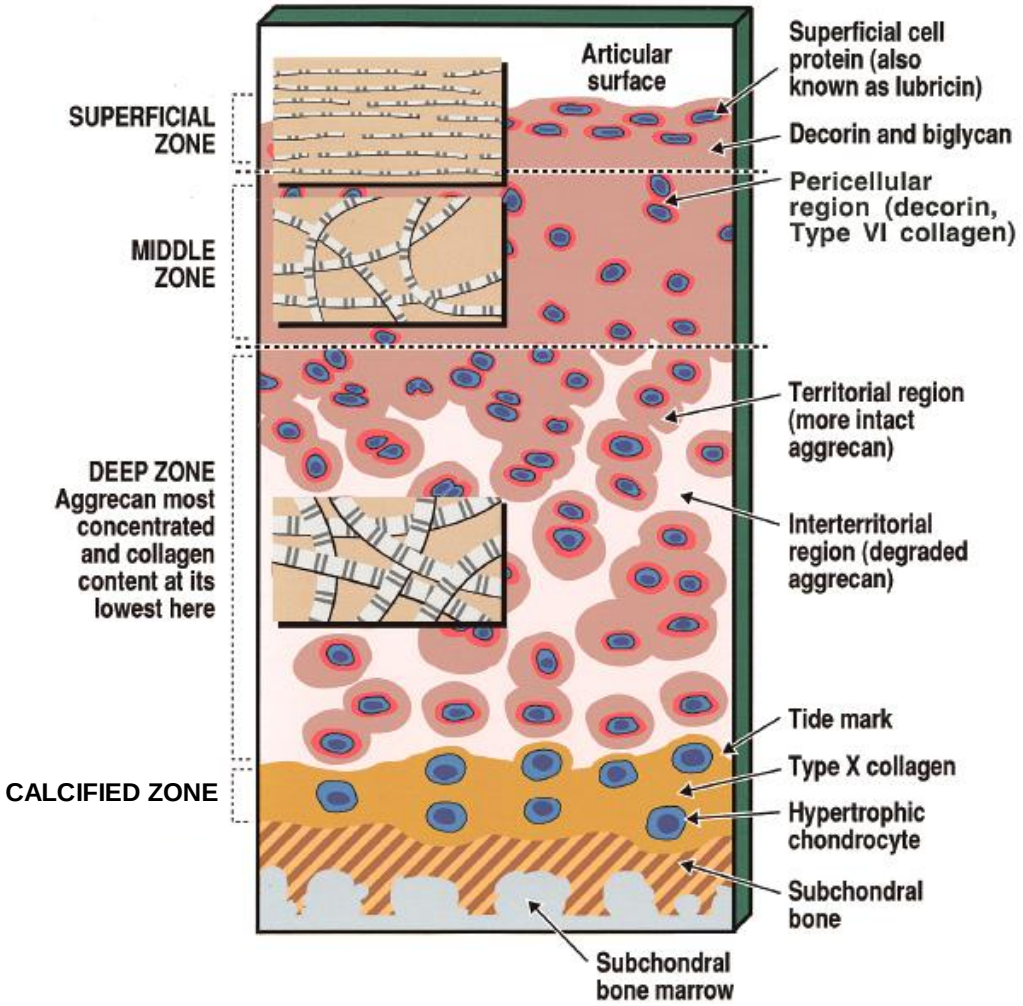

Figure 2: The structure of human adult articular cartilage, showing the zones of cellular distribution and the superficial, middle, and deep regions of matrix organization. *Insets* show the relative diameters and orientations of collagen fibrils in the different zones. The positions of the tidemark and subchondral bone and other special features of matrix composition also are noted (Poole *et al.* 2001).

Challenges of *in vitro* chondrocyte culture: It has become obvious from *in vitro* studies that culturing chondrocytes as an adherent monolayer invariably leads to a process of de-differentiation. *In vitro*, the cells acquire a fibroblastic morphology and

lose their chondrocyte specific gene-expression pattern after only a few passages (Lin *et al.* 2008). In particular, when chondrocytes are cultured in monolayer there will be a prompt down-regulation of the expression of cartilage-specific genes, such as those encoding COLII and AGG, and a concomitant initiation or upregulation of the expression of fibroblast associated genes such as those for type I, III and V collagens and versican (Melero-Martin and Al-Rubeai 2007). To understand the process of cartilage physiology in both normal and diseased milieu, it is important to ensure that the expanded cell population retains its phenotypic function in the *in vitro* culture. De-differentiation occurs as soon as the chondrocytes are released from their ECM and when cultured under circumstances that promote flattened cell morphology, such as at low cell density in monolayer and this process is gradual with increasing passage number. De-differentiation can be prevented by culturing chondrocytes under conditions that hold back cell flattening (Watt 1988). Some of the most well-known methods used to redifferentiate chondrocytes are culturing them in high density micromasses and 3- dimensional pellet cultures (Croucher *et al.* 2000; Huch *et al.* 2002; Stanton *et al.* 2004). The detailed procedures of these methods will be described in succeeding chapters.

OSTEOARTHRITIS (OA)

An historical perspective and demographics of OA: OA is probably the planet's oldest known disease as its presence was noticed in the skeletons of dinosaurs and other prehistoric animals (Wells 1973). The disease was a common finding in Egyptian mummies (Braunstein *et al.* 1988) and nearly half the 400 skeletons of ancient Saxons in England examined by Rogers and his colleagues (Rogers *et al.* 1981) had evidence of OA. In a further study (Thould and Thould 1983) in 416 Romano-British skeletons, approximately two-thirds had OA and this was a major cause of disability especially in the elderly (Lawrence *et al.* 1966; Lawrence 1976). All races are affected (Bremner *et al.* 1968; Solomon and Beighton 1975; Solomon *et al.* 1975; Solomon *et al.* 1975; Cronan *et al.* 1989) and the disease is not confined to any particular geographical area (Roberts and Burch 1966). After the age of 50, the increase in incidence of severe OA is exponential (Lawrence *et al.* 1966). OA, can,

however, affect young adults (Lowman 1955). The condition affects approximately 3 million Australians, or approximately 15 % of the population. The economic impact of OA on society is also significant. The direct medical costs to the Australian healthcare budget attributable to OA in 2000–2001 were in the order of $1.5 billion which accounts for 3% of the total health budget. The major component costs were hospitalisation (43% predominantly for joint replacement surgery), visits to general practitioners and specialists (29%), prescription and over-the-counter medications (9%), and allied healthcare (6%) indicating the high prevalence of the disease. The financial burden of OA is also manifested by; its significant contribution to work absenteeism and early retirement (Arthritis research foundation, Australia (www.arthritisfoundation.com.au)).

Factors responsible for OA: The aetiology of OA is multi-factorial and these factors can be broadly divided in to non-genetic and genetic factors. Among the non-genetic factors: age, gender, obesity, inactive lifestyle, joint injury and occupation (certain occupations requiring constant bending of knees and heavy lifting) may have a role in OA development. Among the genetic factors: hereditary and altered gene expression pattern of cartilage and subchondral bone tissues are reported to play a predominant role in the disease onset and progression (Iannone and Lapadula 2003).

Symptoms of OA: The following are some of the common symptoms in OA patients. Changes within the joint may lead to quite severe pain. Pain may even be present at rest as well as in movement. In some people, symptoms may be very mild and occasional, perhaps being brought on by periods of increased use or some minor injury. Joint stiffness may occur after a period of inactivity. Swelling and redness of the joint(s) may sometimes occur. Increased muscle weakness occurs; ultimately leading to the joint feeling unsafe and unstable. A crunching or crackling noise may occur when the joint moves (www.osteoarthritissymptoms.org/).

Current treatment approaches to OA: Most of the patients affected with OA notice the symptoms only when the disease is at the final stage, which often leads to surgery. Joint replacement surgeries especially for hips, knees and less commonly shoulders are now being performed to correct the defect. Paracetamol can be used for the

effective relief of pain and discomfort associated with OA and has no harmful side effects on the stomach. Non Steroidal Anti Inflammatory drugs (NSAIDS) are used to treat inflammation and pain (Dougados 2006; Kneitz *et al.* 2006; Jawad and Irving 2007; Sun *et al.* 2007). Glucosamine sulfate (GS) and chondroitin sulfate (CS) are derivatives of glycosaminoglycans found in articular cartilage and some pateints reported the effectiveness of this analgesic for the OA pain relief (Brief *et al.* 2001; McAlindon 2001; Clegg *et al.* 2006). Some studies with varying levels of evidence suggest that sodium hyaluronan, doxycycline, matrix metalloproteinase (MMP) inhibitors, bisphosphonates, calcitonin, diacerein, cyclo-oxygenase 2 specific inhibitors (CSIs) and avocado-soybean unsaponifiables may modify disease progression (Abramson *et al.* 2006, Takemoto *et al.* 2008). Non-pharmacological management strategies include educational and intervention, weight loss, exercise, physiotherapy and mechanical aids (Balint and Szebenyi 1997; Hsieh and Dominick 2003).

OA cartilage: OA initially is treated as a disease of cartilage. But the growing evidence suggests OA as an organ level failure, involving cartilage, bone, synovium and capsule. The comprehensive subchondral bone changes that are associated with OA are discussed in coming sections. The macroscopic OA cartilage changes include chipping or forming tiny cracks. In advanced cases, there is a total loss of the cartilage cushion between the bones of the joints, which causes friction between the bones, leading to pain and restriction of joint mobility. In knee OA, the medial compartment more often shows prominent OA changes compared to the lateral compartment. This phenomenon is explained as that the medial compartment is susceptible to more weight bearing compared to lateral compartment under certain conditions, for example obesity and strenuous work. This unequal distribution of mechanical load has been reported to direct the biological activation leading to wear and tear of cartilage (Li and Aspden 1997; Burr and Radin 2003). A typical illustration of OA changes, more prominent in medial compartment is depicted in ***figure 3.***

At a molecular level, OA chondrocytes undergo several changes. The predominant chondrocyte phenotypic changes that are commonly associated with OA include production of type I and III collagens, which could be suggestive of the de-

differentiation process of these cells (Sandell and Aigner 2001). Furthermore, OA chondrocytes are also capable of synthesizing typical terminal differentiated markers which point towards the fact that OA articular chondrocytes are undergoing differentiation towards hypertrophic phenotype (Pullig *et al.* 2000; Iannone and Lapadula 2008). At present, the factors inducing this phenotype shift in OA chondrocytes for the most part remain unidentified. A brief account on the importance of hypertrophic changes during the endochondral ossification and its pathological role in different diseases and in OA cartilage will be discussed in the next section.

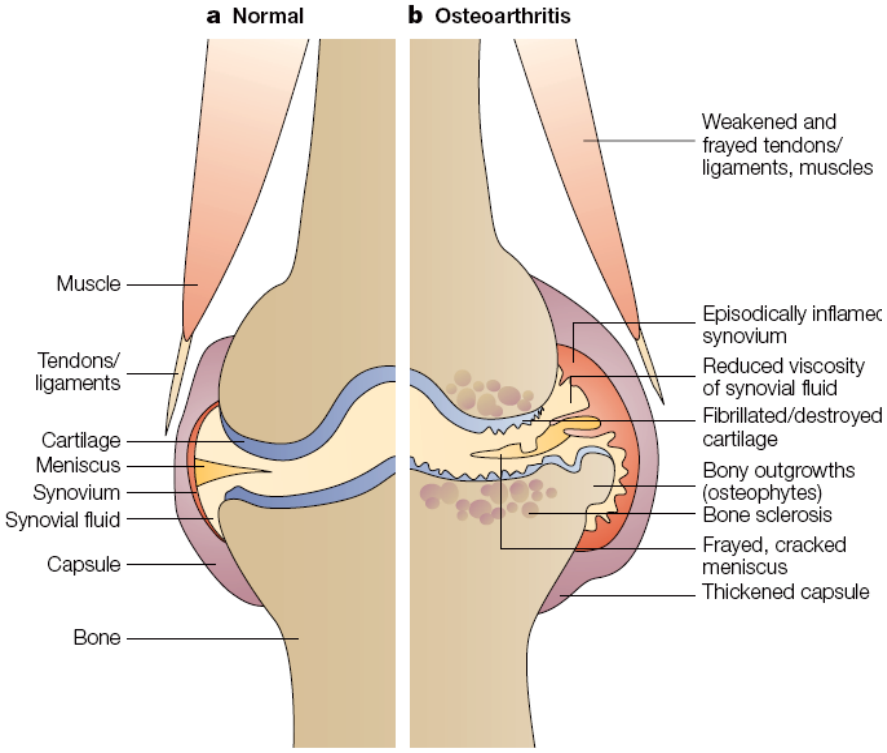

Figure 3: **Articular structures that are affected in OA. (A)** Healthy tissue of lateral compartment is shown: normal cartilage without any fissures, no signs of synovial inflammation. **(B)** Early focal degenerate lesion and fibrillated cartilage, as well as remodelling of bone, is observed in medial compartment in OA patients. This can lead to bony outgrowth and subchondral sclerosis (Wieland *et al.* 2005).

Chondrocyte hypertrophy in growth plate and pathological diseases: Chondrocyte hypertrophy/ mineralization is typically seen in the growth plate

chondrocytes, that are destined to form bone. In endochondral ossification, an initially avascular cartilaginous template undergoes hypertrophy and mineralization, vascular invasion, MMP (Matrix metalloproteinases) or osteoclast-dependent remodelling, and ultimately bone deposition by osteoblasts (Anderson 2002). In the growth plate, hypertrophic chondrocytes acts as a template for bone formation. It has been reported that hypertrophic chondrocytes send signals for: osteoblast recruitment and differentiation (Nurminskaya *et al.* 2003), vascularisation (Gerber and Ferrara 2000) and MMPs expression (Tchetina *et al.* 2003) which plays a vital role in cartilage resorption and subsequent longitudinal growth (Ortega *et al.* 2003). Any disturbances in the hypertrophic differentiation phase of growth plate chondrocytes lead to growth arrest (Zelzer and Olsen 2003).

Except in the last phase of growth plate cartilage, the cells present in the soft tissues such as skin, tendons, blood vessels and articular cartilage never undergo hypertrophy related changes in normal conditions. However, such hypertrophic changes are reported to be initiated in certain pathological conditions such as atherosclerosis (Kapustin and Shanahan 2009), cutaneous calcification (Rivet *et al.* 2006), cardiovascular diseases (Van Campenhout and Golledge 2009) and OA (Pullig *et al.* 2000). X-ray images of some of the common diseases associated with soft tissue mineralization are shown in ***figure 4***.

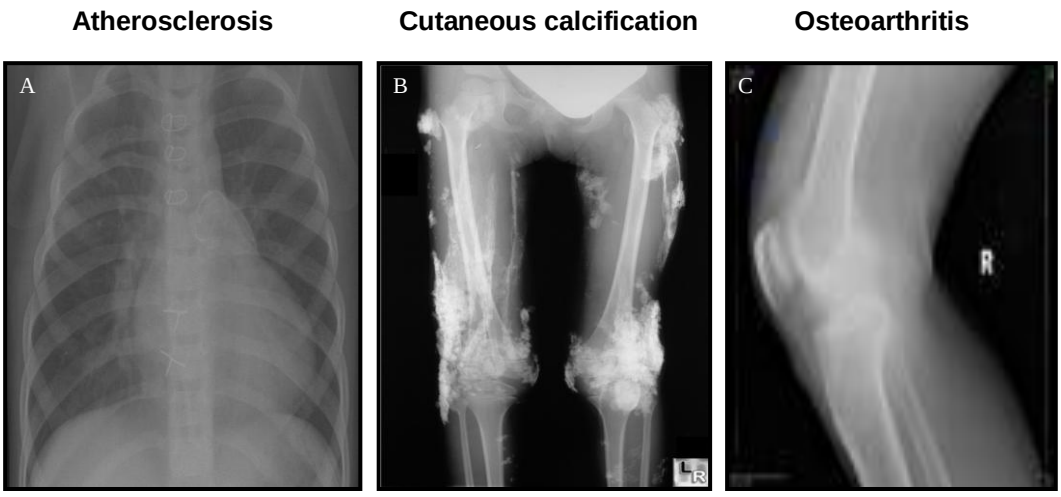

Figure 4: X- ray images showing different types of pathological soft tissue mineralization. **(A)** Chest X-ray of 54- year old patient showing the calcification of

arterial valves (med-ed.psu.ac.th/.../srp/srp8/case_srp8_1.html). **(B)** X-ray image of 32-year-old female showing the skin calcification (tmcr.usuhs.mil/tmcr/chapter5/05-020A.jpg). **(C)** X-ray image of 72 year old OA patient sowing articular cartilage mineralization (www.learningradiology.com/.../cow207.jpg).

As yet it is unclear why soft tissue cells have the tendency to undergo hypertrophic changes in various pathological conditions. In the beginning it was thought that pathological mineralization was the result of physiochemical precipitation of calcium and phosphates (Huitema and Vaandrager 2007). However, recent studies into various pathological mineralization conditions have provided evidence that soft tissue mineralization is a regulated process showing many similarities with gowthplate hypertrophic cartilage cells (Rutsch and Terkeltaub 2003). Accumulating evidence now points toward a competition between factors promoting hypertrophy and inhibitors of hypertrophy (Rutsch and Terkeltaub 2005). Besides the biochemical factors, the proteins like Annexin II, V and VI (Kirsch 2005), ankylosis (ANK) protein (Zaka and Williams 2006), transglutaminases (Heinkel *et al.* 2004) and osteopontin (OPN) (Pampena *et al.* 2004) have been reported to mediate the hypertrophic changes in the cells during endochondral ossification and in pathological diseases. Because of these common denominators, endochondral ossification has been proposed as a developmental model to understand soft tissue hypertrophy in different diseases (Olivotto *et al.* 2008).

Hypertrophic chondrocyte change in OA cartilage: It has been suggested that normal chondrocytes are kept in a state of maturational arrest (Drissi *et al.* 2005). In contrast to this phenotype, it has been shown that OA chondrocytes express typical hypertrophic differentiation related markers such as, Type X collagen (COLX), alkaline phophotase (ALP) and OPN, an indicative that these cells have undergone differentiation to a matured phenotypic state (Pullig *et al.* 2000; Pullig *et al.* 2000; Iwamoto *et al.* 2003).

It has been suggested that MMP expression by hypertrophic chondrocytes plays a crucial role in degrading the cartilage matrix (ECM) components during the final phase of endochondral ossification (Sandell and Aigner 2001). MMPs are responsible

for catabolism and turnover of the matrix, and are induced during the process of chondrocyte hypertrophic phase of endochondral ossification (Tchetina *et al.* 2003, D'Angelo *et al.* 2000). Without these proteases the cartilage remains and the mature bone layers will not form correctly (Inada *et al.* 2004). This suggests that cartilage chondrocyte hypertrophy is in part responsible for MMP regulation during endochondral ossification. Given that cartilage hypertrophy is a feature of OA cartilage it is likely that these phenotypic changes can increase expression of degenerative enzymes such as MMPs several fold, thereby accelerating cartilage degradation similar to that seen during early phases of bone development. Indeed, it has been reported that induced mineralization in articular cartilage up regulates the MMPs expression several fold (Cheung 2005). These results together indicate that alterations in the phenotypic changes of articular cartilage chondrocytes to a hypertrophic phenotype could be pathological to the structure, health and integrity of the cartilage leading to its degeneration.

However, not all studies are in agreement with the above studies with respect to alterations in phenotypic changes of OA articular chondrocytes. It has been shown that the processes leading to chondrocyte hypertrophy was a consequence of age rather than an integral part of OA itself (Mitsuyama *et al.* 2007). On the other hand, studies showed no evidence of generalized hypertrophy related changes in OA chondrocytes (Brew *et al.* 2008). Because of these discrepancies in the existing literature, it is not clear whether chondrocyte hypertrophy is a common finding or just merely an incidental finding in some patients suffering with OA. It is therefore, important to first authenticate whether or not chondrocyte hypertrophic changes are fundamental to OA cartilage pathology. Further studies are required to completely demonstrate this phenomenon in relation to OA patho-physiology, by localizing a broad array of hypertrophy markers both at tissue and cellular level, avoiding the variations related to age, lifestyle, gender and genetics during the data analysis.

BIOGRAPHY OF SUBCHONDRAL BONE

Formation of bone: With the exception of clavicles and some bones of the cranium, which develop without a cartilage template through *intramembranous ossification*, all the other bones in the body, develop through the process of *endochondral ossification*, which makes use of a cartilage template (Steele *et al.* 1988). Endochondral ossification takes place in the growth plate, a specialized cartilaginous structure located in the area between epiphysis and the diaphysis of infants and adolescents (Carter 2001).

Throughout the entire growth period, chondrocytes present in the growth plate undergo distinct transformations and the cartilage model is continually replaced by bone as length increases (Anderson 2002). As the skeleton reaches maturity the pace of longitudinal bone growth ceases as proliferation of growth plate chondrocytes reduces. Decreased growth rate proliferation is coupled with structural changes in the epiphysis leading to condensed height of the proliferative and hypertrophic zones and reduced hypertrophic cell size and column density (Weise *et al.* 2001). In humans and some other mammals, the growth plate is completely resorbed following puberty, resulting in fusion of the epiphysis to the metaphysis. A detail of phases involved during the bone formation is shown in the ***figure 5***.

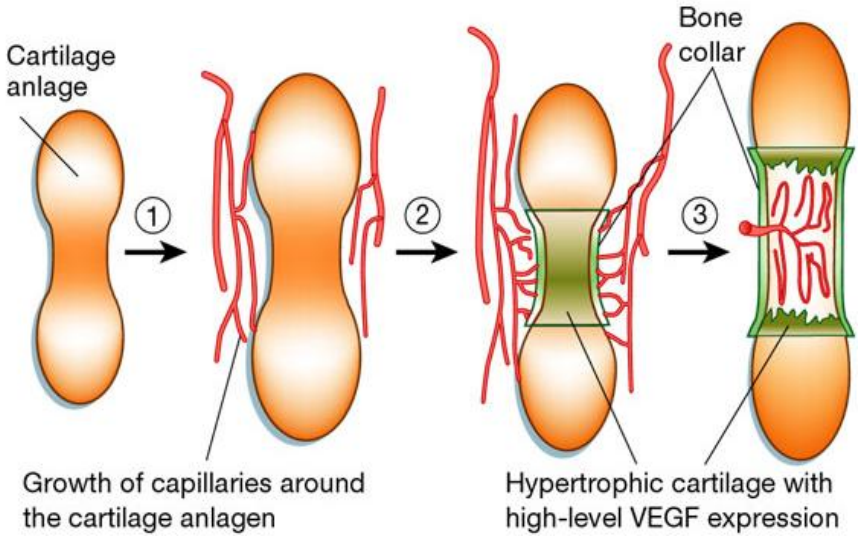

Figure 5: Illustration of steps involved in endochondral bone formation. Initially mesenchymal stem cells condense to form a cartilage template, followed by replacement of the models by bone. In step one; capillaries grow around the cartilage anlagen. In step two, cells around the cartilage differentiate to osteoblasts and produce a collar of bone around the middle of the cartilage. Chondrocytes in the centre of the cartilage mature to hypertrophy and express high levels of vascular endothelial growth factor (VEGF), a factor needed for invasion of capillaries into the cartilage. In step three, hypertrophic cartilage is replaced by marrow leading to bone formation (Zelzer and Olsen 2003).

Structure of subchondral bone: The calcified layer of mature articular cartilage lies on the surface of subchondral bone. The subchondral bone is composed of two types of lamelle: concentric layers around osteons and flat layers typical of appositional new bone formation (Clark and Huber 1990). It has been shown that the mineral in calcified cartilage is bound to the collagen fibrils in bone (Duncan *et al.* 1987). The subchondral bone plate is corticalized, it is not very porous and may not be very vascular (Burr 2004). Beneath the subchondral bone plate is subchondral trabecular bone. Though the molecular differences between subchondral bone and trabecular bone are not clear, these two tissues are structurally organized differently, to adapt to mechanical loading (Choi *et al.* 1990; Martin *et al.* 1998).

OA subchondral bone: The predominant typical feature of OA is the degeneration of articular cartilage; hence for several decades biochemical investigations in to the pathogenesis of OA have concentrated on the mechanisms involved in the destruction of articular cartilage. Radin and Rose were first to postulate that thickening of subchondral bone plate as an important clinical manifestation in OA patients. These authors hypothesized that the "health and integrity of the overlying articular cartilage depends on the mechanical properties of its bony bed, and stiffening of the subchondral bone can affect joint conformation, which involves deformation of articular cartilage and bone to create maximum contact areas under load (Radin and Rose 1986)". Recent studies re-emphasized the importance of subchondral bone changes such as composition, architecture, quality and regulation as an important distinguishing feature of OA (Burr 2004). The typical structure of OA subchondral bone is shown in ***figure 6***.

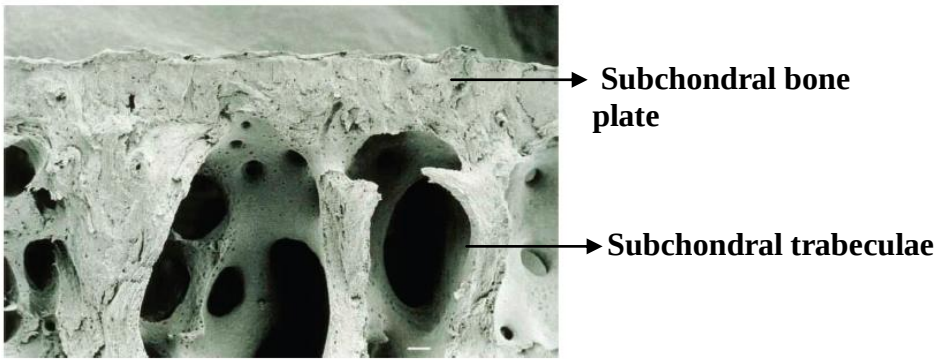

Figure 6: A scanning electron micrograph of subchondral bone, from a 75-yr-old patient, suffering with OA, showing the porous and dense texture of the bone matrix. (Scale bar 100 μm) (Aspden 2008).

Biochemical and mineral composition of OA subchondral bone tissue: Biochemical studies have demonstrated an abnormal behaviour of OA subchondral bone compared to normal. Increased bone activity in patients with severe OA, was observed using labeled bi-/phosphate in a scintigraphic study (Dieppe *et al.* 1993), while in an other study (Mansell and Bailey 1998) an increase in collagen metabolism of the OA bone was observed. It has been shown that subchondral bone explants from OA patients, secrete higher levels of TGF-β1, ALP, IL (interleukin)-6, uPA (urokinase plasminogen activator), prostaglandin and IGF (insulin growth factor)-1

compared with normal bone tissues (Lajeunesse *et al.* 1999; Bailey *et al.* 2004). All these biochemical factors favour bone formation, therefore these results point towards the fact that in OA, there is an increase of bone cell, *i.e* osteoblast, anabolic activity, an opposite scenario to what we see in osteoporosis patients. Indeed, several studies have demonstrated that OA patients are resistant to osteoporosis (Sambrook and Naganathan 1997, Shen *et al.* 2009). With respect to the mineralization content of OA bone, some argument exists in the literature, with reports on an increase of bone mineral density with progression of OA (Dequeker *et al.* 1997; Dequeker *et al.* 2003), while other studies describe an abnormal low mineralization (Grynpas *et al.* 1991). The detailed osteoblast changes reported to subsist in the OA compared to normal subchondral bone at the cellular level were summarized in the next section.

Altered subchondral bone cell metabolism in OA patients: Recent findings suggest that, at cellular level, function of subchondral osteoblasts may be altered in OA (Massicotte *et al.* 2002). OA subchondral bone osteoblasts (SBOs) express higher levels of ALP mRNA than those from normal individuals (Truong *et al.* 2006). Likewise, when osteocalcin (OC) expression was evaluated at the protein level it was clearly elevated in OA SBOs (Gevers *et al.* 1989; Hilal *et al.* 1998). An anomalous expression with respect to Type 1 collagen (COL1) has also been reported in OA SBOs (Mansell and Bailey 1998). The growth factors such as IGF-1, IGF-2 and TGF-β levels have been reported to be overproduced by OA SBOs (Hilal *et al.* 2001). In a recent study ALP, Transglutaminases, C-terminal type 1 procolagen propeptide, OC, OPN, IL-6, IL-8, and TGF-β1 were reported to be significantly increased in sclerotic OA SBOs compared to patient matched non sclerotic SBOs (Sanchez *et al.* 2008). These findings collectively emphasise that the OA SBOs have enhanced osteogenic capabilities and produce abnormal cytokine and growth factors compared to normal SBOs. Furthermore, it has been proposed that this abnormal behaviour of SBOs can influence the overlying cartilage properties (Lajeunesse and Reboul 2003).

Mechanisms responsible for abnormal subchondral bone remodelling in OA: The mechanism by which the subchondral bone in OA undergoes change is unclear. The excessive loading and as a result the micro-fractures in the bone are thought to be important factors (Frost 1994). Micro-fractures can be accompanied by activation of

remodelling and osteoblast activity, a process bone adopts to heal these cracks, leading to increased bone density and metabolism. Some authors hypothesized the role of altered cytokine expression (Fazzalari *et al.* 2001) and apoptosis of osteocytes (Verborgt *et al.* 2000) in stimulating bone remodelling. Nevertheless, this phenomenon was just a hypothesis and has yet to be demonstrated. Vascular disturbances that cause subchondral bone necrosis may also be involved in abnormal bone remodelling (Imhof *et al.* 1997). Furthermore, a positive correlation between cartilage degeneration and subchondral bone remodelling was observed, indicating changes in cartilage may affect the underlying bony properties (Botter *et al.* 2008).

Based on the above literature it is apparent that the OA changes include significant cartilage and subchondral bone changes. However, the exact mechanism by which these structural changes occur during the development of OA is not completely understood. In this respect, some recent studies have shown extensive cross-talk between bone and cartilage in maintaining the normal joint homeostasis and also in the development of OA. These findings from the literature and the current knowledge gap in understanding these interactions will be reviewed in the next section.

CROSS-TALK OF BONE AND CARTILAGE CELLS

Terminology of cell interactions: Cell interaction is a term refering to the mechanism whereby one cell population influences the development of neighbouring cells. The interaction of cells can be unidirectional or bi-directional. The cross-talk of two types of cells can direct each others differentiation and any alterations in the cross-talk may lead to an undesirable pathway leading to pathological diseases (Zalatnai 2006). Cells are also able to communicate at different thresholds. For example, a low concentration of an inductive signal causes a cell to presume fate A, but a higher concentration can direct the cell to fate B (Berezhkovskii *et al.* 2008). Cell communication occur mainly by membrane bound factors or soluble factors (Grellier *et al.* 2009). Membrane bound factors include specialized intercellular channels (Barbe *et al.* 2006), adhesion molecules (Norman *et al.* 2008), connexins (Serre-Beinier *et al.* 2002), integrins (Wang *et al.* 2007) and gap junctions (Meda

1996). Additionally, the communication through soluble factors are mediated by growth factors (Ko *et al.* 2009; Zhang *et al.* 2009), cytokines (Sundararaj *et al.* 2009) and chemokines (Zujovic and Taupin 2003) which consecutively bind to receptors in the recipient cell. This binding of soluble factors to receptors triggers the signalling pathway coding to a specific phenotype or function in a cell. However, the recent data suggest that transcription factors - in particular, homeoproteins - can be transferred from cell to cell and code for a particular function and activity (Prochiantz and Joliot 2003). The model of cell-to-cell interactions was shown in the **figure 7**.

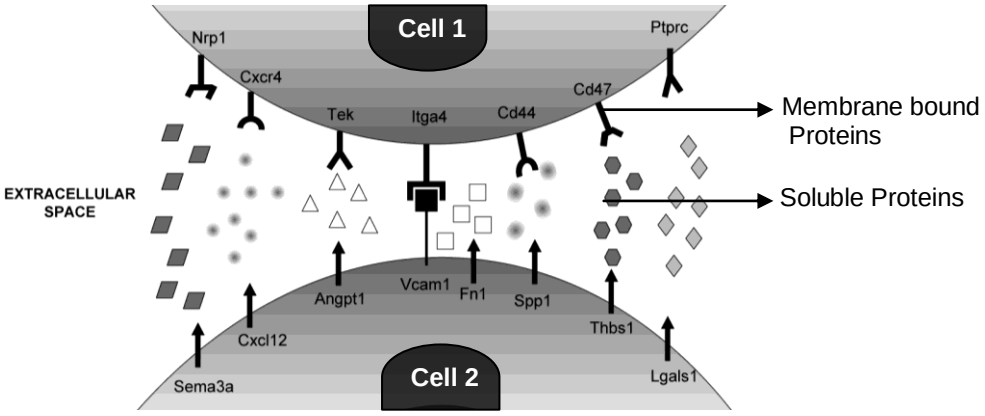

Figure 7: Model depicting that the cell-to-cell interactions can occur via both membrane bound and soluble factors which intun can transude in to cell fate determinations (Pedemonte *et al.* 2007).

How bone and cartilage cross-talk could occur in the joint?

Since the physical separation between articular cartilage chondrocytes and subchondral bone osteoblasts is generally significant at invivo level, how would it be possible that paracrine regulation could be occurring? Given the nature of the matrix in adult bone and cartilage, the radius of a potential morphogen gradient was also belived to be quite small. However, ruling out these theories, several recent studies

have demonstraed the presence of connections such as microcracks (Sokoloff 1993), vascular channels (Shariff *et al.* 1995) and noevascularization between subchondral bone and cartilage giving rise to speculations that mediators produced by subchondral bone or *vice versa* may pass through these channels thereby directing the cell-to-cell interactions. Moreover, it has been suggested that the products derived from subchondral bone or cartilage are readily secreted into the joint space, as evidenced by their detection in synovial fluid. Therefore, it is likely that the molecules that influence the cartilage or bone will gain access with each other through the synovial fluid (Westacott *et al.* 1997). Furthermore, it has been belived until recent times that adult articular cartilage receives its nutrition from snovial fluid. Neverthless, the recent observations in animals gave rise to suggestions that nutrients from the medullary cavity in bone may nourish cartilage via channels that connect bone with cartilage as well as the blood vessels indicating that communication between the two structures are essential for healthy joint (Imhof *et al.* 1999, Malinin *et al.* 2000).

Interactions of bone and cartilage cells during endochondral ossification: The pathways that regulate chondrocyte hypertrophy and matrix mineralization are linked to signalling by systemic hormones and growth factors (Van der Eerden *et al.* 2003) during endochondral bone development. Besides these signals, interactions of osteoblasts and chondrocytes play an important role for the coordinated regulation of the matrix calcification and eventual integration of cartilage with the bone. Cartilage hypertrophy during endochondral ossification appears to be the result of signals derived from various cells such as osteoblasts and haematopoietic cells (Johnstone *et al.* 2000). In another study it was demonstrated that hypertrophy in ectopic cartilage was enhanced in the presence of osteoblasts or their conditioned medium, but inhibited by co-culture with skin fibroblast-like cells (Zimmermann *et al.* 1990). On the other hand, it was demonstrated that the signals from the chick hypertrophic cartilage chondrocytes direct osteoblast differentiation and subsequent bone matrix deposition (Nurminskaya *et al.* 2003). These results collectively emphasize the important role of bi-directional co-ordinated communication between chondrocytes and osteoblasts during early bone development.

Cross-talk of subchondral bone and articular cartilage – At tissue level: Subchondral bone and articular cartilage play balancing roles to bear mechanical load across the joints, thereby maintaining normal joint homeostasis. In explaining the relationship between cartilage degeneration and subchondral bone in OA development, Radin and Rose proposed a "cushion-and-chair analogy: subchondral bone (represented by a chair) present under cartilage (represented by a cushion) is partially weakened, and additional stress on this condition may induce cartilage degeneration" (Radin and Rose 1986). In some OA animal models, alterations of the subchondral bone changes preceded cartilage changes (Quasnichka *et al.* 2006). It was observed that an increase in the stiffness of subchondral bone lead to progression of cartilage degeneration in a rabbit OA animal model (Serink *et al.* 1977). Similarly, it has been demonstrated that mechanically induced injury in subchondral bone led to increased remodelling and subsequent loss of cartilage (Wei *et al.* 1998). These studies together support that the subchondral bone changes precede cartilage degeneration and these changes may be responsible for the alterations in the cartilage properties.

Other studies have demonstrated that the cartilage changes occur first and can influence the subchondral bone density in OA animal models (Bobinac *et al.* 2003; Burger *et al.* 2007). Histomorphometric and MRI studies have shown that increase in cartilage degradation is correlated to enhanced subchondral bone remodelling (Yamada *et al.* 2002). These findings indicate the likelihood of cartilage influencing bone metabolism.

In contrast to the above studies, some authors have demonstrated that the changes in subchondral bone and cartilage occur concurrently in many animal models (Ding *et al.* 1998; Botter *et al.* 2009; Stok *et al.* 2009). Altogether, these studies strongly suggest that the articular cartilage and subchondral bone can influence each others structural properties possibly by communicating with each other. Indeed, this communication of bone and cartilage was further strengthened by recent *in vitro* studies as discussed in the next section.

Cross-talk of subchondral bone and articular cartilage – At cellular level: So far only two studies are present in the literature, demonstrating the role of osteoblasts and chondrocytes interactions in regulating each others phenotype and differentiation in order to maintain normal joint homeostasis (Jiang *et al.* 2005; Nakaoka *et al.* 2006). However, these studies showed inconsistent findings. For example, osteoblasts were reported to enhance the phenotype of the chondrocytes under direct co-culture conditions (Nakaoka *et al.* 2006). In contradiction to the above observed results it has been demonstrated that the GAG deposition was lower in the co-cultured group containing osteoblasts compared to non co-cultured chondrocytes (Jiang *et al.* 2005). Hence, based on the available literature it is difficult to draw any conclusions on the reciprocal interactions that are required to maintain joint homeostasis.

Even less is known about the communication between subchondral bone and cartilage in the development of OA. So far only three studies are available in the literature, reporting that OA SBOs decrease the cartilage specific phenotype (GAG, AGG, COL2) upon co-culture with ACCs (Westacott *et al.* 1997; Sanchez *et al.* 2005; Sanchez *et al.* 2005). However, the molecular mechanism(s) by which these interactions occur during the cross-talk of SBOs and ACCs in normal or OA joints has not been investigated to date. Also, the effect of ACCs on the adjacent SBOs in the development of OA has not been studied. Therefore, further studies are required to understand, how articular cartilage cells and subchondral bone cells are interacting in the healthy joint in the comparison to the diseased OA joint, to examine whether altered interactions between these two cells are the primary cause of OA development.

Methods review in studying cell-to-cell interactions: Cell-to-cell interactions can be best studied by co-culturing the cells in the same environment *via* either direct or indirect contact between the cells. The most common method used to study direct cell interactions is mixed cultures (two types of cells mixed in 1:1 ratio). This approach allows the study of cell-cell interactions, and gives the ability to compare the effects of both membrane bound and soluble mediators. Indirect cell interactions *via* soluble factors can be studied using commercially available cell culture inserts or conditioned media transfer. This paradigm consists of culturing one type of cell on inserts and

placed below the other type of cell. The osteoblasts and the chondrocytes share the same medium but no direct cell-cell interactions are possible due to the physical separation of the cells by a polycarbonate membrane. The pore size of 0.4µm is commonly used to separate cells, as this size doesn't allow any cell migration through the membrane. In the conditioned media transfer method, the media used by one type of cell is transferred to the other type of cell to observe the role of soluble factors (Walker 2006). However, a significant challenge in studying osteoblasts and chondrocytes interactions is that, unlike osteoblasts, which are anchorage dependent, chondrocytes de-differentiate and lose their phenotype in monolayer cultures. There is an absence of well established co-culture model to study both direct and indirect interactions which supports/ preserves the phenotype of chondrocytes.

MATRIX METALLOPROTEINASES (MMPS)

General information: The MMP family consists of at least 26 members, numbered in order of discovery. A typical MMP consists of a propeptide of approximately 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable size and a hemopexin (Hpx) domain of about 200 amino acids (Marc *et al.* 2003). A detailed structure of MMPs and the variability between different MMPs is shown in ***figure 8***. The major function of MMPs is to degrade both matrix and non-matrix proteins of the ECM to play a house keeping role. The role of MMPs is also apparent in many functions such as morphogenesis; wound healing, tissue repair and remodelling in response to injury and in progression of diseases such as atheroma, arthritis, cancer and chronic tissue ulcers (Malemud 2006).

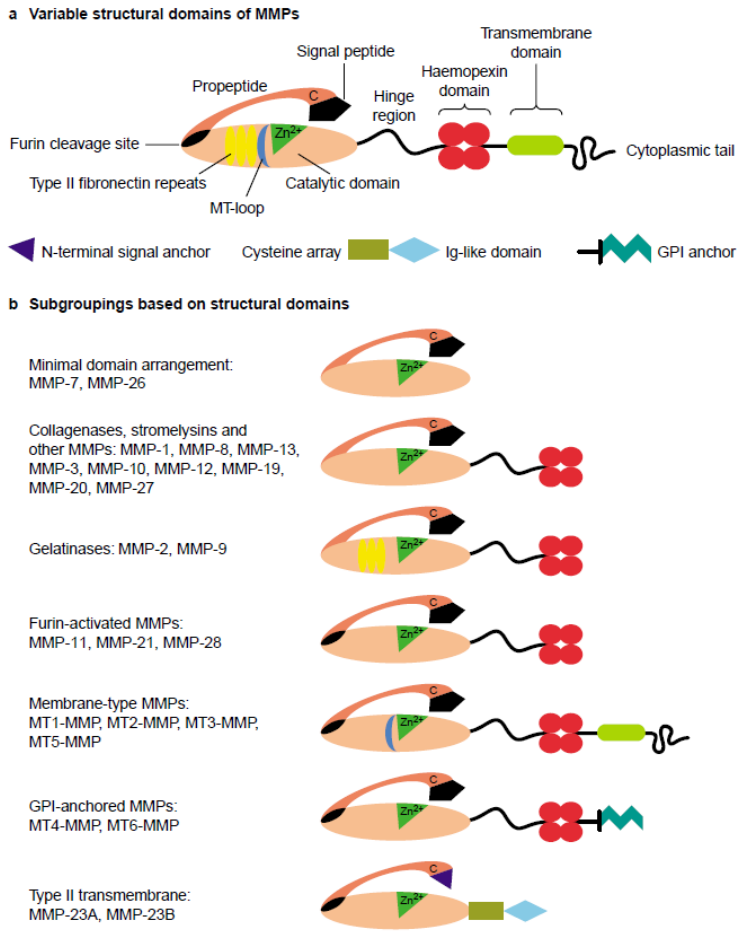

Figure 8: Structure-dependent subgrouping of the matrix metalloproteinases (MMPs). (A) Variable structural domains of MMPs. All MMPs possess a signal peptide that targets the MMPs for secretion, a propeptide domain (containing a conserved Cys residue) and a catalytic domain. All regions/features showing modifications within the family are shown. **(B)** Subgroupings based on structural domains. Most MMPs (with the exception of MMP-7, MMP-23A/B and MMP-26) contain a C-terminal haemopexin domain and a hinge region. Other MMP subgroups contain unique features such as: a transmembrane domain, cytoplasmic tail and a membrane-type (MT)-loop (MT1-, MT2-, MT3- and MT5-MMP; also known as MMP-14, MMP-15, MMP-16 and MMP-24, respectively); a glycosylphosphatidylinositol (GPI) anchor (MT4-MMP and MT6-MMP; also known as MMP-17 and MMP-25, respectively); a furin recognition site (MT-MMPs, and MMP-11, -21, -23A/B and -28); fibronectin type II repeats (MMP-2 and -9); and an N-terminal signal anchor, a cysteine array and an Ig-like domain (MMP-23) (Marc *et al.* 2003).

MMPs function in normal bone and cartilage biology: Genetic analyses using transgenic mice that are engineered to enhance or repress the function of MMPs and pharmacogenetic studies with chemical inhibitors have greatly helped to elucidate the roles that MMPs play in cartilage and bone development and remodeling (Wu *et al.* 2002; Wang *et al.* 2003). Growth and development of the tissues are associated with brisk cell movement by reformation and remodelling of ECM. A number of studies have provided evidence for involvement of MMPs and their inhibitors during this developmental process (Birkedal-Hansen *et al.* 1993; Murphy and Nagase 2008). During endochondral ossification, in the absence of MMPs the cartilage remains intact and adult bone layers will not form properly (Ahmed *et al.* 2007). MMPs are responsible for the catabolism and turnover of the cartilage matrix and are induced during the process of chondrocyte hypertrophy and are necessary for angiogenesis in the growth plates, and therefore critical for normal chondrocyte hypertrophy and bone formation (Ballock and O'Keefe 2003).

In particular, MMP-9 null mice compared with their wild-type littermates displayed substantially more hypertrophic cartilage, impaired endochondral ossification and a delay in the formation of the bone marrow cavity, indicating the important role of MMP-9 as a key regulator of early growth plate angiogenesis and apoptosis of hypertrophic chondrocytes (Vu *et al.* 1998). On the other hand, MMP-2 (gelatinase A) is diffusely expressed in osteoblasts, bone marrow cells and periosteum but significantly expressed in the hypertrophic chondrocytes. MMP-2 mutant displayed an osteolysis and arthritis syndrome characterized by destruction and resorption of affected bones (Martignetti *et al.* 2001; Mosig *et al.* 2007). On the other hand, in contrast to MMP9 null mice, MT1-MMP null mice revealed its importance during secondary ossification and chondrocyte proliferation (Zhou *et al.* 2000). Additionally, MMP-13 null mice showed profound defects in growth plate cartilage with markedly increased hypertrophic domains as well as delay in endochondral ossification and formation and vascularization of primary ossification centres (Inada *et al.* 2004).

Once growth formation ceases, the rates of MMP activities will be decreased significantly. However, certain low levels of MMPs are still active and are required for the regulation of cell behaviour by maintaining ECM integrity in both cartilage

and bone (Clark *et al.* 2008). Nonetheless, the recent studies have demonstrated the role of MMPs far behind the cleaning of surplus ECM. Numerous studies have shown that MMPs help to release growth factors from the ECM or from the cell surface, which are later important for the cellular differentiation process of both cartilage and bone (Maeda *et al.* 2002). Also, it has been reported that MMPs are a part of coupling mechanism to fine-tune the actions of osteoblasts and osteoclasts during the bone remodelling process (Ortega *et al.* 2003). The following **figure 9** illustrates some of the important functions of MMPs required for normal physiological functions.

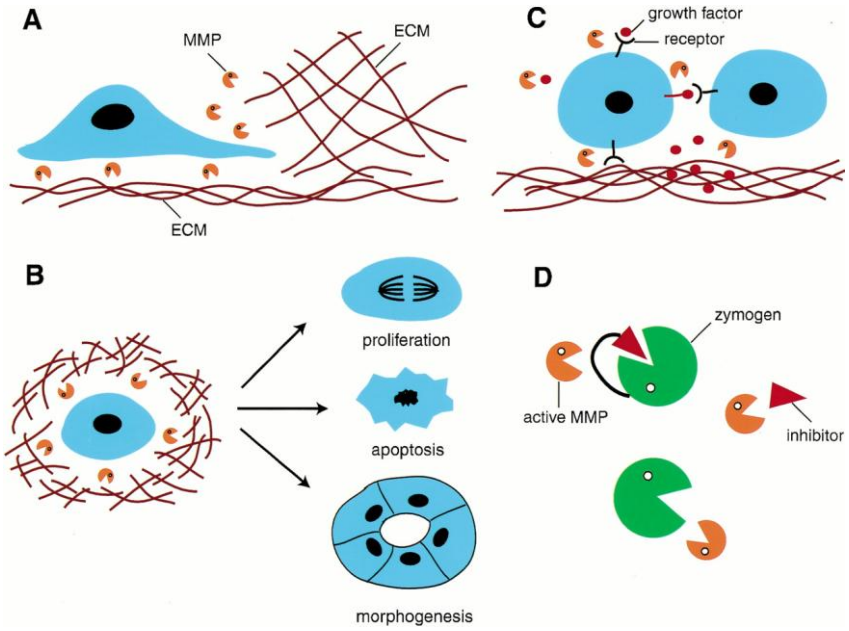

Figure 9: Modes of action of the matrix metalloproteinases. (A) MMPs may affect cell migration by changing the cells from an adhesive to non-adhesive phenotype and by degrading the ECM. **(B)** MMPs may alter ECM microenvironment leading to cell proliferation, apoptosis, or morphogenesis. **(C)** MMPs may modulate the activity of biologically active molecules such as growth factors or growth factor receptors by cleaving them or releasing them from the ECM. **(D)** MMPs may alter the balance of protease activity by cleaving the enzymes or their inhibitors. (Reference: Vu T. H., Werb Z. Genes Dev. 2000; 14: 2123-2133).

Pathological role of MMPs in OA cartilage and bone biology: Timely degradation of ECM is an important feature of development, morphogenesis, tissue repair and remodelling. It is precisely regulated under normal physiological conditions, but when dysregulated it becomes a cause of many diseases including OA (Nagase *et al.* 2006). Reduction of proteoglycans from articular cartilage (degradation of proteoglycans) is a frequent early change in the OA joint disease with subsequent degradation of the collagen fibrils. Therefore, understanding of how AGG is normally degraded and whether this process can be forestalled is critical in preventing OA. Degradation of aggrecan is known to be attributed by MMPs (MMP-2 and -9) (Mott and Werb 2004) and aggrecanases (ADAMTS 1, ADAMTS 4, and ADAMTS-5) (Huang and Wu 2008). AGG is a glycosaminoglycan-containing molecule with three globular (G1, G2 and G3) domains. Several studies projected that the MMPs play an important role during in degrading the AGG during the OA pathological process (Prehm 2005). It has been demonstrated that the MMP-2 and -9 family members are able to degrade AGG, by cleaving interglobular G1-G2 domain, the major aggrecan fragments and release it from the AGG-hyaluronan network (Burrage *et al.* 2006; Sumer *et al.* 2007).

Additonally, aggrecanase-mediated aggrecan degradation is a significant event in early-stage OA (Bondeson *et al.* 2008). Aggrecanases belong to a disintegrin and metallo-proteinase with thromboSpondin motifs (ADAMTS) family of proteinases. There has been considerable interest in the possible role of these aggrecanases, especially ADAMTS-4 and ADAMTS-5, as therapeutic targets in OA. The aggrecanase generated cleavage sites in the aggrecan molecules were identified as distinct from those generated by MMPs (Asu341-Phe342) (Huang and Wu 2008). Moreover, it had been recently revealed that aggrecanase-1 can secondly cleave the aggrecan molecule at an MMP site (Glu373-Ala374) and this activity is considered a hallmark of cartilage degradation during inflammatory joint diseases like OA (Bondeson *et al.* 2008; Huang and Wu 2008). Recent knockout mouse studies have shown that deletion of ADAMTS-5 provided significant protection against proteoglycan degradation and decreased the severity of OA (Glasson *et al.* 2004). To investigate the importance and effects of a complete absence of ADAMTS-4 and ADAMTS-5 aggrecanase activity on the progression of OA, Majumdar et al generated mice with dual deletion of both genes. When the ADAMTS-4/5-double- knockout

mice were surgically induced with joint instability, it was encouraging to note that these mice were physiologically normal and showed a decrease in the progression of OA (Majumdar *et al.* 2007). These results emphasise the critical role of MMPs and ADAM proteases in proteoglycan degration during OA pathogenesis.

On the other hand COLII is a major fibrillar interstitial collagen in cartilage and is very resistant to most proteinases because of its triple-helical structure (Perumal *et al.* 2008). MMP-1, MMP-2, MMP-8 and MMP-13 are reported to be capable of degrading the COL2 (Chung *et al.* 2004). Once the collagen molecules are cleaved, the helical structure is unwound at body temperature and becomes denatured into gelatin. Gelatin is then digested into smaller peptides by gelatinases (MMP-2 and MMP-9) (Hausser *et al.* 2004).

In human OA, it is now apparent from many studies that MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 are significantly upregulated compared to normal cartilage tissue, and this phenomena has been viewed to be responsible for cartilage degeneration (Burrage *et al.* 2006). The immunohistochemical expression levels of MMPs such as MMP-2, MMP-3, MMP-9 and MMP-13 in chondrocytes are reported to correlate directly with the histological destruction score of the articular cartilage (Aigner *et al.* 2001; Martin *et al.* 2001; Stove *et al.* 2001). In an experimental OA animal model, MMP-9&13 and type X collagen are co-expressed in the degenerative cartilage suggesting the close connection between the hypertrophic differentiation and MMP production by chondrocytes (Huebner *et al.* 2009). Many studies have shown that the inflammatory cytokines are the culprits responsible for abnormal MMP activation. However, the use of anti-inflammatory drugs showed no significant benefit in the treatment of OA patients (Daheshia and Yao 2008). This observation indicates that the activities of MMPs are far beyond the control of cytokines and additional studies are required in order to elucidate the exact triggering factors leading to abnormal MMP activities in OA patients. Similarly, one study has reported that the levels of MMP-2, 9 and -13 are significantly higher in the OA subchondral bone compared to normal subchondral bone (Hulejova *et al.* 2007). MMPs have the ability to cleave native helical type I collagen, therefore increased MMPs can contribute to

bone collagen remodelling in OA patients leading to changes in structure of subchondral bone (Reinhardt *et al.* 2005). However, the mechanism(s) responsible for this uncontrolled release of MMPs in OA cartilage and bone are not completely understood.

It has been suggested that MMP activities are accurately regulated according to cellular differentiation status of osteoblasts and chondrocytes (Mizutani *et al.* 2001; Bertram *et al.* 2009). These findings logically indicate that an alteration in differentiation capability of the cell would modify the MMP levels, which may lead to imbalance in the anabolic and catabolic metabolism in a cell. Given the potential interaction of osteoblasts and chondrocytes in regulating each other's differentiation we can rationally assume that the MMPs are also precisely regulated during the cross-talk of bone and cartilage cells. However, so far it is not known how the cross-talk of chondrocytes and osteoblasts regulate MMPs in normal *vs.* OA joint.

Tissue inhibitors of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3 and TIMP-4 have the ability to inhibit MMPs, since these TIMPs play an important role in the regulation of MMP activity, they are crucial in controlling the destruction of tissue (Pelletier *et al.* 1990). Previous studies have shown that TIMPs, once activated, can inhibit MMPs, and it is now widely accepted that the balance between MMPs and TIMPs is very important in maintaining joint cartilage homeostasis (Visse and Nagase 2003). Recently, it was reported that TIMP-3 can inhibit aggrecanase activity in vitro (Kashiwagi *et al.* 2001). Interestingly, it has been shown that both TIMP-1 and TIMP-2 each have growth-promoting activity (Nagase *et al.* 2006). Such activity may influence matrix turnover in OA cartilage. It is noteworthy that the level of latent MMP synthesis in OA cartilage greatly exceeds the up-regulation of gene expression for TIMP-1, -2, -3, and -4 (Murphy and Lee 2005). Although TIMP-1 mRNA expression by chondrocytes in OA cartilage was higher than that by chondrocytes derived from normal cartilage, the amount and TIMP isoform produced by OA chondrocytes is insufficient to inhibit the level of MMPs. It has also been reported that the activity of TIMP-1 was upregulated in OA subchondral bone (Hulejova *et al.* 2007) indicating that the altered expression of TIMPs may also play a role in abnormal subchondral bone remodelling typical of OA. In addition, once activated

and not effectively inhibited by TIMPs, MMPs degrade both the endogenous and newly synthesized ECM molecules.

Methods to detect MMPs: The expression of MMPs in the cells or tissues can be determined using several techniques. MMP activity is regulated at multiple levels, such as at the level of gene transcription and the synthesis of pro-MMP. However, when analysing the regulation of MMPs during the normal or diseased physiological process it is important to profile the activities rather than the overall abundance (Clark 2001). The most common methods to detect activities of MMPs are zymography, ELISA and western blot (by using antibodies specific to both active and inactive MMP forms).

MITOGEN ACTIVATED PROTEIN KINASES (MAPK) SIGNALLING PATHWAY

General information: MAPK cascades are found in almost all eukaryotic organisms, and are expressed in virtually all tissues types. MAPKs are activated by dual phosphorylation of conserved threonine and tyrosine residues within the activation loop (denoted T-X-Y) and they phosphorylate targets on serine and threonine residues within a consensus PXT/SP motif (X can depend on the MAPK) (Songyang *et al.* 1996). They play a very important role to regulate various cellular activities, such as gene expression, differentiation, cell survival and apoptosis (Pearson *et al.* 2001). The illustration of activation of different MAPK signalling cascades by different extracellular stimuli is illustrated in ***figure 10***. Five families of MAPKs have been identified in mammalian cells: extracellular signal-regulated kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2 and JNK3); p38 kinase isozymes (p38-α, p38-β, p38-γ and p38-δ); ERK3/ERK4; and ERK5 (Davis 2000; Chen *et al.* 2001; Chang *et al.* 2002; Roux and Blenis 2004). MAPKs are synchronized and regulated at several levels, these include kinase-kinase and kinase-substrate interactions, and inhibition of cross-talk/output by the MAPKs themselves (Chen and Thorner 2005). The vast majority of defined substrates for MAPKs are transcription factors which in

turn regulate the functions such as proliferation, differentiation and cell fate. However, MAPKs also have the ability to phosphorylate many other substrates including protein kinase, phopholipases and cytoskeleton associated proteins (Bardwell 2006).

MAPK signalling subtypes cross-talk: Although the three MAPK subtypes such as p38, ERK1/2 and JNK run in parallel, there is a considerable degree of cross-talk between them, which leads to fine-tuning of responses to different signals (Krishna and Narang 2008). The type and degree of cross-talk depends upon extrinsic and intrinsic factors, including the length, intensity timing of signal, type of cell and cell-specific receptor distribution at the plasma membrane (McClean *et al.* 2007). Such existence of cross-talk among MAPK pathways themselves is exemplified by a study showing that the pro-survival ERK1/2 pathway and pro-apoptotic JNK pathway possibly act in dynamic balance, with the ERK1/2 pathway acting to inhibit the JNK pathway or vice versa (Xia *et al.* 1995). A similar see-saw like balance between p38 and ERK1/2 pathways has been demonstrated in regulating diverse functions in various types of cells (Houliston *et al.* 2001; Sharma *et al.* 2003, Cheng *et al.* 2009) including osteoblasts (Shimo *et al.* 2007) and chondrocytes (Kim *et al.* 2002; Reilly *et al.* 2005). The detailed cross-talk among MAPK subtypes in chondrocytes will be discussed in chapter 5.

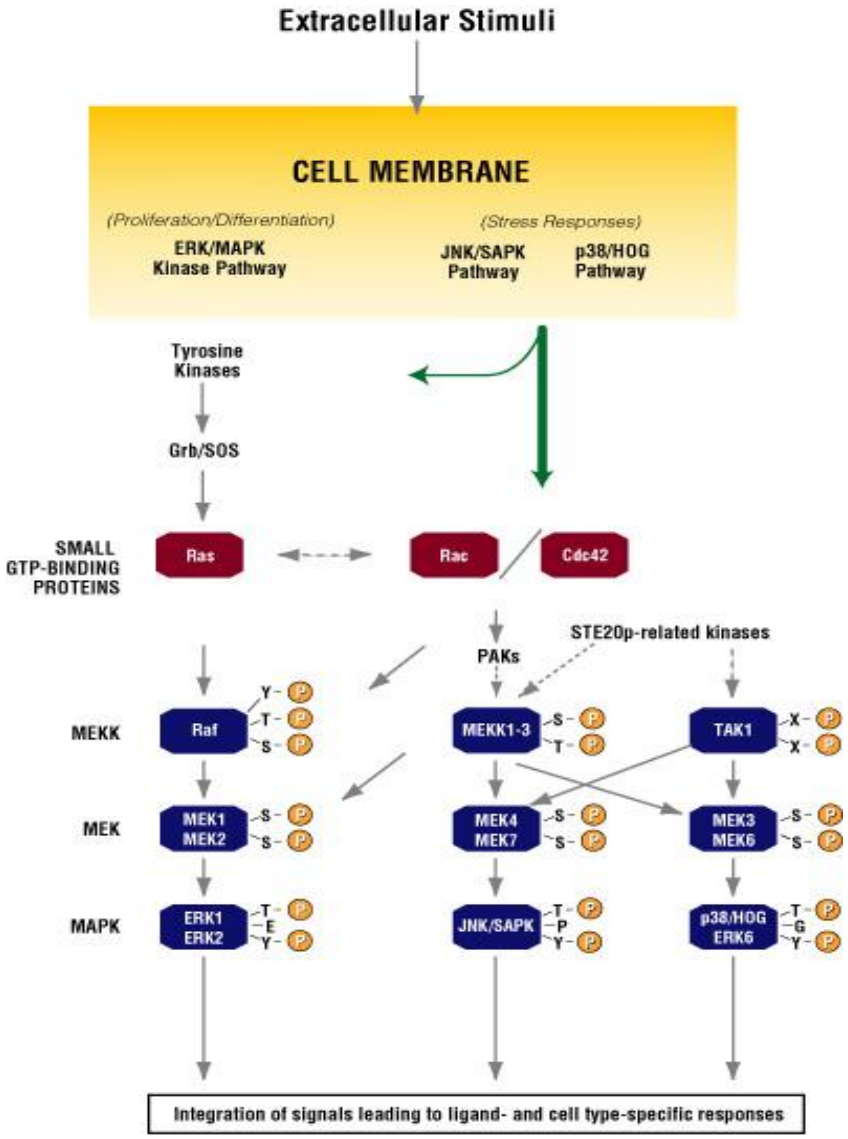

Figure 10: Activation of different MAPK signalling cascades by different extracellular stimuli: The ERK, JNK and p38 cascades all contain the same series of three kinases. A MEK Kinase (MEKK) phosphorylates and activates a MAP Kinase Kinase (MEK), and then MEK phosphorylates and activates a MAP Kinase (MAPK). (Resource: www.promega.com/paguide/chap7.html).

MAPK role in normal cartilage: MAPKs are typical signal transducers in chondrocytes (Stanton *et al.* 2003). In chondrocytes, increase of p38 phosphorylation and decrease of ERK1/2 activation was reported during chick mesenchymal cells chondrogenic differentiation (Oh *et al.* 2000). A similar kind of positive role of p38

and negative role of ERK1/2 pathway has also been demonstrated in chondrogenesis of human mesenchymal stem cells (hMSCs) (Han *et al.* 2004). Inhibition of chondrogenesis by epidermal growth factor (Yoon *et al.* 2000), rapamycin (Oh *et al.* 2001) and retinoic acid (Bhasin *et al.* 2004) was accompanied by decrease of p38 signalling pathways. Conversely, it was reported that inhibition of ERK1/2 pathway increased cartilage specific gene expression (Bobick and Kulyk 2006). These studies collectively, suggest that, in chondrocytes, the ERK1/2 pathway communicates a negative chondrogenic signal, whereas p38 signalling gives a positive chondrogenic differentiation signal. However, not all studies are in agreement with the above studies. It has been demonstrated in one study that the ERK1/2 pathway is required for chondrogenic differentiation of the C3H10T1/2 cell line (Seghatoleslami *et al.* 2003). It was also demonstrated that the transcriptional regulation of SRY (sex determining region Y)-box 9 (SOX9), a typical, marker of chondrogenesis is under the control of ERK1/2 pathway (Zakany *et al.* 2005). These discrepancies in the results may probably relate to use of different cell lines, different extra cellular stimuli and the duration of upstream MAPK signals. A positive role of ERK1/2 signalling pathway during chondrocyte hypertrophic differentiation in the growth plate has also been demonstrated (Provot *et al.* 2008). With respect to JNK signalling it was demonstrated that the inhibition of JNK phosphorylation during hMSC chondrogenic differentiation partially blocked the chondrogenesis, indicating a minor role of this pathway during chondrogenic differentiation (Tuli *et al.* 2003). The involvement of JNK was also reported in proteoglycan synthesis of chondrocytes following cyclical mechanical stimulation (Zhou *et al.* 2007).

MAPK signalling role in OA cartilage: In OA progression abnormalities in signalling pathways are thought to play a major role that occurs *via* modifying the phenotype of cartilage cells thereby leading to imbalance in the anabolic and catabolic pathways (Saklatvala 2007; Daheshia and Yao 2008). From the above review, it is apparent that the MAPKs play an important role in cartilage biology; therefore it is likely that these changes in OA cartilage might be occurring via abnormalities in the expression (up or down regulation) of MAPK pathways. Indeed in clinical human OA cartilage samples, the levels of phosphorylated ERK1/2 and JNK have been shown to be hyper-activated in OA cartilage compared to normal's (Fan *et al.* 2007).

Furthermore, activated ERK1/2 and JNK were highly upregulated in a surgically induced OA dog animal model signifying the pathological role of this pathways during OA development (Boileau *et al.* 2005). Nevertheless, the exact means by which these abnormalities in the signalling pathways are taking place in OA cartilage compared to normal are not completely understood. However, its known that MAPKs are abnormally activated by a diverse range of stimuli including cytokines, growth factors, and matrix proteins that bind to various receptor such as tyrosine kinases, G-protein coupled receptors, cytokine receptors, and integrins (Haneda *et al.* 1999). Once activated, the MAPKs, in turn, activate other protein kinases and several transcriptional regulatory proteins (Fiil *et al.* 2009). The transcriptional activation is known to trigger the host genes relevant to inflammatory response, abnormal differentiation and production of matrix degrading enzymes such as MMPs leading to OA related destructive changes in the cartilage. Therefore, inhibiting the altered signalling pathways that regulate these phenotypic changes might theoretically restore homeostasis in the joint (Loeser et al. 2008).

MAPKs function in normal *vs.* OA bone: The differentiation of bone cells is dependent upon the temporal regulation of multiple interacting signalling pathways. Runt-related transcription factor 2 (RUNX2), which is an essential osteoblastic transcription factor for bone formation (Franceschi *et al.* 2009) was reported to be controlled by MAPK signalling subtypes. In osteoblasts, activation of ERK1/2 has been reported in response to various growth factors such as fibroblast growth factor (FGF) (Miraoui *et al.* 2009), platelet- derived growth factor (PDGF) (McCarthy *et al.* 2009), insulin-like growth factor (IGF)-1 (Celil and Campbell 2005), and epideramal growth factor (EGF) (Matsuda *et al.* 1998). MC3T3-E1 cells and primary cultures of hMSCs induced RUNX2 phosphorylation and osteocalcin expression by a process requiring ERK1/2 activation (Xiao et al., 2002b). These studies together suggest an important role of MAPKs in osteoblast function. In view of the fact that abnormal subchondral bone metabolism is intimately involved in the genesis of this OA disease; it is possible that these changes in the SBOs might occurr through the MAPK dependent pathway. However, until now the role of MAPKs in relation to OA subchondral bone pathogenesis remains unknown.

MAPKs as a therapeutic approach: Pharmacological inhibitors have been identified for targeting ERK1/2 with PD98059, SB203580 for p38 isoforms, and SP600125 for JNK protein kinases (Bain *et al.* 2007). These inhibitors have been successfully applied to block the progression of diseases such as rheumatoid arthritis (Meyer and Pap 2005), and cancer (Wagner and Nebreda 2009). In addition ERK1/2 and JNK inhibitors are also successfully used at clinical level to decrease the neuropathic pain that acts *via* reversing the inflammatory pathways (Borsello and Forloni 2007). In OA the use of MAPK inhibitors as a therapeutic strategy has been proposed (Malemud *et al.* 2003). Despite studies suggesting the use of MAPK inhibitors as a potential OA therapy there is little research into their potential clinical application.

Methods to detect MAPKs: Many of the MAPK cascade components are activated by phosphorylation, therefore detection of MAPKs usually based on comparing the relative strength of phospho levels in treated vs. untreated cells/tissues. Chromatography, and gel kinase methods have been used to detect the amount of kinase. Hitherto, these methods endure with a lack of reproducibility. The recent development of phospho specific commercial antibodies enabled the detection of phosphorylated proteins within a variety of cell culture and tissue sample lysates with good sensitivity, detection range and reproducibility (Yoav and Rony 2006). The direct pathway involvement can be determined by using MAPK pharmacological inhibitors. Biochemical inhibitors are highly specific and highly selective for individual enzymes in the pathway and are widely used for understanding the MAPK cascade (Margutti and Laufer 2007). The most common inhibitor is SB203580, a specific inhibitor of p38. Likewise, PD098059 and SP600125 are widely used to inhibit ERK1/2 and JNK phosphorylation. Determination of appropriate concentrations that minimize the cytotoxicity levels and at the same time blocking the required activated levels are important considerations in using these inhibitors in *in vitro* and *in vivo* situations (Seger 2004).

Chapter 3

Post-natal changes in osteoarthritis development: Histology and Immunohistochemistry

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Suggested Statement of Contribution of Co-Authors for Thesis by Published Paper:

Contributor	Statement of contribution*
Prasadam I	Performed laboratory experiments, data analysis and interpretation. Wrote the manuscript.
Signature	
Date	
Forsythe M	Involved in sample collection and processing and assisted in manuscript preparation
Shi W	Involved in the conception and design of the project, performed some Immunostaining experiments
Crawford R	Involved in the conception and design of the project, assisted in collection of clinical samples and reviewed the manuscript
Xiao Y	Involved in the conception and design of the project, technical guidance, Manuscript preparation and review

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name	Signature	Date

ABSTRACT

Aim: To reveal whether osteoarthritic (OA) cartilage undergoes hypertrophy related phenotypic changes.

Methods: Osteochondral sections were prepared from tibial plateaus taken from OA patients undergoing knee replacement surgery. Sections were divided into two groups: score 0 (normal) and score over 3 (arthritic), according to the Mankin scale. Comprehensive histological analysis was performed to detect any morphological variations. Immunostaining techniques were applied to observe the relative expression strength of hypertrophic and chondrogenic differentiation markers in cartilage graded according to disease severity.

Results: Normal cartilage differed from arthritic cartilage both in terms of morphology and molecular markers. There was a significant site-specific expression of hypertrophic differentiation markers in arthritic cartilage tissues compared to patient matched relatively normal tissues. Interestingly, in some arthritic tissues cartilage islands were found embedded deep inside the subchondral bone matrix. The islands stained positive for type 2 collagen, but did not stain for any bone markers, confirming that these islands were of cartilaginous origin.

Conclusions: The observations reported here suggest that cartilage hypertrophy changes are an integral part of OA cartilage pathogenesis. The presence of cartilage islands in arthritic, but not in normal bone tissues, is a notable phenomenon. Based on our results, we conjecture that, the articular cartilage in the OA is undergoing post-natal changes leading to the cartilage being replaced with bone.

INTRODUCTION

Throughout the entire growth period, chondrocytes present in the growth plate undergo distinct transformations through the sequential process of cell proliferation, ECM synthesis, hypertrophy, matrix mineralization, vascular invasion and are consequently replaced with bone (1-3). During the hypertrophic phase the chondrocytes undergo dramatic phenotypic changes characterized by expression of bone specific markers and mineralized matrix deposition (4). In broad terms there are two prevailing theories regarding the fate of hypertrophic chondrocytes, one being that they undergo apoptosis, the other that they trans-differentiate into osteoblasts (5-7).

Permanent articular cartilage under normal conditions tends not to undergo hypertrophic differentiation. OA cartilage has many distinguishable features compared to normal non-diseased cartilage. Expression of type X collagen (COL10) and osteocalcin (OC) has been demonstrated, indicating that OA cartilage is undergoing hypertrophic related changes (8, 9). These observations suggest that they are common biological bystanders in both the endochondral ossification and OA, which in the former is vital for bone formation, whereas in the latter appear to be deleterious to the integrity of the cartilage. The most recent studies, however, suggest that the processes precipitating the hypertrophic changes in OA can be seen to reflect the process of aging rather than an integral part of the OA condition itself (10). Importantly, some studies showed no evidence of hypertrophic related changes in the OA cartilage (11). Given the discrepancies in the existing literature, in the present study, we intended to test if hypertrophic changes are an integral part of OA pathogenesis or whether this phenomenon is merely an incidental finding. For this reason, we analysed specific clinically graded regions in an attempt to produce the most comprehensive and clinically relevant data possible, and to exclude the variations related to age, lifestyle and genetic factors during data analysis. For this purpose we investigated the following biochemical markers: bone sialoprotein (BSP), osteopontin (OPN), alkaline phosphotase (ALP), bone morphogenetic proteins-2/4 (BMP -2/4), osteocalcin (OC), type 1 collagen (COL1), Matrix metalloproteinases-9 (MMP-9), Matrix metalloproteinases-13 (MMP-13) and vascular endothelial growth

factor (VEGF), all of which are well characterized markers of chondrocyte hypertrophy during the endochondral ossification. As well as this, the expression of the chondrogenic markers type II collagen (COL2) and aggrecan (AGG) were also studied. Standard haematoxylin and eosin (H&E) staining was also carried out to view the apparent morphological differences between the grade 1 and grade 4 from patient matched osteochondral junctions.

METHODOLOGY

Patients: Five patients undergoing total knee replacement for OA were recruited for this study after informed consent was given. The study had the approval of the Queensland University of Technology and Prince Charles Hospital ethics committees. Patient variables of height, weight and age were recorded and preoperative radiographs of the knee, anteroposterially and laterally, were taken. OA was classified according to the Mankin score (12). The patients selected had score over 3 **(Arthritic)** medial compartments and score 0-1 **(normal)** OA lateral compartments. The degree of arthritis was confirmed by intraoperatively classifying both compartments according to the Outerbridge classification (13).

Tissue Preparation: Four 1cm samples were taken from tibial plateau region covered by meniscus to the anterior from each knee: two from the arthritic medial compartment and two from the relatively normal lateral compartment. The samples included any overlying cartilage and subchondral bone to an approximate depth of 1cm. All of the samples were prepared for histology and immunohistochemical analysis by washing in saline and immediately fixed in 4 % paraformaldehyde for 24 hours. The samples were washed in 1% phosphate buffer solution (PBS) for one hour and then placed in 10% ethylene diamine tetraacetic acid (EDTA) for decalcification. The EDTA solution was changed weekly for four weeks and decalcification was confirmed by radiographic analysis. Tissue specimens were embedded in paraffin wax once the decalcification was complete. Tissue slices of 5 μm thick, were sectioned by microtome, placed on 3-aminopropyltriethoxy-silane coated glass slides, air-dried and stored at 4^{o}C prior to analysis. Each specimen was H&E stained to visualize the general morphology.

H&E staining: Tissue slices were dewaxed in xylene and dehydrated in descending concentrations of ethanol (100% to 70%). After that, sections were stained with Mayers's haemotoxylin for 1-2 minutes before washing with tap water for 5 minutes. The slides were dehydrated in ascending concentration of ethanol (70% to 100%) and stained with eosin for 15 seconds and cleared with xylene and mounted on DePeX mounting medium (BDH Laboratory Supplies, England).

Immunohistochemistry: Immunohistochemistry was carried out using an indirect immunoperoxidase method. Tissue slices were dewaxed in xylene and dehydrated in ethanol. Endogenous peroxidases were blocked by incubation in 0.3% peroxide in methanol for 30 min following repeated washing in PBS. The sections were then incubated with proteinases K (DAKO Multilink, CA, USA) for 20 min for antigen retrival. Next, all sections were treated with 0.1% bovine serum albumin (BSA) with 10% swine serum in PBS. Sections were then incubated with optimal dilution of primary antibody overnight at 4^{o}C. Optimum concentration of antibodies was determined by using a series of dilutions. Next day, sections were incubated with a biotinylated swine-anti-mouse, rabbit, goat antibody (DAKO Multilink, CA, USA) for 15 min, and then incubated with horseradish perioxidase-conjugated avidin-biotin complex for 15 min. Antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 min and the reaction was stopped by immersion and rinsing of the sections in PBS. Sections were lightly counterstained with Mayer's haematoxylin and Scott's blue for 40 sec each, in between 3 min rinses with running tap water. Following this, they were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a coverslip using DePeX mounting medium. Controls for the immunostaining procedures included conditions where the primary antibody or the secondary (anti-mouse IgG) antibodies were omitted. In addition, an irrelevant antibody (anti CD-15), which was not present in the test sections, was used as a control. A list of antibodies sources and dilutions used for this study are summarized in ***Table 1***.

Statistical analysis: Paired Student's t-test was used to determine if there was any significance between normal *vs.* arthritic cartilage specimens, by counting the % positive staining cells. $P \leq 0.05$ was considered significant.

RESULTS

Patient Demographics: The mean age of the five patients was 72 years (range 63 – 82), mean weight was 93.4 kg (range 74-124) and mean height was 173 cm (range 162-185). As samples for each group were taken from the same patient, there were no differences between the groups, therefore a paired t-test was performed to acertain any statistical differences. A representative radiograph and tissue is shown in **fig 1** with complete joint space obliteration in the medial compartment and normal appearing lateral compartment.

H&E staining: *Normal*: All specimens had normal appearing articular cartilage with underlying subchondral bone. The deep (radial) layer of noncalcified cartilage (NC) was separated from the underlying calcified cartilage (CC) layer by the tidemark (TM). Immediately adjacent to the CC was the subchondral bone (SB) **(fig 1A).** *Arthritic*: All arthritic specimens showed cartilage loss with a small region on the edge of the slide where there was some preservation of the deep and middle zone cartilage layers. The NC was much thinner and the CC was relatively thicker when compared to the normal specimens. Therefore, the distance between the TM and the SB was thicker than in the normal specimens. SB advancement in the arthritic specimens was seen in all the patients indicating extensive bone remodeling **(fig 1B).** Some arthritic specimens collected from the medial compartment, had islands of cartilage (CI) deeply embedded within the bone matrix at various locations in three out of five patients samples **(fig 1C).**

Expression of cartilage specific markers in normal *vs.* arthritic cartilage

COL2 and AGG: Both CC and NC stained evenly for COL2 in extracellular matrix (ECM) of both normal and arthritic sections with out any significant visual differences. Similarly, the expression of AGG also looks visually identical in both normal and arthritic cartilage specimens **(fig 2A&B vs. fig 3A&B)**.

Expression of hypertrophic and mineralization markers in normal *vs.* arthritic osteochondral tissues

ALP: *Normal:* In all specimens there was an even staining of osteoblasts and SB ECM. The staining pattern was very distinctive in that the TM stained stronger against a background of lighter staining throughout the rest of the CC. No cellular staining was observed in chondrocytes of NC layer, with the exception that one patient showed a light staining in chondrocytes **(fig 2C)**. *Arthritic:* The staining pattern was similar to normal specimens within the bone matrix and osteoblasts. However, staining in the CC layer was much stronger than in the normal specimens. In contrast to the normal specimens, all the tested samples showed a strong staining of the chondrocytes covering almost all the cells in the remaining layers of NC **(fig 3C).**

OPN: *Normal:* In all specimens, there was strong OPN staining of the bone matrix with less staining of the osteoblasts. There was no apparent staining of osteocytes or chondrocytes. In contrast for ALP there was a strong and even staining of OPN in the CC matrix, and light staining at the TM **(fig 2D).** *Arthritic:* OPN staining patterns similar to the normal samples were observed throughout the matrices of the bone and cartilage. However, in contrast to normal samples all arthritic specimens showed strong staining of the majority of chondrocytes within the remaining NC (**fig 3D)**.

BSP: *Normal:* There was light cellular BSP staining in osteoblasts present in SB. However, a strong staining in the bone and CC matrix was noticed. The pattern of staining within the CC layer was homogenous and granular in appearance from the osseous junction to the TM. There was no staining of the NC matrix or the chondrocytes **(fig 2E).** *Arthritic:* Most BSP staining in the arthritic specimens was found in the bone ECM. There was a strong BSP stain within the CC matrix and a moderate to strong staining of the chondrocytes in the NC layer in all five samples **(fig 3E).**

COL1: There was robust COL1 staining of bone matrix in all the samples, both normal and arthritic. There was mild staining of osteoblasts surrounding marrow spaces but no staining of osteocytes was observed. COL1 expression was not seen in

the CC matrix of either normal or arthritic samples. However, four out of five patients showed a weak staining in the chondrocytes present in NC layer. This pattern was not seen in the normal samples **(fig 2F and 3F).**

BMP 2&4: *Normal:* There was light to moderate staining in the bone matrix and osteoblasts in all the samples. BMP2/4 expression was not observed in CC matrix nor was there any apparent staining of chondrocytes in NC layer **(fig 2G)**. *Arthritic:* BMP2/4 staining of the arthritic specimens was similar to normal specimens in all aspects with the exception that all five specimens showed moderate to strong staining of chondrocytes in NC **(fig 3G)**.

OC: *Normal:* In all five specimens, there was strong OC staining in the bone matrix and less staining in the osteoblasts. There was no obvious staining of osteocytes or chondrocytes, but there was a lighter staining in the CC matrix, which was homogenous in appearance **(fig 2H).** *Arthritic:* A similar staining pattern to the normal samples was seen throughout the matrices of the bone and cartilage of the arthritic samples, except that all five samples showed strong staining in most of the chondrocytes within the residual chondrocytes present in NC **(fig 3H).**

Vascularization in OA specimens: Blood vessel penetration from SB to the TM was seen in all of the arthritic specimens **(fig 4A)**, but not in the patient matched normal samples. Compared to the normal samples, expression of VEGF, MMP-9 and MMP-13 was stronger in the arthritic NC chondrocytes **(fig 4B-D)**.

Results from the immunostaining are summarized in ***Table 2***.

Characterization of cartilage islands in subchondral bone: Three out of the five samples collected from arthritic sites, showing complete cartilage degeneration exposing the bone, had islands of cartilage deeply embedded within the subchondral bone in several places. These islands stained evenly for COL2, but revealed very slight bone marker expression, indicating a cartilaginous origin **(fig 5B)**. The surrounding bone matrix, however, stained consistently for all the bone markers tested **(fig 5C-F)**.

DISCUSSION

There are few studies, which look in any detail at the histochemical properties of the osteochondral junction region. The objective of this study was to further analyse the osteochondral junction by histology and immunohistochemistry to learn more about the morphological and molecular changes taking place during OA development. Our results revealed site-specific immunolocalization of a broad array of hypertrophic and also mineralization markers within the OA cartilage when compared to patient matched lateral compartment cartilage. These results indicate that the amount of hypertrophy/mineralization of the cartilage is a more true reflection of the magnitude, extent and severity of OA. The consistencies of these results suggest that hypertrophic changes are an integral part of OA pathogenesis. Similar results are also seen at a cellular level, which will be covered in chapter 5. Also in this study, we identified the presence of cartilage islands deeply embedded within the subchondral bone in the arthritic, but not in the normal specimens. These characteristics of arthritic cartilage observed are typical of post-natal cartilaginous changes; therefore subsequent discussion of this paper will focus on data relating post-natal changes to molecular changes in OA pathogenesis.

During the process of endochondral ossification hypertrophic chondrocytes release high levels of ALP, which is a requirement for matrix mineralization (14). We observed strong ALP staining within the chondrocytes of the remaining middle layers of cartilage in the arthritic specimens. In the normal specimens the staining was observed only up to the calcified zone. It has been reported that the chondrocytes in mineralized hypertrophic growth plate cartilage tissues are similar to *in vivo* cells in the deep calcified articular cartilage (15). The expression of ALP in the non calcified cartilage of arthritic tissue is therefore a clear indication that the OA chondrocytes are undergoing changes that are similar to deep zone articular cartilage.

In vitro studies show that both OPN and BSP are the most effective apatite nucleators required for hypertrophy and subsequent mineralization of cartilage (16-18). The expression of these proteins have been demonstrated in hypertrophic chondrocytes in the growth plate and it has been suggested that these factors have a role in the

biological mineralization and resorption of cartilage matrix (19, 20). In our study we found that the expression of both BSP and OPN were strongly localized to the arthritic chondrocytes of NC, but not in NC of normal specimens. These observations suggest that the expression of these non collagenous proteins might be responsible for the formation of hydroxyapatite and calcium pyrophosphate dihydrate minerals, a phenomenon which has previously been described in OA cartilage (21). The deposition of these minerals may influence the expression of several degenerative pathways involving inflammatory cytokines and MMPs, all of which have been implicated in OA pathogenesis leading to subsequent cartilage resorption (22).

Although osteocalcin expression has been reported in the growth plate mineralized cartilage its precise function there is not known and no growth plate abnormalities were noted in osteocalcin knockout animal models (23). In the present study there was evidence of strong OC staining in the degenerative arthritic cartilage, mirroring that of hypertrophic growth plate cartilage. However its direct role in the pathology of OA needs further investigation.

COL1 is the most abundant protein in the ECM of bone (24). The antibody used in this study was specific for bone tissue and our results showed a consistent staining of bone extracellular matrix in both arthritic and normal specimens. In the arthritic samples there was light COL1 expression in chondrocytes present in the NC cartilage, which was not the case in the normal specimens. This was indicative of a dramatic alteration of the phenotype of arthritic chondrocytes with respect to extracellular matrix synthesis capabilities.

The expression of BMP-2 and 4 was evenly expressed within the subchondral bone, but not beyond, in normal specimens. By contrast, there was strong BMP-2 and 4 expression in the chondrocytes of all arthritic cartilage specimens. BMP-2 and 4 are part of the TGF-β family. TGF-β stimulates the differentiation and proliferation of chondrocytes at moderate levels but at high levels TGF-β is known to induce arthritic changes in murine joints (25). BMP 2&4 are known to induce the ectopic bone formation by means of a process similar to endochondral ossification (26), and can also help to stimulate the expression of hypertrophic markers such as COL10 and

VEGF (27) in the growth plate. Based on this information BMP mediated pathways might therefore have a role in regulating these hypertrophic phenotypic changes in OA cartilage.

The last phase of endochondral ossification depends on the action of MMP 9 and 13 to degrade hypertrophic cartilage to facilitate the invasion of blood vessels (28, 29). In the absence of these proteases the cartilage remains intact and adult bone layers will not form properly. MMP-9 null mice exhibited defective angiogenesis, reduced chondrocyte apoptosis, widening of the hypertrophic zone, and decreased mineralization of the matrix, demonstrating the important role of MMPs during the cartilage hypertrophic phase (28). The process of hypertrophy within the growth plate appears to induce MMP-9 and MMP-13 expression (30), and their expression is only found in the hypertrophic and calcifying cartilage of mammalian growth plate, suggesting these proteases are restricted mainly to the hypertrophic cartilage (31, 32). In light of this, the strong MMP-9 and MMP-13 expression in OA cartilage can be viewed as hypertrophy-induced events which lead to cartilage degradation by cleavage of type-II collagen, which contrasts with endochondral ossification where the cartilage is replaced by bone.

During the final phase of endochondral ossification, MMP-9 and -13 expression by hypertrophic chondrocytes play a crucial role in degrading the extracellular matrix components which ingress space for vascularization (28, 29, 33). Articular cartilage is considered an avascular tissue, but both our own and other studies have demonstrated the presence of blood vessels into the OA cartilage (34). Angiogenesis is believed to be regulated by a VEGF dependent pathway and these pathways are essential for skeletal regeneration and suggestive of initiation of *de novo* bone formation (35, 36). We found evidence of strong VEGF staining of nearly all the chondrocytes in the cartilage that was present in the arthritic tissues, which is an indication that this pathway might be responsible for recruiting blood vessels in OA pathogenesis.

There are two dominant theories pertaining to the fate of hypertrophic chondrocytes of growth plate: (i) that they undergo apoptosis, or (ii) that they trans-differentiate into osteoblasts (37). In the present study, we found the presence of cartilage islands in the

arthritic, but not in normal specimens, which seems to support the notion that new bone is growing into and replacing the degrading cartilage by a process of endochondral ossification. Following experimentally induced trauma to the growth plate, it was found that intrusion into the injury site of the metaphyseal vessels led to failure of endochondral ossification. As a result, portions of cartilage from the growth plate become fixed in the metaphysis while growth of bone continued, leaving small islands of cartilage trapped in the epiphysis (38, 39). We therefore hypothesise that the hypertrophic chondrocytes in OA may be eventually remodelled into the subchondral bone; the cells in the cartilage islands may therefore be the result of defective or improper bone formation. Alternatively, it has been proposed that the cartilage islands seen in the subchondral bone may have been derived from the articular cartilage and/or from the marrow (viz., mesenchymal stem cells) (40, 41). However, further studies on a larger sample size are required in order to understand the aetiology, origin and relation of these islands to OA patho-physiology.

It is noteworthy that many of the changes in chondrocyte staining appear to be in surface zone of the remaining cartilage. However, histomorphometric evidence and our results (chapter 4&6) suggest that the factors from subchondral bone cell can influence the cartilage cell changes (42). It is possible that that the passive factors released by the OA subchondral bone osteoblasts can penetrate deep to superficial layers of the cartilage. Since, the cells from superficial cartialge layer are always under constant shock absorption and mechanical stress compared to deep zone chondrocytes, coupled with further release of degradative factors from subchondral bone may lead to further worsen the situation causing the degeneration of cartilage.

Conclusions: The arthritic, but not the matching normal cartilage, showed strong expression of hypertrophy and mineralization markers, which is a clear indication that alterations in the phenotypic properties are a part of OA development. The presence of cartilaginous islands within the OA subchondral bone also support the hypothesis that the articular cartilage being replaced by subchondral bone.

TABLES, FIGURES AND LEGENDS

Protein	Dilution	Source
COL1	1:5000 (polyclonal)	Kind gift from Dr LW Fisher, NIH, Bethesda, USA
ALP	1:300 (monoclonal)	Sigma-Aldrich, St Louis, Missouri, USA
BMP-2/4	1:100 (polyclonal)	Santa Cruz biotechnology, Santa Cruz, California, USA
OC	1:50 (polyclonal)	Biomedical Technologies Inc, Stoughton, Massachusetts, USA
OPN	1:100 (polyclonal)	Kind gift from Dr LW Fisher, NIH, Bethesda, USA
COL2	1:500(monoclonal)	Bio Scientific Pty Ltd, Gymea, New South Wales, Australia
AGG	1:200(polyclonal)	Millipore, New South Wales, Australia
VEGF	1:100(polyclonal)	Labvison, Fremont, CA
BSP	1:400 (polyclonal)	Kind gift from Dr LW Fisher, NIH, Bethesda, USA
MMP-9	1:100(polyclonal)	Labvison, Fremont, CA
MMP-13	1:200(polyclonal)	Labvison, Fremont, CA

Table 1: Antibodies source and working dilutions used for this study.

	Arthritic (n=5)					Normal (n=5)				
Protein	**OB**	**Bone ECM**	**CC**	**NC**	**CH**	**OB**	**Bone ECM**	**CC**	**NC**	**CH**
COL1	++	+++	-	-	*+ (4/5)	++	+++	-	-	-
OCN	+	+++	++	-	*+++ (5/5)	+	++	++	-	-
OPN	+	++	+++	-	*+++ (5/5)	+	++	+++	-	-
ALP	++	++	++	-	*+++ (5/5)	++	++	+++	-	+ (1/5)
BSP	+	+++	+++	-	*++ (5/5)	+	+++	+++	-	-
BMP 2/4	++	++	-	-	*++ (5/5)	++	++	+	-	-
COL2	-	-	+++	+++	++ (5/5)	-	-	+++	+++	++
AGG	-	-	++	++	+++ (5/5)	-	-	++	++	+++
VEGF	*+++	++	++	-	*+++ (5/5)	+	+	++	+	+
MMP-9	*+++	++	+	-	*+++ (5/5)	+	-	+	-	+
MMP-13	*+++	++	+	-	*+++ (5/5)	+	-	+	-	+

Table 2: Summary of immunostaining results. +: Light staining, ++: Moderate staining, +++: Strong staining. (OB: Osteoblasts, ECM: Extra cellular matrix, CC: Calcified cartilage, NC: Non-calcified cartilage, CH: Chondrocytes). * indicates a significant difference in normal *vs.* arthritic samples.

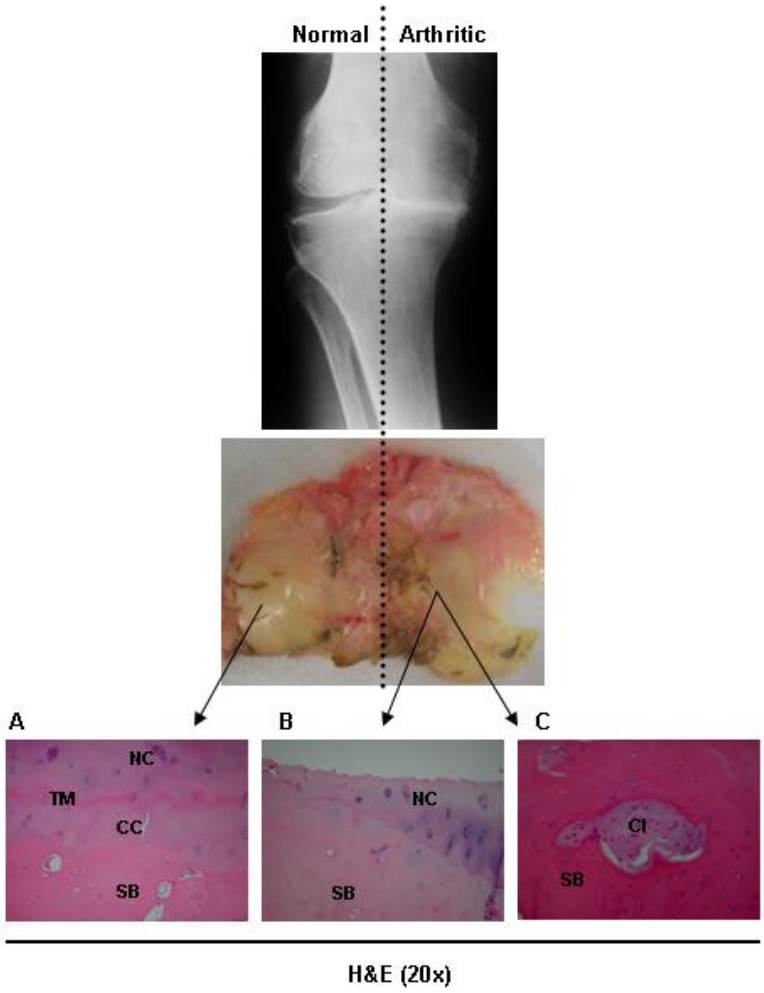

Figure 1: Location of sample collection: X-ray image show the sites of sample collection as indicated. Cartilage degeneration was more prominent in the sections collected from medial compartment and considered as "arthritic", whereas the lateral compartment sections were considered "normal". Some sections were prepared from the sites where the cartilage was completely worn out exposing the sclerotic bone. **(A-B)** Histological appearance of osteochondral sections isolated from normal and arthritic osteochondral compartments. **(C)** H&E staining of sections collected from exposed sclerotic subchondral bone (20X). Note the presence of cartilage islands (CI) embedded deep in the subchondral bone (40X).

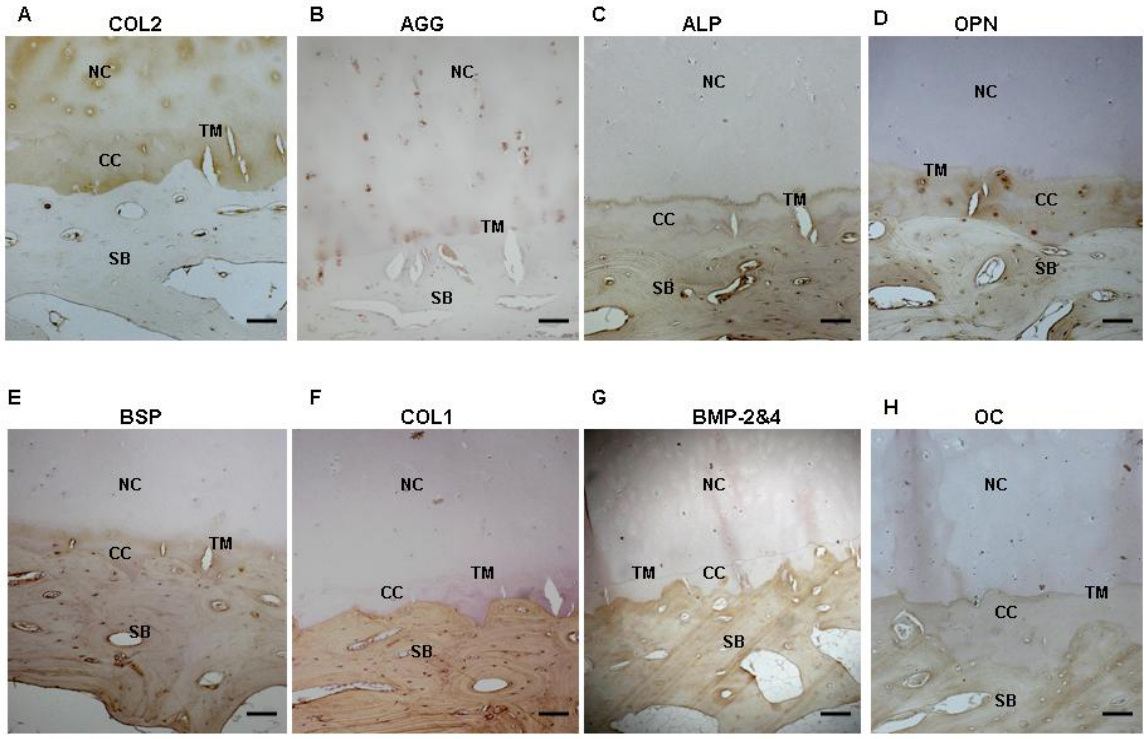

Figure 2: Immunohistochemical localization of cartilage specific (COL2 **(A)** and AGG **(B)**), hypertrophic and mineralization markers (ALP **(C)**, OPN **(D)**, BSP **(E)**, COL1 **(F)**, BMP-2&4 **(G)** and OC **(H)**) in the osteochondral sections collected from normal sites (Scale bar-10X). NC: Non-calcified cartilage, CC: Calcified cartilage, TM: Tide mark, SB: Subchondral bone.

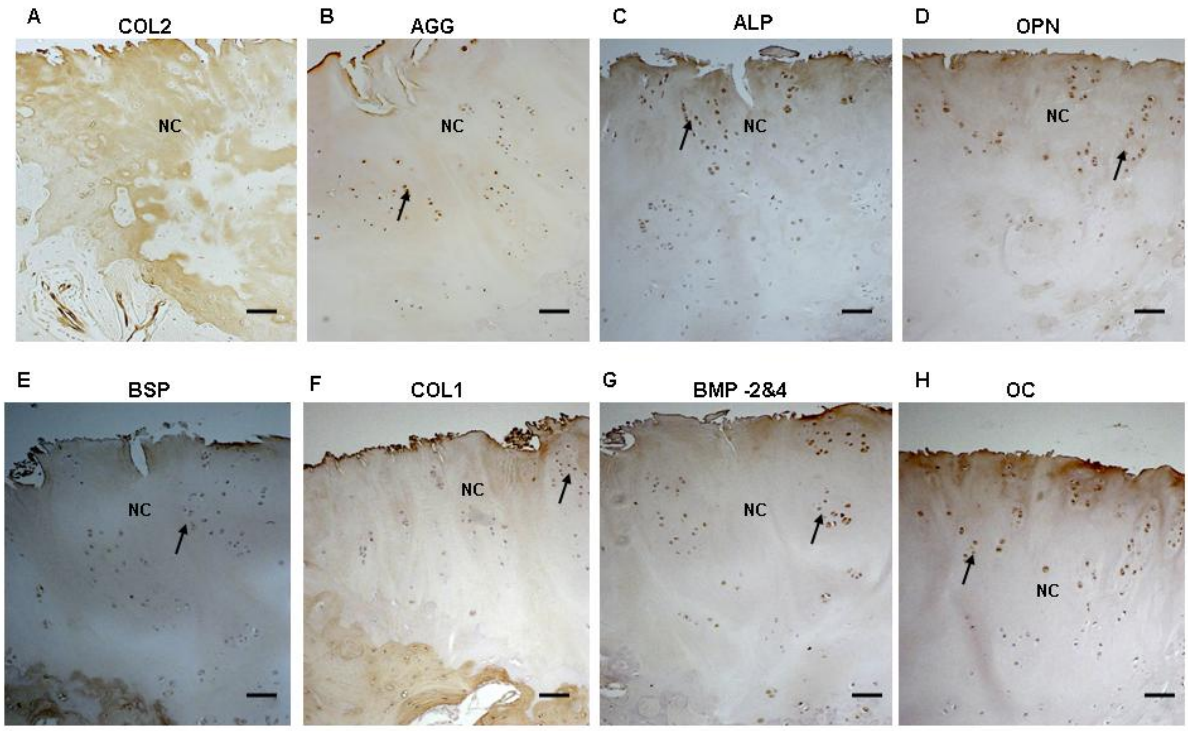

Figure 3: Immunohistochemical localization of cartilage specific (COL2 **(A)** and AGG **(B)**), hypertrophic and mineralization markers (ALP **(C)**, OPN **(D)**, BSP **(E)**, COL1 **(F)**, BMP-2&4 **(G)** and OC **(H)**) in the cartilage collected from arthritic sites (Scale bar- 10X). NC: Non-calcified cartilage.

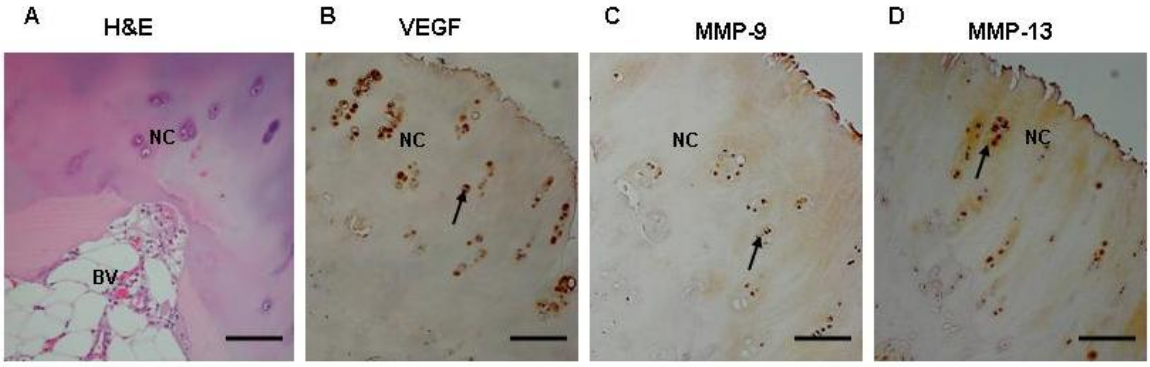

Figure 4: Vascularization- H&E **(A)**, VEGF **(B)**, MMP-9 **(C)** and MMP-13 **(D)** expression in the cartilage sections collected from arthritic sites (Scale bar- 20X). NC: Non-calcified cartilage, BV: Blood vessel.

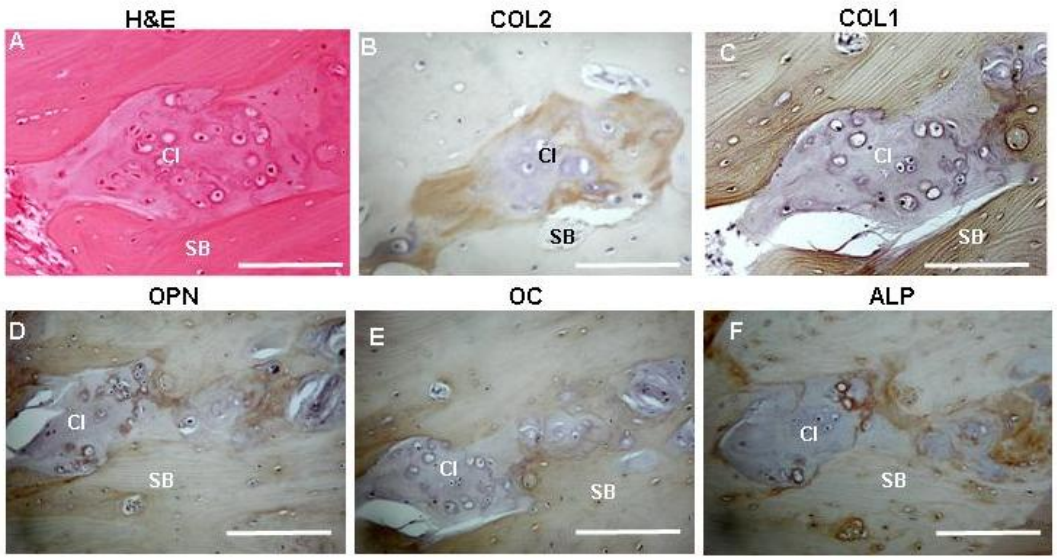

Figure 5: Characterization of cartilage islands (CI) in subchondral bone. (A) H&E staining of the cartilage island embedded in the subchondral bone. **(B**) Immunostaining for COL2 antibody was positive for the CI, but not in the SB matrix. **(C-F)** Immunostaining for bone markers such as COL1, OPN, OC and ALP markers are strong and even in the subchondral bone matrix, but very faintly stained in the cartilage island matrix and chondrocytes. (Scale bar– 40X). NC: Non-calcified cartilage, SB: Subchondral bone.

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Chapter 4

Osteoarthritic cartilage chondrocytes alter subchondral bone osteoblast differentiation via MAPK signalling pathway involving ERK1/2

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Suggested Statement of Contribution of Co-Authors for Thesis by Published Paper:

Contributor	Statement of contribution*
Prasadam I	Involved in the conception and design of the project, Performed laboratory experiments, data analysis and interpretation. Wrote the manuscript.
Signature	
Date	
Friis T	Assisted with manuscript preparation
Shi W	Involved in the conception and design of the project
Gennip S	Involved in the conception and design of the project
Crawford R	Involved in the conception and design of the project, assisted in collection of clinical samples and reviewed the manuscript
Xiao Y	Involved in the conception and design of the project, technical guidance, Manuscript preparation and review

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name Signature Date

ABSTRACT

Osteoarthritic subchondral bone is characterized by abnormal bone density and enhanced production of bone turnover markers, an indication of osteoblast dysfunction. Several studies have proposed that pathological changes in articular cartilage influence the subchondral bone changes, which are typical of the progression of osteoarthritis; however, direct evidence of this has yet to be reported. The aim of the present study was to investigate what effects articular cartilage cells, isolated from normal and osteoarthritic joints, may have on the subchondral bone osteoblast phenotype, and also the potential involvement of the mitogen activated protein kinase (MAPK) signalling pathway during this process. Our results suggest that chondrocytes isolated from a normal joint delayed osteoblast differentiation, whereas chondrocytes isolated from an osteoarthritic joint enhanced osteoblast differentiation, both via a direct and indirect cell interaction mechanisms. Furthermore, the interaction of subchondral bone osteoblasts with osteoarthritic chondrocyte conditioned media appeared to significantly activate ERK1/2 phosphorylation. On the other hand, conditioned media from normal articular chondrocytes did not affect ERK1/2 phosphorylation. Inhibition of the MAPK-ERK1/2 pathways reversed the phenotype changes of subchondral bone osteoblasts, which would otherwise be induced by the conditioned media from osteoarthritic chondrocytes. In conclusion, our findings provide evidence that osteoarthritic chondrocytes affect subchondral bone osteoblast metabolism via an ERK1/2 dependent pathway.

INTRODUCTION

Osteoarthritis (OA) is a common musculoskeletal disorder and 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 [1, 2]. The mechanisms responsible for the abnormal subchondral bone activity remains elusive and questions remain unanswered as to whether subchondral bone changes precede cartilage degeneration or vice versa or maybe run parallel with each other. In some OA animal models, it has been shown that a thickening of subchondral bone precedes fibrillation of the cartilage [3-5], 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 remodeling of the subchondral bone [6, 7]. The changes in OA cartilage lead to the activation and promotion of subchondral bone remodelling [8, 9]. These findings hint at the possibility that signals transmitted from articular cartilage chondrocytes (ACCs) to subchondral bone osteoblasts (SBOs) may trigger phenotype changes in the subchondral bone. There is, however, no conclusive evidence to support the hypothesis that ACCs affect the phenotype of adjacent SBOs during the development of OA.

The role of the mitogen activated protein kinase (MAPK) signalling network in osteogenesis and bone homeostasis has been demonstrated in several studies [10-12]. MAPKs are proline-directed kinases, and 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 [13]. 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 [14, 15]. MAPKs phosphorylate specific transcription factors, which affect the transcription levels of target genes; one such gene is core-binding factor 1 (CBFA1), a marker of osteoblast differentiation [16]. *In vitro* studies have shown that MAPK signalling can either promote or prevent osteoblastic differentiation depending on the nature of extracellular stimuli [17, 18]. The present study was designed to determine the effects

of normal and OA ACCs on the SBOs phenotype and the involvement of MAPK signaling pathways during these processes. Cell-cell interactions can occur both distally and in close proximity, direct and indirect co-culture systems were therefore designed to study the cross-talk of SBOs and ACCs.

MATERIALS AND METHODS

Reagents: *Cell culture:* Dulbecco's Modified Eagle's Medium (DMEM), antibiotics (penicillinG and streptomycin) and collagenase type II 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, Australia).

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: Ethics approval for this project was granted from the Queensland University of Technology and Prince Charles Hospital Ethics Committees and informed consent was given to all patients involved. **OA ACCs** (n=5) were sourced from the main defective area of the medial compartment cartilage showing degenerative changes from the patients undergoing knee replacement surgery. The average age of the OA patients participating in this study was in the range of 65.20 ± 5.94. **Normal ACCs** (n=5) were obtained from trauma patients, where knee tissue was available. 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), none of whom were taking

medications which might affect cartilage or bone metabolism. The patients selected for this study had all ceased taking anti-inflammatory medication at least one month prior to surgery. In order to eliminate early OA changes, patient samples showing any evidence of cartilage changes were excluded. Such changes include softening of the hyaline articular cartilage, thining and fibrous dislocation, ulcerations of the cartilage, and light sclerosis of the subchondral bone. At the microscopic level, the cartilage was classified according to the Mankin score, where score 0 indicated normal cartilage and score over 3 indicated degenerative OA cartilage [19-21]. Chondrocytes from the cartilage tissues were isolated following a method described previously with minor modifications [22]. Briefly, cartilage is dissected to small pieces with sterile scalpel, and washed several times with 1X PBS (phosphate buffered saline). Chondrocytes are released by digesting the tissues in 0.2% collagenase type II mixed in high glucose DMEM at 37°C for approximately 16 hrs. The cell suspension was filtered (70µm mesh) and centrifuged at 1000g for 10 min and resuspended in complete DMEM media containing 10% FCS and 50u/ml penicillin and 50µg/ml streptomycin and plated at a density of 2500 cells/cm^2.

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 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 trauma patients mentioned above, with no evidence of subchondral bone erosion or cartilage degeneration on top of the bone according to the criteria of American college of Rheumatology [23]. SBOs were isolated according to the method described by Beresford [24, 25]. Briefly, bone is minced to 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. 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 (See appendix 1).

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

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 600 × *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 high glucose 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 pellets 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 bottom plates in complete DMEM medium (DMEM supplemented with 10% FBS and 50 u/ml penicillin and 50 µg/ml streptomycin) and allowed to settle for

24 hr to avoid accidental mixing of cells. High-density micromass droplets were prepared as described previously [26]. Briefly, normal or OA ACCs were resuspended in growth media at final cell density of 2.5×10^7 cells/ml and spotted as 10 µL/well droplets on cell culture inserts, incubated for 2 hr at 37^oC, after which the inserts were placed above the normal SBOs monolayer and the co-cultures were incubated in high glucose 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 every 3 days. After 2 weeks of chondrogenic differentiation, ACC pellets were incubated with 2 mL of complete DMEM media (DMEM supplemented with 10% FBS and 50 u/ml penicillin and 50 µg/ml streptomycin) for 48 hr. Conditioned medium (CM) from normal or OA ACCs pellets was collected and centrifuged at speed of 500 × *g* for 15 min and the supernatants are stored at -80^0C 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 (pre-incubated at 37^0C and frozen) and osteogenic supplements (at concentrations described above). SBOs were replenished with fresh CM every 2 days. Control SBOs were incubated in DMEM supplemented with 10% FBS, 50 u/ml penicillin, 50 µg/ml streptomycin and osteogenic supplements which was pre-incubated at $37\ ^0C$ and frozen and treated similarly as CM. The SBOs were harvested for total cell lysate, RNA and protein on days 3, 7, and 14 of co-culture. 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 triplicate 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; 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 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 (Appendix 2). 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 (table 1) 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 4ºC. The membranes were washed three times in TBS-Tween buffer, and then incubated with an anti-rabbit secondary antibody at 1:2,000 dilution 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: Each normal sample of SBOs was cocultured with either normal (n =5) or OA (n =5) ACCs and the study was repeated in 5 normal SBO samples. Results are presented as a mean ± SEM. 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 ($p \geq 0.05$) were observed in either direct or indirect co-culture systems at any of the time points (3, 7, and 14 days) (Appendix 3).

ACCs pellet characterization: Chondrogenic characterization of normal and OA ACCs was evaluated by the expression of markers such as 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 cell lines collected from both normal and OA patients (***Figure 3A***) confirming the phenotypic stability of these cells used in this study. 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 quantitative PCR studies (data shown in chapter 5). These results indicate that the subpopulation of normal and OA ACCs used for co-culture studies have different phenotypic properties.

OA ACCs enhanced SBOs differentiation in both direct and indirect cell co-cultures: 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 we assessed whether this 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. qRT-PCR 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 levels 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 were 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 ($p \leq 0.05$) 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 relevant to OA pathogenesis, the basal levels of phosphorylated ERK1/2 were measured by immunoblot method in freshly isolated normal and OA SBOs. The phenotype of normal and OA SBOs was characterized subsequently and shown in figure 2. The results revealed 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 detected 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, 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.

DISCUSSION

Histomorphometric evidence suggests progression of cartilage degeneration leads to changes in bone parameters affecting deeper levels of subchondral bone, which hints to the cartilage influencing the subchondral bony changes [8]. 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, which is in agreement with the previous reports in the literature [27-29]. However, the question as to how these changes occur at the cellular level, and therefore the nature of molecular mechanisms involved, remain unclear.

Applying *in vitro* co-culture models, two earlier studies have shown that interactions between osteoblasts and chondrocytes modulate cell phenotypes. Co-culturing bovine chondrocytes with osteoblasts leads to decreased osteoblast differentiation compared to the osteoblasts that were cultured alone, indirect evidence that the chondrocytes produce factors that are capable of delaying osteoblast differentiation [30, 31]. Using both direct and indirect co-culture models, we have demonstrated that ACCs, isolated from a healthy joint, decreased the differentiation potential of the SBOs, a finding consistent with these earlier studies. 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 is not known, however, whether this cell-cell interaction mechanism is the same as when OA ACCs are co-cultured with normal SBOs. Here we reported 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 interest, since they suggest that dysregulation of SBOs, by OA ACCs, can result in increased bone metabolism, leading to changes to subchondral bone architecture and subsequent bone sclerosis, a characteristic feature of OA.

A number of studies have demonstrated an increase in bone mineral density in human OA subchondral bone [32]. However, more recent studies have shown that OA subchondral bone, besides being sclerotic, is mechanically weak due to hypomineralization, despite a significantly greater expression of osteogenic markers such as *ALP*, *OC* and *OPN*, in the OA compared to normal SBOs. In fact, certain animal models have shown thinning of subchondral bone thickness in OA [33]. The explanation for these phenomena may be an abnormal type I collagen production by OA SBOs coupled with a lower affinity for calcium [34, 35]. In our study we found that OA ACCs significantly increased the mineralized matrix formation of SBOs. *In vivo* changes in bone density could likely be the linked process of osteoblast and osteoclast functions in the subchondral bone. Further studies should therefore focus on the interactions of OA ACCs with whole subchondral bone remodelling to better

understand how these changes induced by OA ACCs in SBOs are translating into bone remodelling process.

It has become apparent that chondrocytes from OA cartilage have an altered phenotype, characterized by a decrease in cartilage matrix expression and increase of the terminal differentiated phenotypic changes [36, 37]. During endochondral ossification calcified cartilage functions as an osteoconductive substrate, permitting the osteoblast precursors to attach and initiate deposition of bone matrix [38]. A co-culture study by Nurminskaya and colleagues demonstrated that factors, derived from growth plate hypertrophic chondrocytes, promote osteoblast maturation via transglutaminases and protein kinase C signaling [39]. 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 initiated in the course of pathological conditions such as OA [37, 40]. The observations presented here, that OA, but not normal ACCs, increase SBOs differentiation is therefore most probably a result of the phenotypic changes in the OA chondrocytes. Indeed, the ACCs from OA patients we used in this study showed strong mRNA expression for hypertrophic specific genes such as *COLX*, *ALP*, *CBFA1* and *MMP-13 (*data shown in chapter 5).

Westacott showed that conditioned media from OA osteoblasts significantly enhanced glycosaminoglycan release from normal cartilage [41], and in a later study, it was observed that OA osteoblasts decreased the cartilage specific phenotype [42]. Using a similar co-culture model, we were also able to demonstrate that OA ACCs differentially regulated SBO phenotypic properties, compared to normal SBOs, by modifying various signaling pathways (data shown in chapter 5). Taken together these findings suggest that in pathological conditions, such as OA, bi-directional interaction between bone and cartilage cells is altered such that there is an observable phenotypic change in both ACCs and SBOs. Interestingly, several studies have shown direct means of contact between articular cartilage and bone making the way easy for such cross talk [43, 44]. *In vivo,* the medial compartment typically shows evidence of prominent cartilage and subchondral bone changes, which is a clear indication that changes in bone and cartilage are linked, a notion which is supported by a recent

study in which instability induced OA showed synchronized changes to both the subchondral bone and the cartilage [45].

The mechanism by which these interactions are regulated in SBOs, when co-cultured with normal ACCs or OA ACCs, has also been investigated in this study, by elucidating the signaling pathways that are modulated by cellular interactions. Among the signaling factors, MAPK are reported to be involved in the regulation and control of the cellular differentiation events in osteoblasts [10]. Here we have demonstrated that co-culturing SBOs with OA ACCs, leads to a significant activation of the ERK1/2 signaling pathway, whereas normal ACCs failed to activate the same pathway, which indicates that OA ACCs induced SBOs phenotype is transduced via 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 inhibitor. The ERK1/2 inhibition significantly reduced the OA ACCs induced SBOs differentiation. Importantly, we have demonstrated that the basal levels of ERK1/2 phosphorylation increased in OA SBOs compared to normal SBOs, an indication of the relevance of this pathway in OA pathogenesis.

As one of the major components of the MAPK family, ERK is strongly associated with osteoblast differentiation, which mainly operates via activating the transcription factors [17]. 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 inhibitor PD98059. It has been reported, in an *in vitro* model, that activated ERK1/2 physically interacts with *CBFA1* and phosphorylates this transcription factor [16, 46]. The function of *CBFA1* is evident during osteoblast differentiation which is characterized by regulating the expression of major osteogenic genes, such as *ALP* and *OC*, the expression of which is usually followed by an increase of matrix mineralization [46, 47]. The ERK-CBFA1-OC pathway has been documented in osteoblast differentiation in a number of studies [16, 48], and our data suggests that OA ACCs, by the activation of the ERK1/2 signalling pathway, might be responsible for these effects. This, however, does not exclude the possibility that other signalling pathways are involved during this process, for instance the BMP/Smad signalling pathways have been reported to interact with

MAPK and further studies are required to directly demonstrate the role of CBFA1 during this interaction process [15].

The p38 pathway was not activated in our co-culturing systems, which suggests this pathway plays a subordinate role in SBOs differentiation. The JNK pathway, on the other hand, showed significant phosphorylation in co-cultures compared to the non co-cultured SBOs. However, despite this robust activation, the addition of JNK specific inhibitor did not alter SBOs differentiation in co-cultures compared to the non co-cultured controls. It is premature to conclude that p38 and JNK have no effect at all on SBO differentiation, but there were no obvious effects in our experimental model to support this notion. However, it is known that JNK is activated in response to a variety of cellular signals, including cytokines and growth factors [49, 50]; therefore one cannot disregard the likelihood that JNK was induced in responce to these factors upon the co-culture of SBOs with ACCs. Finally, combining our findings and those of previous studies, it is clear that the subchondral bone and cartilage interactions are more complex than was previously thought. Further studies, are warranted to determine how interactions at the cellular level are reflected in the *in vivo* changes and to further strengthen the knowledge of the factors that affect this process.

Conclusions: The data presented in this study supports the hypothesis that OA ACCs play an integral role in SBO phenotypic changes. Normal ACCs appeared to delay SBO differentiation, whereas OA ACCs appeared to enhance SBO differentiation. This cell-cell interaction activated the signalling cascade of ERK1/2 in the disease progression of OA. Specific targeting of these molecular events by ERK1/2 antagonists, as well as with RNA interference, for ERK1/2, may help to control abnormal SBO phenotypic changes and provide a rationale for novel treatment strategies.

Table 1
Primer sequences for real-time PCR.

Gene name	Forward sequence	Reverse sequence
ALP	CGTGGCTAAGAATGTCATCATGTT	TGGTGGAGCTGACCCTTGA
OC	CCACCGAGACACCATGAGAGC	CCAGCGATGCAAAGTGCG
CBFA1	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA
OPN	TCACCTGTGCCATACCAGTTAA	TGAGATGGGTCAGGGTTTAGC
18srRNA	TTCGGAACTGAGGCCATGAT	CGAACCTCCGACTTTCGTTCT
COL2	GTGGGCTTCCTGGTGA	CAGCACCTGTCTCACCA
GAPDH	CGGGAAACTGTGGCGTGATGG	CCTCTTCAAGGGGTCTACATGG
AGG	AGACTTGGTGGGGTCAG	GATGTTTCCCACTAGTG

FIGURES AND LEGENDS

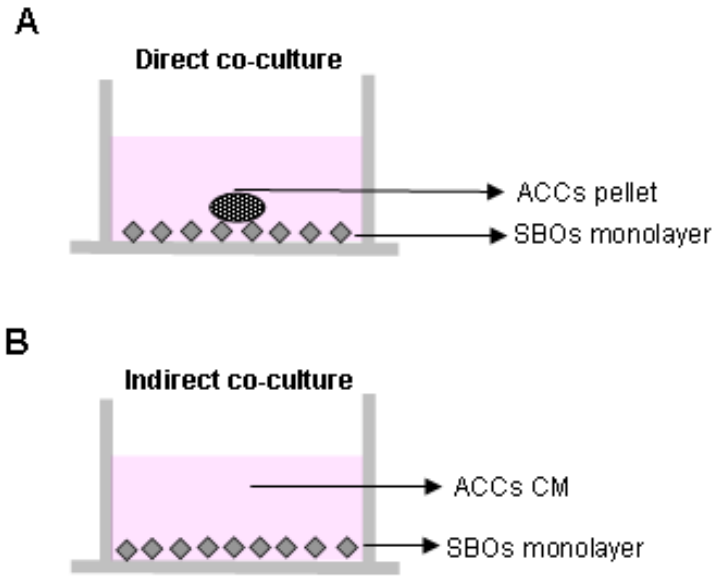

Figure 1: Diagrammatic representation of co-culture models. (A) In the direct co-cultures, ACCs pellets were prepared and placed directly upon the SBOs monolayer in different combinations as described in methods and materials. **(B)** In the indirect co-cultures, ACCs conditioned media (ACCs CM) were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

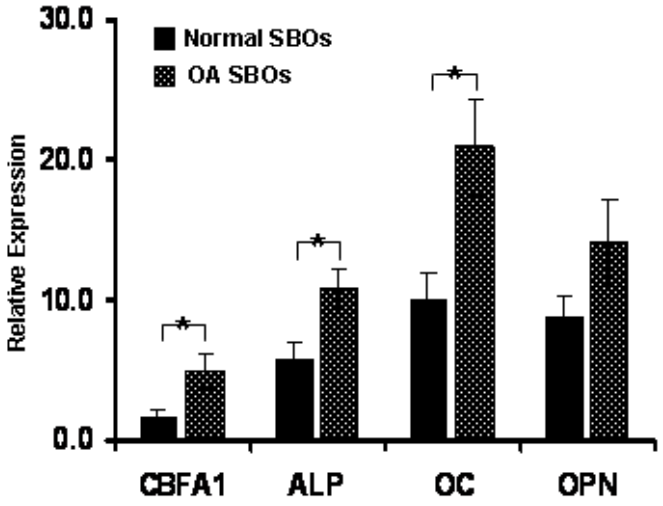

Figure 2: Expression of osteogenic genes in SBOs. The mRNA expression of osteogenic marker genes was compared between normal and OA SBOs after 14 days in differentiation media. The expression of *CBFA1*, *ALP* and *OC* was significantly upregulated in OA SBOs compared to normal SBOs. *OPN* expression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

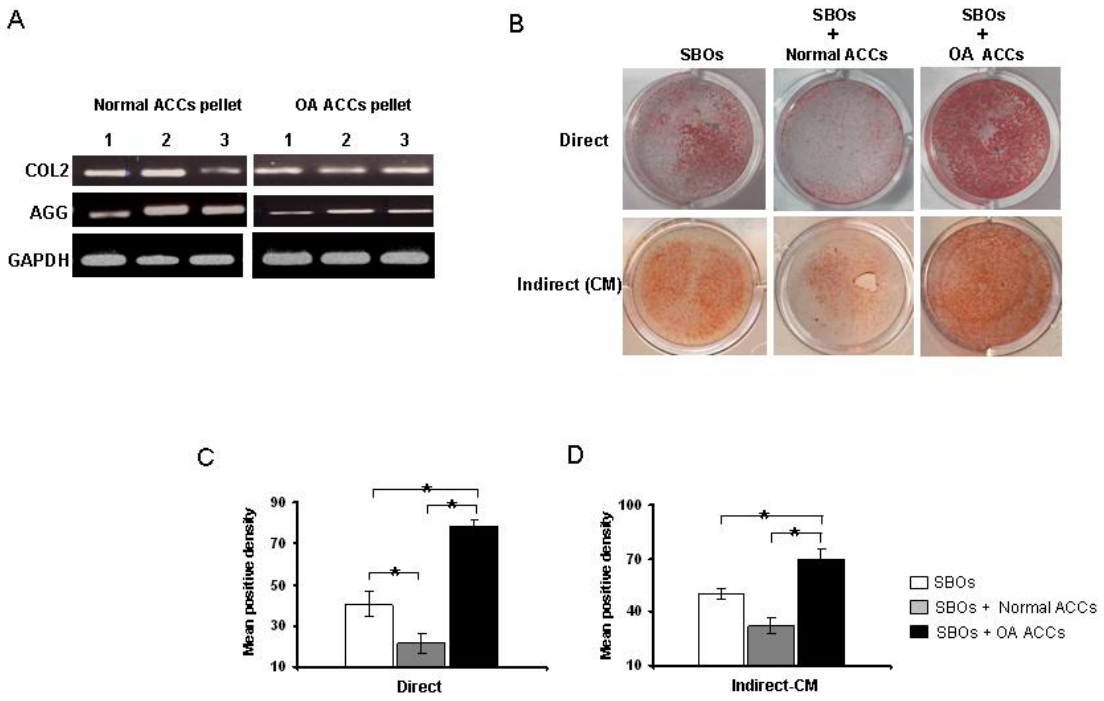

Figure 3: Effects of normal and OA ACCs on SBOs matrix deposition in the direct and indirect co-culture system. (A) Phenotypic characterization of normal and OA ACCs was determined by the mRNA expression of COL2 and AGG after 14 days in the pellet culture. A representative of three normal and three OA ACC pellets results 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 (pictures taken with Sony digital camera and processed using Microsoft power point). 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. *: $p<0.05$.

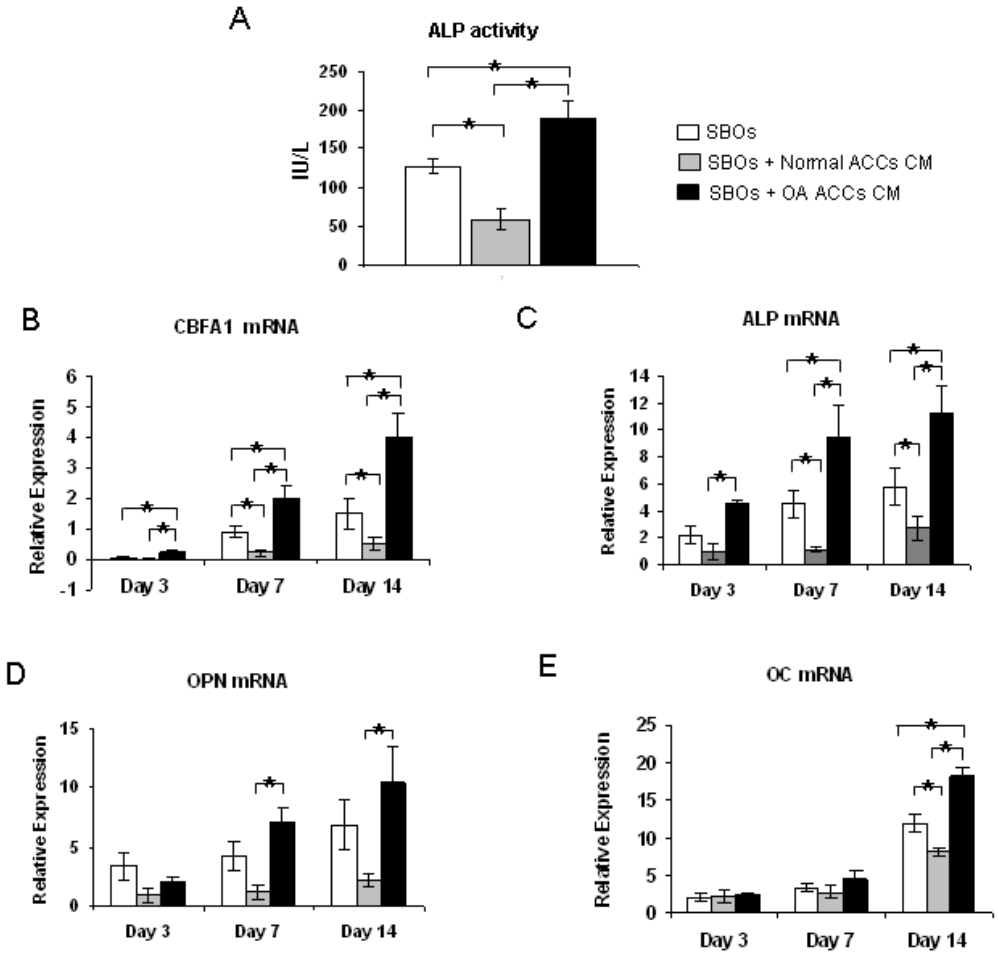

Figure 4: ALP activity and osteogenic gene expression of normal SBOs cultured with normal or OA ACC CM. (A) ALP activity determined by a colorimetric assay in SBOs after culture with CM from normal or OA ACCs for 7 days as indicated in figures. The bars are mean ± SEM. *: $p<0.05$. Expression levels of *CBFA1***(B)**, *ALP***(C)**, OPN**(D)** and *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 and GAPDH. Bar equal mean ± SEM. *: $p<0.05$.

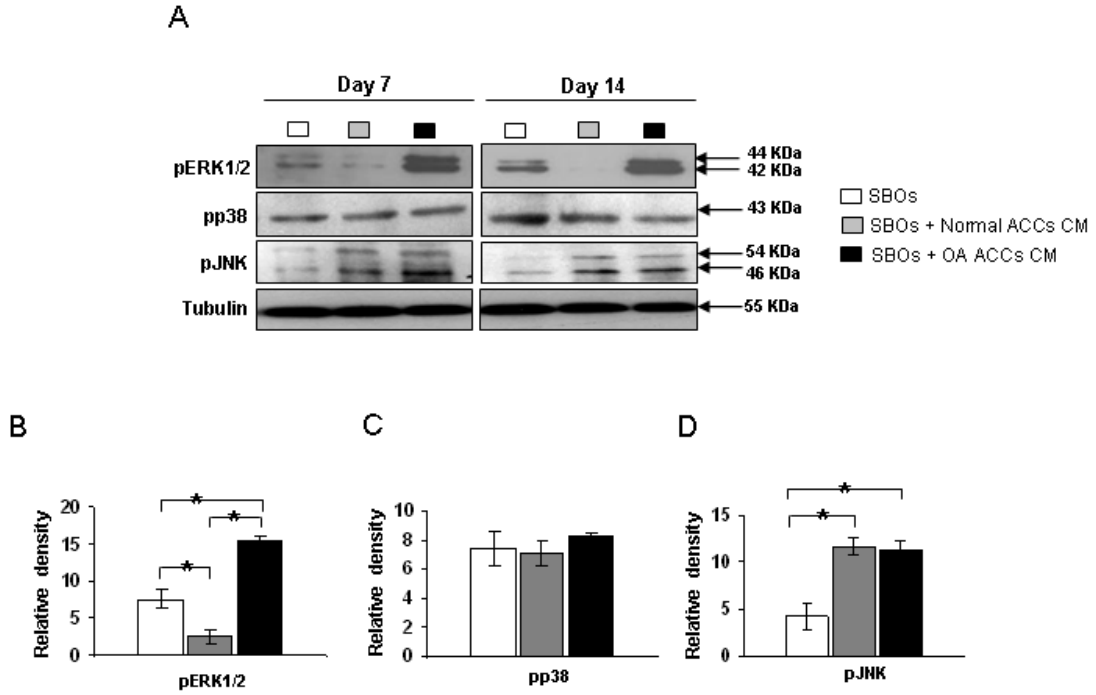

Figure 5: Phosphorylated ERK1/2, p38 and JNK expression in normal SBOs cultured with OA or normal CM. (A) Total cell protein was isolated from SBOs cultured with normal or OA ACCs CM and western blot was performed to determine phosphorylation changes of ERK1/2, p38, and JNK pathways at day 7 and day 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 program. *: $P<0.05$.

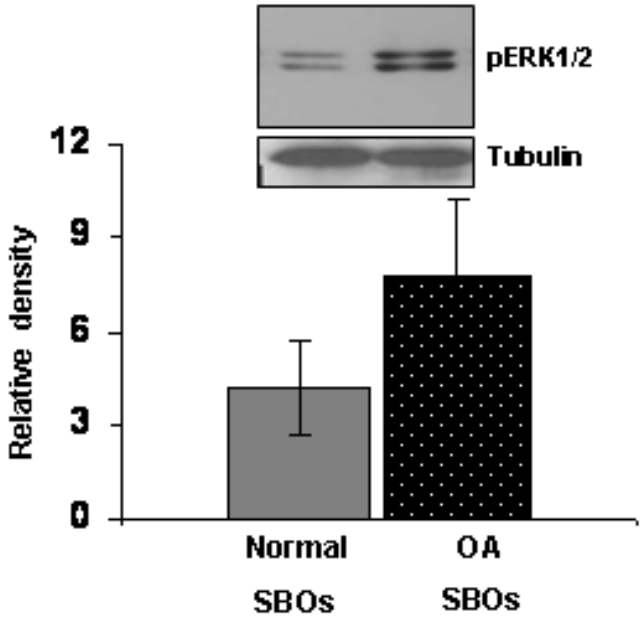

Figure 6: Western blot method was used to measure the basal phosphorylated levels of ERK1/2 in freshly isolated normal and OA SBOs. Higher levels of phosphorylated ERK1/2 were detected in OA SBOs compared to that in normal SBOs. Band density quantification was performed from bands obtained from three individual normal and OA SBOs using imageJ program.

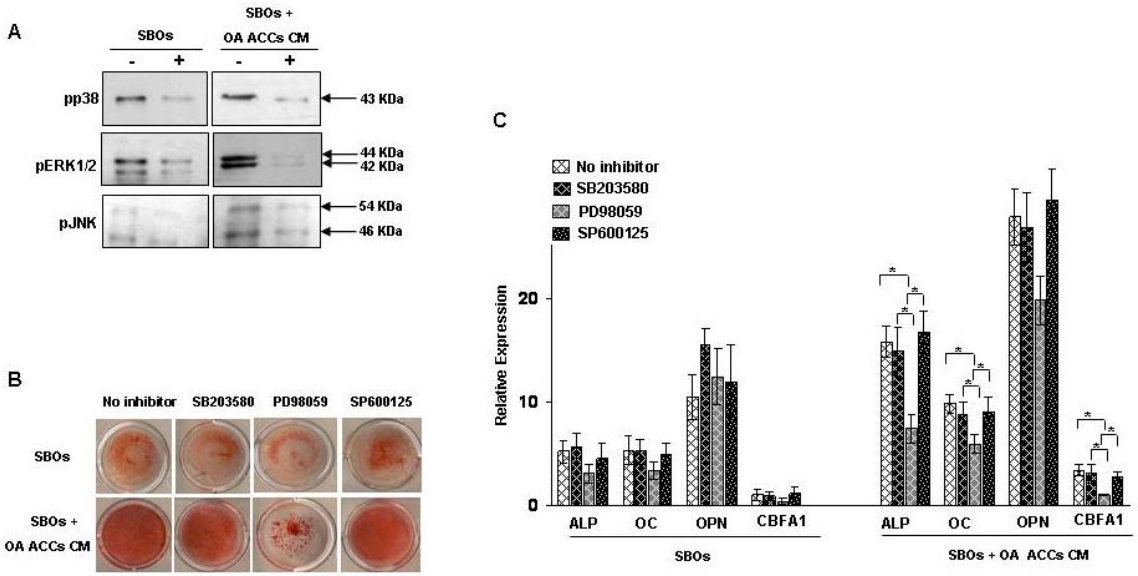

Figure 7: Inhibition of ERK1/2 phosphorylation reversed OA ACCs CM induced SBOs differentiation. Normal SBOs were cultured alone or with OA ACCs CM for 14 days in the presence (+) or absence (-) of SB203580 (5µM) to inhibit p38 phosphorylation, PD98059 (10µM) to inhibit ERK 1/2 phosphorylation or SP600125 (10µM) to inhibit the JNK phosphorylation in osteogenic conditions, respectively. (**A)** Western blot analysis revealed that the addition of the respective inhibitors reduced 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 mineralised extracellular matrix deposition as detected by alizarin red staining 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 GAPDH and the relative expressions were given. *: $p<0.05$.

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Chapter 5

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

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Suggested Statement of Contribution of Co-Authors for Thesis by Published Paper:

Contributor	Statement of contribution*
Prasadam I	Involved in conception and design of the project, Performed laboratory experiments, data analysis and interpretation. Wrote the manuscript.
Signature	
Date	
Gennip S	Performed laboratory experiments in optimising the co-culture models.
Friis T	Assisted with manuscript preparation
Shi W	Performed Immunostaining experiments
Crawford R	Involved in the conception and design of the project, assisted in collection of clinical samples and reviewed the manuscript
Xiao Y	Involved in the conception and design of the project, technical guidance, Manuscript preparation and review

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name Signature Date

ABSTRACT

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

Methods: Knee tissues from the OA patients and normal controls were collected to isolate SBOs and ACCs. Direct and indirect co-culture models were applied to study chondrocyte hypertrophy under the influence of OA SBOs. Mitogen-activated protein kinases in the regulation of the cell-cell interactions were monitored by phosphorylated antibodies and relevant inhibitors.

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

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

INTRODUCTION

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

Recent studies suggest that the adjacent subchondral bone plays a major role in OA cartilage changes, an indication of active communication taking place between the subchondral bone and the cartilage in the progression of OA (4). Bone anabolic factors, such as osteocalcin (OC), osteopontin (OPN), and alkaline phosphatase (ALP) are all upregulated in OA subchondral bone osteoblasts (SBOs) compared to normal SBOs supporting the notion of dysfunction of osteoblast behaviour (5-7). It has been shown in some OA animal models that a thickening of subchondral bone precedes cartilage changes (8, 9), and it has further been demonstrated that *in vivo* factors produced by OA SBOs increase glycosaminoglycan (GAG) release from the cartilage (10) and can influence cartilage specific gene expression (11). It was demonstrated by the application of a co-culture model of bovine explant subchondral bone and cartilage, that excision of subchondral bone from articular cartilage resulted in increased chondrocytes death, thus demonstrating the important role of subchondral bone in maintaining joint homeostasis (12). However, the molecular mechanisms, and in particular signalling pathways, by which normal and OA SBOs regulate articular cartilage phenotype remain unknown.

Activation of the three major classes of mitogen activated protein kinases (MAPKs) - extracellular signal–regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK - has been detected in chondrocytes (13). MAPKs are known to be responsible for the conversion of vast number of extracellular stimuli into specific cellular responses, including chondrocyte proliferation and differentiation (14, 15). The requirement of MAPK signalling pathways, in particular p38 and ERK1/2, during various phases of endochondral ossification has been demonstrated in several studies

(16, 17). MAPK proteins have been shown to play a distinct role in aspects of cartilage biology such as cartilage matrix synthesis and homeostasis (18, 19). The role of MAPK signalling in the biology of cartilage, points towards a possible association of altered MAPK signalling and OA pathogenesis. Indeed, alterations in MAPK signalling pathways are reported to play a prominent role in chondrocyte dysfunction as a part of OA pathogenesis and disease progression (20). Since the OA SBOs are reported to alter the cartilage phenotype, it is possible that these alterations in ACCs may occur via MAPK regulation. The purpose of the present study was to address the question as to how normal and OA SBOs interact with ACCs with respect to hypertrophic changes and to investigate the intermediary role of MAPK signalling pathways during the interactions process.

METHODOLOGY

Articular cartilage sample collection and phenotypic determination: Ethics approval for this project was granted from the Queensland University of Technology and Prince Charles Hospital Ethics Committees and informed consent was given by all patients involved. **OA ACCs** (n=5) were sourced from the main defective area of the medial compartment cartilage showing degenerative changes. The average age of OA patients used in this study was 65.20 ± 5.94. **Normal ACCs** (n=3) were obtained from trauma patients, where knee tissue was available. Normal patients were healthy adults aged 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 macroscopic changes included softening of the hyaline articular cartilage; thinning and fibrous dislocation; ulcerations of the cartilage; and light sclerosis of the subchondral bone. At the microscopic level, cartilage was classified according to Mankin score (21) based on saffranin-O and H&E histology staining, where score 0 indicated normal cartilage and score over 3 indicated degenerative OA cartilage. Chondrocytes from the cartilage tissues were isolated following a method described previously with minor modifications (22). Briefly, cartilage is dissected to small pieces with sterile

scalpel, and washed several times with 1X PBS (phosphate buffered saline). Chondrocytes were released by digesting the tissues in 0.2% collagenase type II mixed in high glucose DMEM at 37°C for 16 hrs. The cell suspension was filtered (70µm mesh) and centrifuged at 1000g for 10 min and resuspended in complete DMEM media containing 10% FCS and 50u/ml penicillin and 50µg/ml streptomycin and plated at a density of 2500 cells/cm^2. Only early passage ACCs (passage 0-2), 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 trauma patients mentioned above, with no evidence of bone erosion or the cartilage degeneration on top of it, as judged by criteria established by the American College of Rheumatology (23). After removing the overlaying cartilage, SBOs were isolated according to a methodology described by Beresford (24, 25). Briefly, bone is minced to 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. Isolated normal and OA SBOs were characterized by differentiating a subpopulation of cells 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) and by analysing the expression of bone specific markers alkaline phosphatase (ALP) and osteocalcin (OC), as well as the staining by 1% alizarin red (Appendix 1).

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 trypsinized and resuspended in a serum free chondrogenic media (serum-free medium-high glucose DMEM (Invitrogen, Mt Waverley, VIC, Australia) supplemented with 10 ng/mL transforming growth factor-β3 (Bio Scientific, Gymea, NSW, Australia), 10nM dexamethasone, 50 mg/mL ascorbic acid, 10 mg/mL sodium pyruvate, 10 mg/mL proline, and an insulin+transferrin+selenium supplement (final concentration: 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL sodium selenite, 0.5 mg/mL bovine serum albumin and 4.7 µg/mL linoleic acid) and centrifuged at 600 x *g* for 20 min to form a pellet. Pellets were allowed to differentiate for two weeks in 3-dimensional conditions in 15 mL Falcon tubes, in chondrogenic medium, which was replenished every 2 to 3 days. After 2 weeks of chondrogenesis ACCs pellets were co-cultured with normal or OA SBOs as described below.

High-density micromass culture: High-density micromass droplets were prepared as described previously (26). Briefly, following trypsinization the ACCs were resuspended in growth media (high glucose DMEM supplemented with 10% FCS, 50 u/ml penicillin and 50 µg/ml streptomycin) 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^oC for 2 hr to allow the cell attachment to the plate. Micromasses were cultured for 1 week in serum free chondrogenic media as described above. 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 (Hypertrophic differentiation media). As controls ACCs pellets are cultured in the same above medium in 24 well plates in the absence of SBOs. After 14 days of co-culture, the ACC pellets were washed three times in PBS and fixed in 4% paraformaldehyde for 10 min and embedded in the paraffin blocks. Paraffin slices of 5 µm thick were

sectioned by microtome, placed on 3-aminopropyltriethoxy-silane coated glass slides, air-dried and stained with 1% alizarin red, 0.5 % alcian blue and immunostained to COL2 antibody 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 pellet cultures (27).

Indirect co-culture: *Preparation of SBOs conditioned medium (CM):* Passage 2, SBOs from normal and OA subchondral bone (2.5×10^5 cells) were cultured in high glucose DMEM supplemented with 1% FCS, 0.5% glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, 50 µM ascorbic acid, 10nM dexamethasone, and 10 mM β-glycerophosphate in 25 cm^2 flasks for 2 days. The conditioned media from these flasks was collected and centrifuged at 500 × *g* for 15 min and the supernatants were transferred to fresh tubes and mixed with an equal volume of fresh (preincubated in 37^oC in the incubator) media with the same supplements to prepare CM. During co-culture experiments, ACCs micromasses, prepared as described above, were grown for 1 week in CM generated from either normal or OA SBOs. Control ACC micromasses were cultured in the same above media composition which was not incubated with SBOs. Media was replenished for every 2 days. At the end of the 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.

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.

Detection of MAP kinase activation: The MAP kinase mediated cellular interactions was evaluated by using the MAP kinase inhibitors SB203580 (Novabiochem, Alexandria, NSW, Australia) and PD98059 (Novabiochem) for p38 and ERK1/2 pathway inhibition respectively. ACC micromasses were incubated with or without

the MAPK inhibitors in CM prepared from normal or OA SBOs. The stock solutions of each inhibitor were dissolved in DMSO; the final concentration of DMSO not exceeding 0.1% (v/v). An equal amount of DMSO vehicle was added to control ACCs. Medium was replenished every 2 days. The optimal concentration for inhibition in ACCs was found to be 10 μM for ERK1/2 and 5 μM for p38. At these concentrations there was no evidence of cytotoxicity, nor was cell proliferation influenced by the addition of the inhibitors. All experiments were performed in triplicate (Appendix 2).

RNA Extraction and Quantitative RT-PCR (qRT-PCR): Total RNA was isolated with TRIZOL reagent (Invitogen), DNase treated and column purified using an RNeasy Mini Kit (QIAGEN Pty Ltd, VIC, Australia). Complementary DNA was synthesized using Superscript III (Invitrogen) from 1μg total RNA following the manufacturer's instructions. PCR primers were designed based on cDNA sequences from the NCBI Sequence database using the Primer Express® software and the primer specificity were confirmed by BLASTN searches. RT-qPCR was performed on an ABI Prism 7000 Thermal Cycler (Applied Biosystems, Scoresby, VIC, Australia) with SYBR Green detection reagent. Briefly, 2 μL of cDNA, 20 pmol of gene-specific primers and 10 μL of 1x Master Mix were used in a 20 μL reaction volume; each sample was performed in duplicate. 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 micrograms 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 4ºC. 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.

Data analysis: Results were presented as a mean ± SD (n=3). 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

Cell proliferation: The cell proliferation of ACCs was assessed in the co-cultured *vs.* non co-cultured ACCs. The results showed no significant differences in either direct or indirectly co-cultured ACCs at any time points. Similarly, no difference in the cell proliferation rates was observed in SBOs after 14 days culture in co-culture media (Appendix 3).

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 in micromass culture at day 7 using serum free media. 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 downregulated in OA ACCs compared to normal ACCs **(*Fig 1A*)**. These results indicated that OA ACCs had greater potential to undergo hypertrophic differentiation compared to normal ACCs. With respect to MAPK phosphorylation, it was observed that the p38 phosphorylation was downregulated in OA ACCs in comparison to normal ACCs (***Fig 1B***). On the other hand ERK1/2 phosphorylation was significantly upregulated in OA ACCs compared with normal ACCs (***Fig 1C***).

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 ACCs co-cultured with OA SBOs compared with normal SBOs **(Fig 2A, upper panel)**. The expression of COL2 immunostaining was decreased in the co-culture groups compared to non co-cultured ACCs pellets. However, the expression of COL2 in ACCs was more prominently decreased in the presence of OA SBOs compared to normal SBOs **(Fig 2A, lower panel)**. In contrast, co-culture of ACCs pellets with OA SBOs led 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 **(Fig 2A, middle panel)**. At the gene expression level, cartilage specific genes such as *COL2* and *AGG* were significantly lower in ACCs co-cultured with OA SBOs compared to ACCs co-cultured with normal SBOs and ACCs alone **(Fig 2B)**. In contrast, OA SBOs induced a significant upregulation of mineralization and hypertrophic markers such as *COL10, ALP* and *CBFA1* compared to co-culture group containing normal SBOs and ACC pellets alone **(Fig 2C)**.

Indirect co-culture: When ACC micromasses were cultured with normal or OA SBO CM, alcian blue staining reveled loss of cartilage matrix deposition upon co-culture with by both normal and OA SBOs CM; however, this cartilage matrix loss was more prominent in the case of OA SBOs CM **(Fig 3A, upper panel)**. Conversely, matrix mineralization was greater in ACCs micromasses grown for 7 days in OA SBOs CM as demonstrated by alizarin red staining **(Fig 3A, lower panel)**. Again at the level of gene expression, the induction of cartilage specific genes *COL2* and *AGG* was significantly downregulated in the presence of both normal and OA SBOs CM compared to ACCs cultured alone, although this decrease was more prominent in ACCs grown in the presence of OA SBOs CM **(Fig 3B)**. In contrast, the expression of cartilage hypertrophy markers *CBFA1, COL10* and *ALP*, was significantly upregulated in ACCs cultured in the presence of OA SBOs CM **(Fig 3C)** compared to both normal SBOs CM and control groups**.**

Phospho p38 and phopho ERK1/2 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 ACCs alone and ACCs co-cultured with normal SBOs. On the other hand, the co-culture of ACCs with OA SBOs leads to a complete attenuation of p38 phosphorylation compared to ACCs cultured with normal SBOs and ACCs alone. These results suggest that upregulation of ERK1/2 and downregulation of p38 phosphorylation are involved in the pathological interaction of OA SBOs with ACCs **(Fig 4A-D)**.

Use of PD98059 reversed hypertrophic gene expression in ACCs induced by OA SBOs CM: Incubation with ERK1/2 inhibitor PD98059 in ACCs cultured with OA SBOs CM had the effect of decreasing the expression of phospho-ERK1/2 in a concentration dependent manner with a co-concomitant increase of phopho-p38 **(Fig 5A).** These findings indicate that the inhibition of ERK1/2 phosphorylation led to a positive feed back to p38 phosphorylation in the ACCs stimulated by OA SBOs CM. ACCs cultured 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. qRT-PCR showed that ERK1/2 pathway inhibition by PD98059 (10µM) reversed the expression of hypertrophic gene such as *CBFA1*, *COL10* and *ALP* in ACC micromasses induced by OA SBOs CM **(Fig 5B)**. By contrast, the expression of *COL2* and *AGG* was upregulated by PD98059 **(Fig 5C)**.

Use of SB203580 induced hypertrophic gene expression in ACCs cultured with normal SBOs CM: Inhibition of the p38 phosphorylation by SB203580 lead to reduction of p38 activity and is accompanied by a co-concomitant increase of ERK1/2 phosphorylation indicating that absence of p38 pathway activates ERK1/2 pathway **(Fig 6A)**. In the presence of SB203580, the hypertrophic genes such as *CBFA1*, *COL10*, and *ALP* were significantly enhanced, whereas, chondrogenic markers of *COL2* and *AGG* were downregulated in ACCs even though co-cultured with normal SBOs CM **(Fig 6C)**. Similar results were also obtained in non co-cultured normal ACCs (data not shown). These results indicate in the absence of continuous p38

signaling, there was a significant shift of ACCs phenotype towards hypertrophy, suggesting an absolute requirement of this signalling pathway to maintain ACCs phenotype.

DISCUSSION

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

It was observed that ACCs isolated from OA patients produced significantly greater levels of the *CBFA1*, *COL10* and *ALP* mRNA when compared to ACCs isolated from healthy patients. This finding indicates that the OA ACCs possessed greater potential to undergo hypertrophic differentiation, results, which corroborated previous works comparing normal and OA ACCs (3, 28).

Applying an *in vitro* indirect co-culture model, Sanchez et al (6) demonstrated that the sclerotic OA SBOs decreased cartilage specific gene expression such as *SOX9* and *COL2*. They also showed that inhibitors of hypertrophic differentiation such as pTHrP and PTH-R were significantly downregulated in ACCs co-cultured in the presence of OA SBOs. These findings are evidence that OA SBOs can decrease the inhibitors of hypertrophic differentiation leading to a subsequent mineralized matrix deposition in cartilage. In the present study, using both direct and indirect co-culture methods, we showed that OA SBOs increased both hypertrophic gene expression and matrix mineralization and these are followed by a simultaneous decrease of the chondrocyte specific phenotype. A characteristic symptom of OA is an up regulation of hypertrophy and mineralization related markers and a down regulation of the chondrocyte specific markers in particular COL2 and AGG (2, 29). The observations in our study suggest that the interaction of OA SBOs may lead to these typical molecular changes in ACCs during the progression of the disease. It has been previously reported that the transition of ACCs phenotype to hypertrophic changes

may contribute damage to the extracellular matrix by triggering degradative enzymes such as Matrix metalloproteinases (MMP) to several fold, which have the ability to cleave the collagen fibrils, a mechanism which inturn is pathological for the health and integrity of articular cartilage leading to its degeneration (30-32). It is also interesting observation that normal SBOs induced significant hypertrophy in ACCs compared to non-cocultured cells although to a lesser extent than compared to OA SBOs. These findings imply that there could be an increase in the secreted soluble factors (yet to be defined) released by the OA SBOs compared to normal SBOs rather than entirely OA-specific factors.

However, the reasons as to why OA SBOs seem to induce these altered ACCs phenotype remain unclear; there are nevertheless several potential pathways responsible. Both our own studies and that from other groups, have demonstrated that OA SBOs produce abnormal levels of osteogenic markers, growth factors and cytokines. Specifically, increased production of growth factors such as Insulin like growth factor (IGF)-1 (33), Bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)-β (34) are reported in OA SBOs compared to normal SBOs. Among these factors BMP-2 (35) and IGF-1 (36) are implicated in inducing cartilage hypertrophic changes in growth plate chondrocytes. In addition, it has been reported that OA SBOs produce abnormal levels of cytokines such as IL (interleukin)-1 & -6 (34), Tumor necrosis factor (TNF)α (37) and MMP-13 (38), the factors which have the ability to activate a diverse array of signalling pathways (39, 40). Therefore, it is possible that the secreted bio-molecules from OA SBOs either individually or co-operatively can communicate with ACCs *via* altering the signalling pathways, thereby mediating the altered communication between bone and cartilage. However, further studies are required to delineate the soluble factors that are induced/up/downregulated in co-culture of ACCs and normal SBOs *vs.* ACCs and OA SBOs.

Among signalling factors, the MAPK subtypes ERK1/2 and p38 play a key role in the signalling process of chondrocyte cellular differentiation and homeostasis depending on the nature of extracellular stimuli (41). This knowledge prompted us to investigate MAPK signalling in the context of what influence normal and OA SBOs have on the differentiation of ACCs. This is the first study of its kind to report that OA SBOs

induce ERK1/2 phosphorylation and suppress p38 phosphorylation in ACCs, indicating that the alterations of these pathways accompany ACCs pathological phenotypic changes. Indeed, we have demonstrated that the basal levels of ERK1/2 phosphorylation increased and p38 decreased in OA ACCs compared to normal ACCs, an indication of the relevance of this pathways with respect to OA pathogenesis.

Furthermore, the inhibition of ERK1/2 signalling prevented acquisition of hypertrophic gene expression and there was a return to the chondrogenic phenotype in ACCs co-cultured with OA SBOs. In contrast, inhibition of p38 signalling pathway potentiates acquisition of hypertrophic gene expression and a weakening of chondrogenic phenotype, thus negating the effects of normal SBOs had on ACCs. Together, this data indicates that the ERK1/2 and p38 signalling pathways exert opposite effects in ACCs co-cultured with normal and OA SBOs, implying that OA SBOs induced altered phenotypic changes in ACCs was transuded via activation of ERK1/2 and de-activation of p38 pathway. The finding that the ERK1/2 activation increased the hypertrophic differentiation in ACCs is consistent with the study showing the strong activation of ERK1/2 pathway in hypertrophic zone of the growth plate (16). Furthermore, it has been demonstrated that the inhibition of ERK1/2 delayed hypertrophic differentiation in growth plate chondrocytes during endochondral ossification (42).

MAPKs are regulated at several levels, including kinase-kinase and kinase-substrate interactions, and inhibition of cross-talk/output by the MAPKs themselves (43). The activities of p38 are primarily governed by extensive cross-talk with ERK1/2, a process that involves protein phosphatase, resulting in a reciprocal bidirectional equilibrium between ERK1/2 and p38 phosphorylation, where an increase in p38 activity suppresses activation of ERK1/2 and vice versa (44, 45). Such cross-talk appears to play a role in OA SBOs regulating ACCs phenotype, the existence of which has been shown in chondrocytes (13). For example, the opposing roles of ERK1/2 and p38 has been demonstrated in chondrogenesis regulation of mesenchymes (46) and the activation of p38 blocks the ERK pathway at the level of MEK through activation of protein phosphotase 2A (47).

It is possible that components of the p38 and ERK1/2 pathways may interact directly in the transcriptional complex (48). The intermediate p38 and ERK1/2 pathway transcription substrates involved in these interaction are not known, but it is interesting that PD98059 (anti ERK1/2) significantly reduced the expression of the transcription factor *CBFA1*, whereas SB203580 (anti p38) activated this transcription factor. 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. Indeed, continuous expression of *CBFA1* in chondrocytes induces hypertrophic differentiation and endochondral ossification, suggestive of an important role of this transcription factor in triggering hypertrophic changes (49). It is therefore possible that OA SBOs induced altered phenotypic changes are triggered in ACCs via the activation of MAPK-CBFA1 pathway. However, further studies are required to define specific role of CBFA1 during the interactions between SBOs and ACCs.

Conclusions: Our results indicate that OA SBOs deactivated p38 phosphorylation and activated ERK1/2 phosphorylation guiding ACCs to undergo hypertrophic gene expression, a phenotype that usually considered as hallmark for OA ACCs. Therapeutic strategies, that alter the relative activation levels of ERK1/2 and p38 signalling routes, may have significant clinical implications.

ACKNOWLEDGMENT

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FIGURES AND LEGENDS

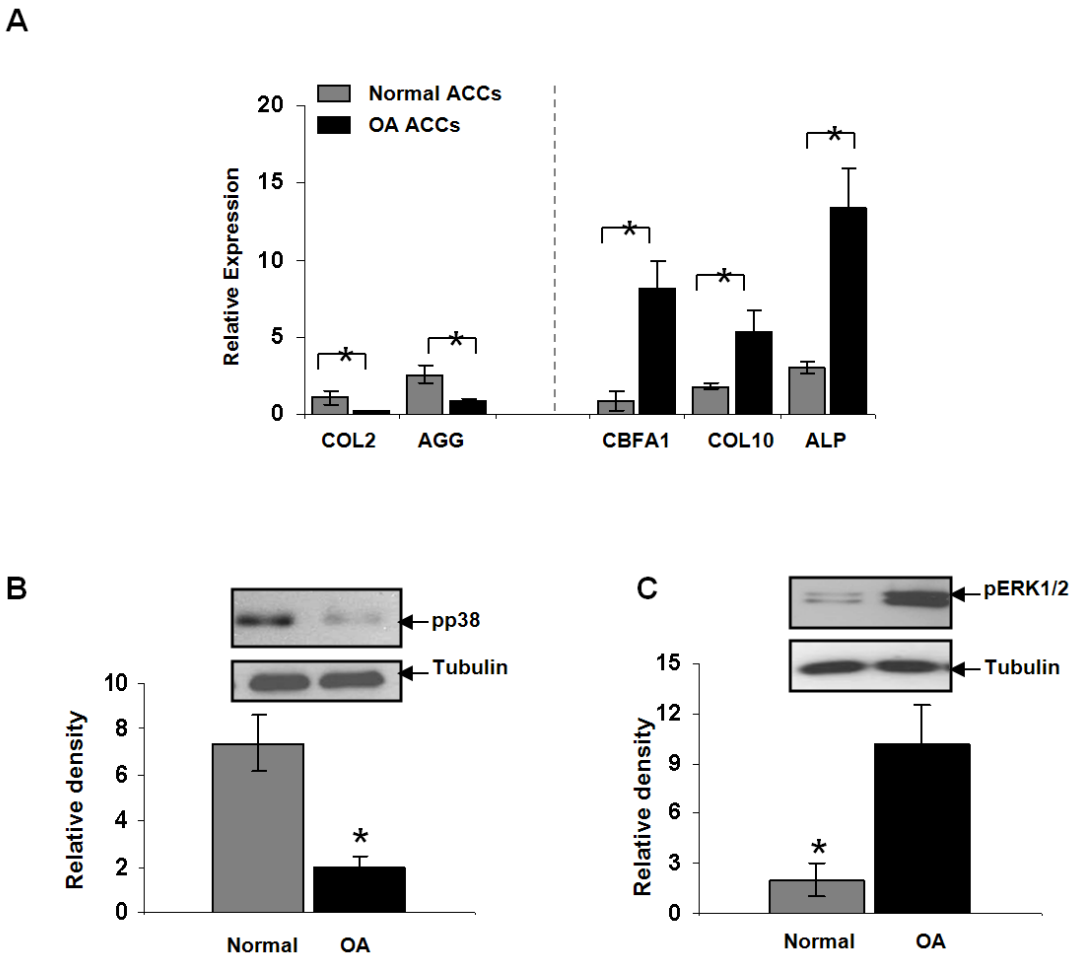

Figure 1: Characterization of normal and OA ACC micromass culture. (A) The mRNA expression of chondrogenic *(COL2* an *AGG)* and hypertrophic marker (*CBFA1*, *COL10* and *ALP)* genes are compared between normal and OA ACC micromasses Results are shown as mean ± SD for three pateints. *: P≤0.05. **(B-C)** Western blot analysis was performed to determine phosphorylation changes of p38 **(B)** & ERK1/2 **(C)** in normal and OA ACC micromasses after 7 days culture in hypertrophic differentiation media. Tubulin was used as a loading control. The bar graphs are representative of protein bands from three separate experiments. * Represents a significant difference (p≤0.05)**.**

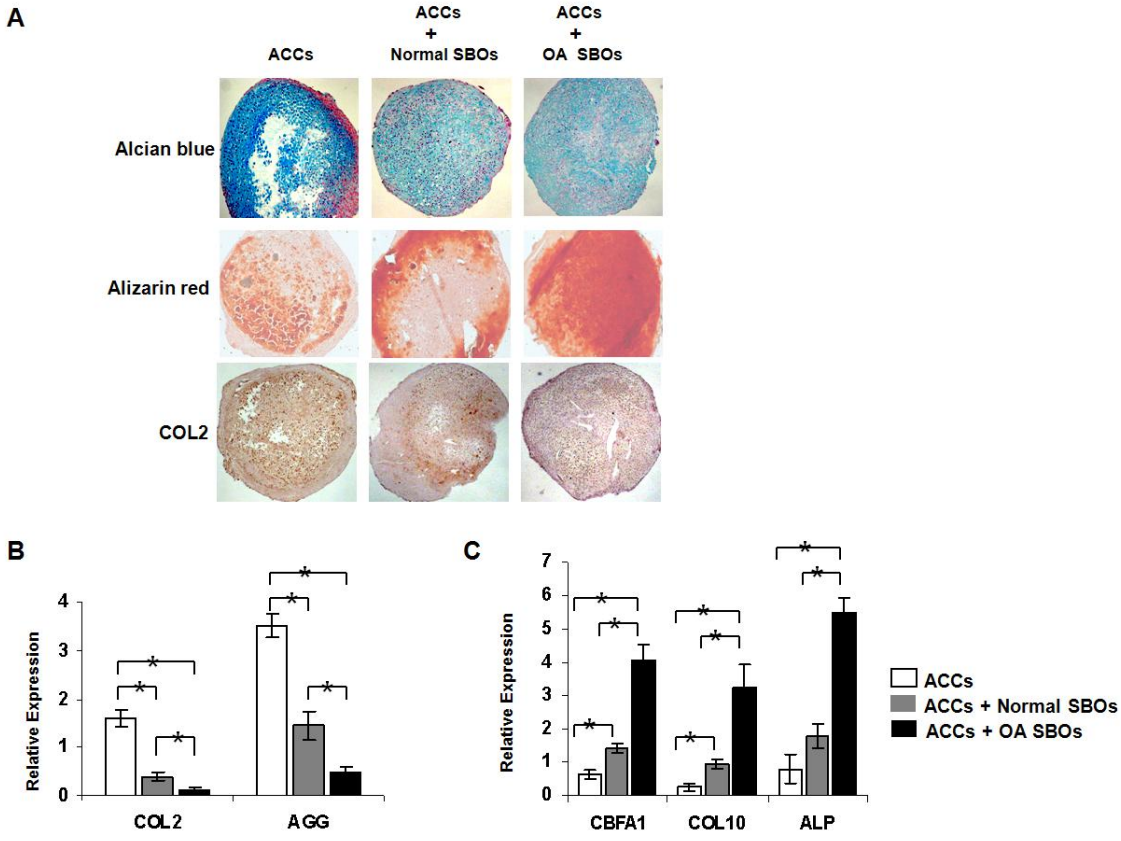

Figure 2: Effects of normal and OA SBOs on normal ACCs matrix deposition and gene expression in the direct co-culture experiments. (A) Alcian blue staining for GAG deposition and immunostaining for COL2 showed a decreased staining pattern in ACCs co-cultured with OA SBOs. On the other hand, mineralized matrix stained by Alizarin red was increased in ACCs pellets co-cultured with OA SBOs. Control ACCs pellets were cultured in the same media, in the absence of SBOs (10X magnifications). **(B-C)** qPCR showed that chondrogenic markers such as *COL2* and *AGG* were downregulated and hypertrophic markers such as *CBFA1*, *COL10,* and *ALP* were significantly upregulated in ACCs pellets co-cultured with OA SBOs. mRNA levels were normalized against GAPDH and 18s and the relative gene expression is presented. Results are shown as mean ± SD (n=3). * Represents a significant difference ($p \leq 0.05$).

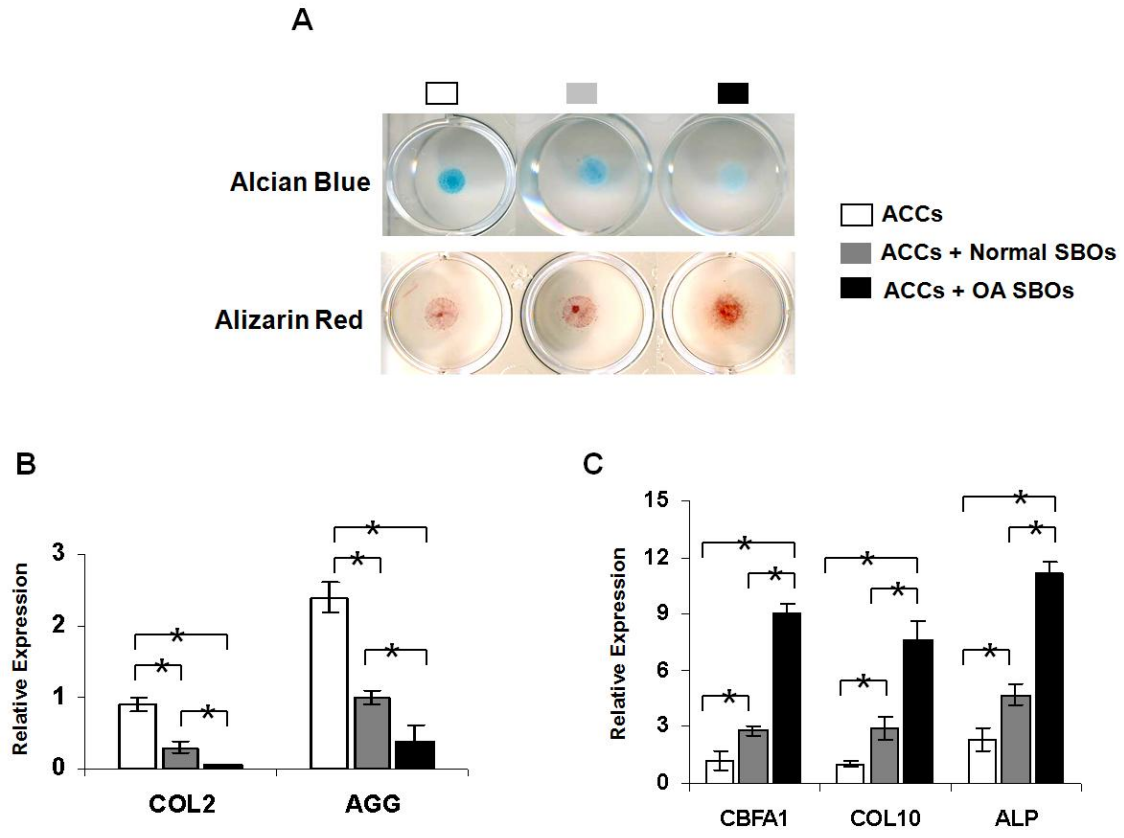

Figure 3: Effects of normal and OA SBOs conditioned media (CM) on normal ACCs matrix deposition and gene expression in indirect co-culture experiments. (A) ACC micromasses were cultured with CM prepared from normal or OA SBOs and control ACC micromasses were cultured in the same media that was not conditioned by SBOs. GAG deposition was decreased and matrix mineralization was increased in ACCs co-cultured in the presence of OA SBOs CM after 7 days (pictures taken with sony digital camera and processed using Microsoft power point). **(B&C)** qRT-PCR showed that *COL2* and *AGG* decreased and *CBFA1*, *COL10,* and *ALP* levels are increased in ACC micromasses cultured in the presence of OA SBOs CM for 7 days. Results are shown as mean ± SD (n=3). * Represents a significant difference ($p \leq 0.05$).

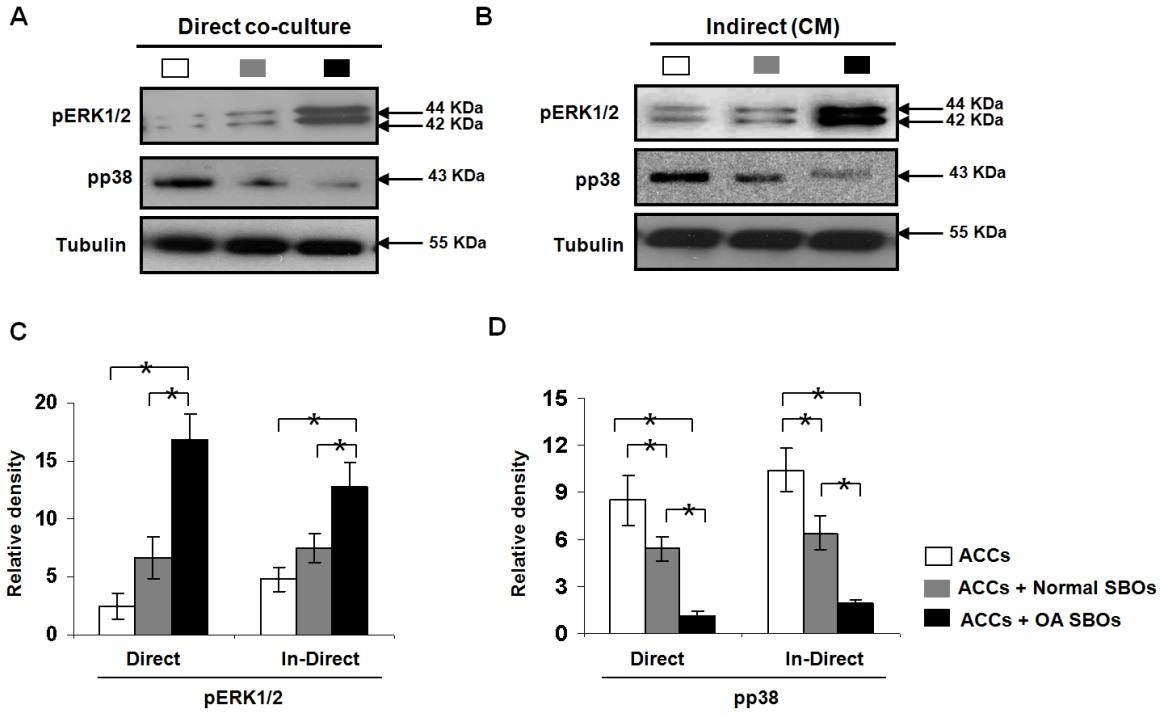

Figure 4: MAPK signalling pattern in the direct and indirect co-cultures of normal ACCs. (A) ACCs pellets were co-cultured directly with normal or OA SBOs monolayers as described in the methodology. After 14 days protein was isolated from ACCs pellet. Western blot analysis showed an increased phospho-ERK1/2 and decreased phopho-p38 in ACCs upon co-culture with OA SBOs. As controls ACC pellets are cultured in the same media, in the absence of SBOs. Tubulin was used as a loading control. **(B)** ACCs micromasses were cultured in the presence or absence of CM generated from normal or OA SBOs for 7 days. Increased phospho-ERK1/2 and decreased phopho-p38 were detected in ACCs micromasses cultured in the presence of OA SBOs CM. 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 ($p<0.05$).

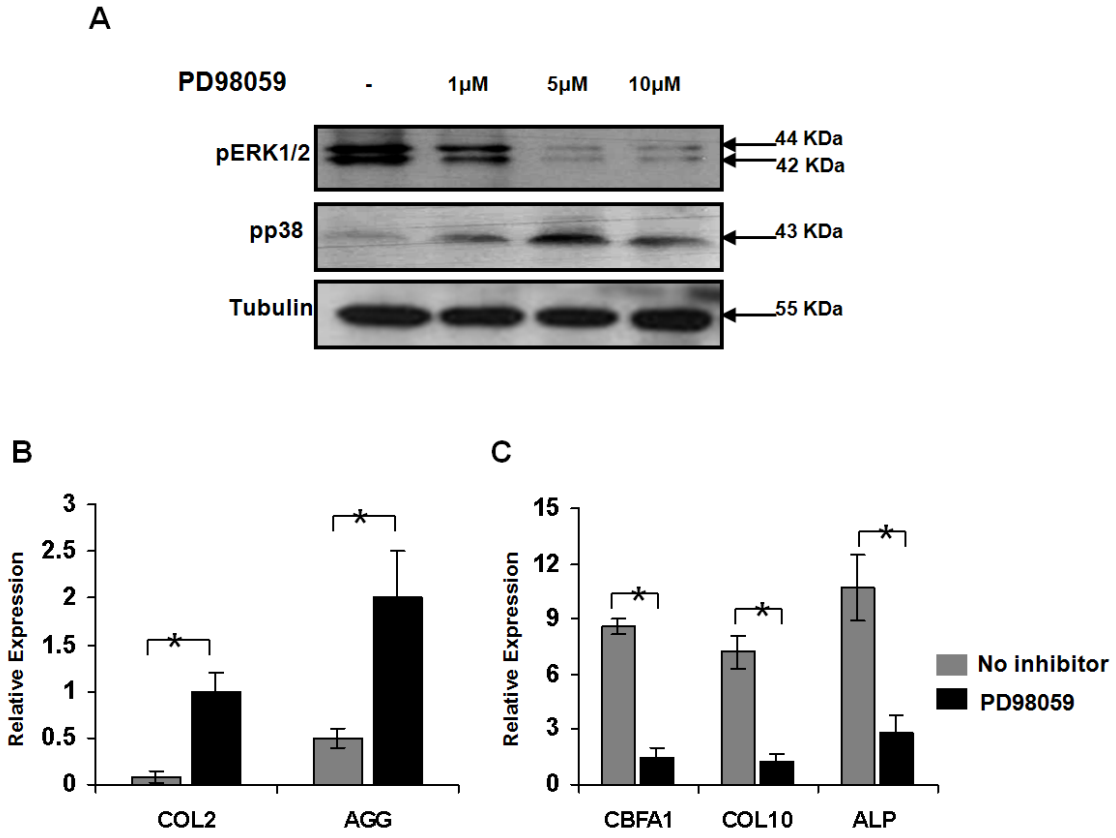

Figure 5: Use of PD98059 reversed the expression of hypertrophic genes in normal ACCs cultured with OA SBOs conditioned media (CM). (A) ACC micromasses were cultured with OA SBOs CM with or without the ERK1/2 inhibitor PD98059 at different concentrations. After 7 days, total cell protein was isolated from ACC micromasses and phosphorylation changes in ERK1/2 and p38 were measured. PD58059 decreased the levels of pERK1/2 in a concentration dependent manner, with a concomitant increase of pp38 levels. **(B-C)** PD98059 (10μM) significantly upregulated *COL2* and *AGG* and significantly downregulated *CBFA1*, *COL10* and *ALP* mRNA levels respectively in ACCs cultured in the presence of OA SBOs CM. mRNA levels were normalized against *GAPDH* and 18s and the relative expression is shown. Results are shown as mean ± SD (n=3), in ACCs co-cultured with OA SBOs CM +/- PD98059. * Represents a significant difference ($p<0.05$).

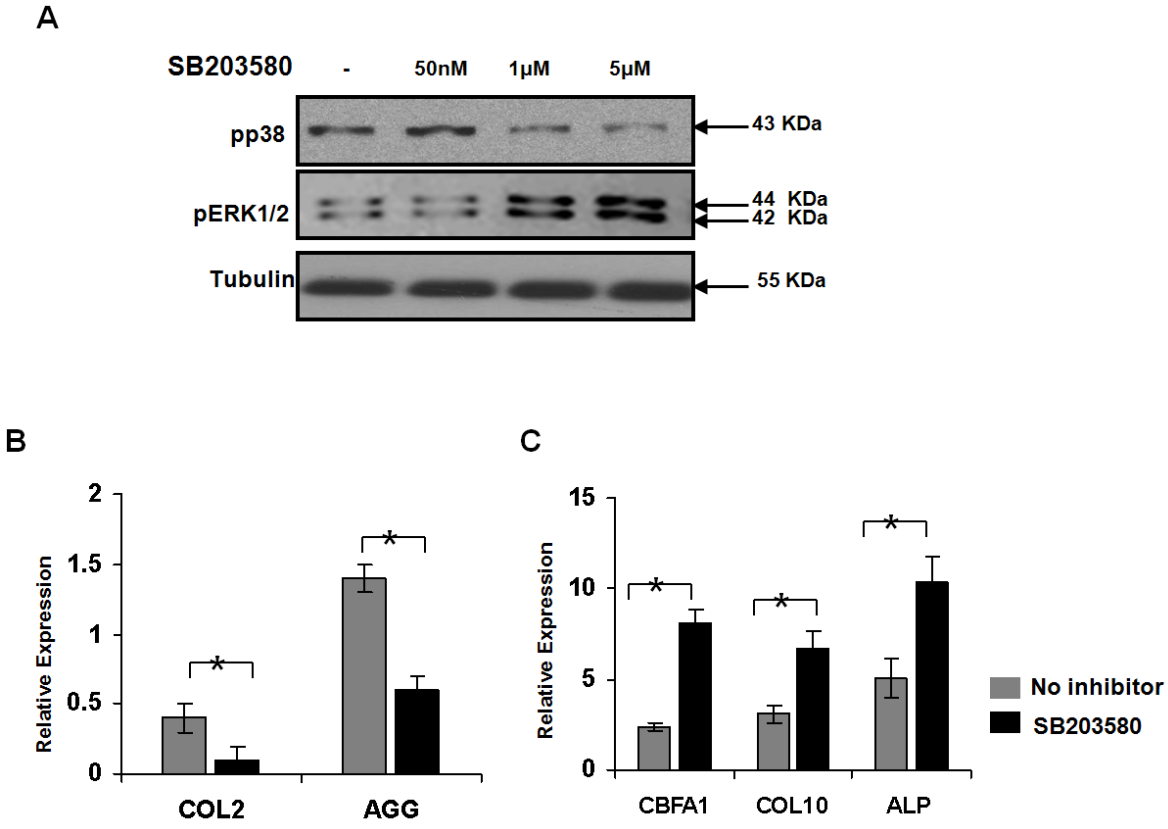

Figure 6: Use of SB203580 induced the expression of hypertrophic genes in normal ACCs cultured with normal SBOs conditioned media (CM). (A) ACCs micromasses were cultured with normal SBOs CM with or without the p38 inhibitor, SB203580. After co-culture for 7 days, total cell protein was isolated from ACCs and changes to p38 and ERK1/2 phosphorylation was measured. SB203580 inhibited pp38 phosphorylation, however enhanced pERK1/2 activity. **(B-C)** Addition of SB203580 (5μM)**,** decreased COL2 and AGG, but increased CBFA1, COL10, and ALP mRNA level in ACC micomasses cultured with normal SBOs CM. mRNA levels were normalized against *GAPDH* and *18s* and the relative expression is shown. Results are shown as mean ± SD (n=3), in ACCs co-cultured with normal SBOs CM +/- SB203580. * Represents a significant difference ($p<0.05$).

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Chapter 6

Cell interactions of osteoarthritic subchondral bone osteoblasts and articular chondrocytes aggravate MMP-2 and MMP-9 production through the mediation of ERK1/2 and JNK phosphorylation

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Suggested Statement of Contribution of Co-Authors for Thesis by Published Paper:

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ABSTRACT

Matrix Metalloproteinases (MMP) play a key role in osteoarthritis (OA) development. The aim of the present study was to investigate whether the cross-talk between subchondral bone osteoblasts (SBOs) and articular cartilage chondrocytes (ACCs) in OA alters the expression and regulation of MMPs, and also to test the potential involvement of mitogen activated protein kinase (MAPK) signalling pathway during this process. As determined by zymography, ELISA and western blotting methods, our results revealed that the direct and indirect co-culture of OA SBOs with ACCs significantly aggravated the proteolytic activity and increased the expression of MMP-2 and MMP-9 in ACCs. In turn, co-culture of OA ACCs led to abundant MMP-2 expression in SBOs. Furthermore, addition of the ERK1/2 inhibitor, PD98059, and the JNK inhibitor, SP600125, reversed the abnormal MMP-2 and MMP-9 production that was induced during the interactions of OA SBOs and OA ACCs. In conclusion, this is the first study to document that signals transmitted between SBOs and ACCs in OA can lead to altered bi-directional interaction which may be related to OA progression.

INTRODUCTION

The key patho-physiological features of OA joints include abnormal subchondral bone metabolism and articular cartilage degeneration. It has been proposed that the changes in the density and the architecture of the underlying subchondral bone have a profound effect on the initiation of cartilage degeneration (1). On the other hand, a strong correlation has been found with articular cartilage changes and abnormal bone density in OA (2-4). These findings hint that both articular cartilage and subchondral bone influence each other's metabolism, leading to an altered bi-directional signal transmission which results in OA pathogenesis. An ideal therapeutic approach would therefore be directed at regulating these altered interactions. In order to achieve this outcome, specific pathological cascades that are triggered during the interactions and the molecular mechanisms that govern those events should first be identified and then targeted.

The excessive cartilage degeneration and abnormal bone remodelling, which both characterize OA, prompted us to address the potential involvement of MMPs in the interaction between articular cartilage and subchondral bone in the OA joint. Several independent groups have previously demonstrated that MMPs are the major culprits responsible for the degeneration of the articular cartilage in OA (5). More importantly, MMP-2 and -9 have the substrate specificity for type II collagen (COL2) and proteoglycans, the major constituents of articular cartilage, and are the dominant enzymes responsible for the cartilage degradation seen in OA (6). On the other hand, upregulated expression of MMPs has also been reported in OA SBOs compared to normal's relating to the abnormal osseous tissue remodeling (7, 8). Although it is known that ACCs and SBOs express abnormal levels of MMPs in OA tissues, substantial inroads remain to be gained to understand the exact mechanism by which this abnormal production of MMPs taking place.

The influence of OA SBOs increases MMP-3 and MMP-13 expression in ACCs (9), but it is not known if the activity of gelatinases such as MMP-2 and MMP-9 are also altered during this process. The bi-directional interaction of ACCs and SBOs has yet to be studied, in particular the specific signalling mechanism(s) underlying altered

SBOs and ACCs cross-talk stimulated activation in the OA compared to the normal joint has not been identified so far. Mitogen activated protein kinase (MAPK) cascades; including ERK1/2, JNK and p38 mediate a number of cell responses both in osteoblasts and chondrocytes (10, 11). There is accumulating evidence that activation of MAPKs contributes to the induction of MMP expression by a variety of extracellular stimuli in different cell types including chondrocytes (12) and osteoblasts (13). The aim of the present study was to examine if the interaction between SBOs and ACCs is a critical event that orchestrates the abnormal production of gelatinases MMP-2 and –9 in OA development and to investigate the potential molecular mechanism involved during this process. For this purpose we used an *in vitro* co-culture model which allowed us to study both direct and indirect cell interactions. Specific MAPK inhibitors were used to delineate the pathway involvement during this interaction process.

METHODOLOGY

Reagents: *Cell culture:* Dulbecco's Modified Eagle's Medium (DMEM) and antibiotics (penicillin 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) and Tri-reagent from Invitrogen. *Inhibitors:* MAPK pathway specific inhibitors for ERK1/2 (PD98059), for p38 (SB203580) and for JNK (SP600125) were purchased from Calbiochem (Novabiochem, Alexandria, NSW, Australia). *ELISA Assays*: MMP-2 and MMP-9 assay kit was from Raybio systems (Bioscientific Pty Ltd, NSW 2232, and Australia).

Antibodies: phospho ERK1/2, phospho JNK, phosphor p38, MMP-2 and MMP-9 antibodies were purchased from Cell Signalling Technology (Genesearch, Arundel, QLD, Australia) or AbCam (Sapphire Bioscience Pty Ltd, Redfern, NSW, Australia).

Articular cartilage sample collection and phenotypic determination: Ethics approval for this project was granted from the Queensland University of Technology and Prince Charles Hospital Ethics Committees and informed consent was given by patients involved. Normal ACCs (n=3) were obtained from knees of the trauma patient's. Normal patients were healthy adults with an mean age of 53.56 ± 10.76, with no clinical signs or symptoms of joint, metabolic or hormonal diseases (osteoporosis). None of the pateints were taking medications which might affect cartilage or bone metabolism. The patients selected for this study had all ceased taking anti-inflammatory medication at least two weeks prior to surgery. Early stage OA patients were excluded if the samples showed any evidence of cartilage changes such as: softening of the hyaline articular cartilage; thinning and fibrous dislocation; ulcerations of the cartilage; and light sclerosis of the subchondral bone. OA ACCs (n=4) were sourced from the main defective area of the medial compartment from patients undergoing total knee replacement surgery. The mean age of OA patients used in this study was 65.20±5.94. All radiographs were reviewed, and the patient samples were classified according to two categories, depending on the Mankin score. Mankin score 0 indicated *normal cartilage* and a score greater than 3 indicated *degenerative OA* cartilage (14). Chondrocytes from the cartilage tissues were isolated following a method described previously with minor modifications (15). Briefly, cartilage is dissected to small pieces with sterile scalpel, and washed several times with 1X PBS (phosphate buffered saline). Chondrocytes are released by digesting the tissues in 0.2% collagenase type II mixed in high glucose DMEM at 37°C for 16 hrs. The cell suspension was filtered (70µm mesh) and centrifuged at 1000g for 10 min and resuspended in complete DMEM media containing 10% FCS and 50u/ml penicillin and 50µg/ml streptomycin and plated at a density of 2500 cells/cm^2.

Subchondral bone sample and phenotypic determination: Knee bone specimens were taken within 5 mm of the subchondral bone plate. OA SBOs (n=4) were cultured from bone sourced from the weight bearing sites of knee from the patients suffering advanced OA, where the cartilage was degraded and showing prominent subchondral bone erosion and density. Normal SBOs (n=3) were cultured from bone collected from the trauma pateints with no evidence of subchondral bone erosion or cartilage degeneration. The criteria for this diagnosis were those established according

to American College of Rheumatology criteria (16). None of normal patients had any muscoloskeletal disorders such as osteoporosis. SBOs were isolated according to the method described by Beresford and only cells from passage 3-4 were used in this study (17, 18). Briefly, bone was minced to 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. The bone cell phenotype was confirmed by determining the production of early bone markers alkaline phosphatise (ALP) and osteocalcin (OC). All bone cell population tested negative, by flow cytometry, for the hemopoietic cell marker CD45. Isolated SBOs showed a strong staining for alizarin red (Appendix 1) and positive expression for ALP and OC under osteogenic induction media, confirming that the cells isolated from the bone samples were of an osteogenic lineage. None of the patients had received any anti-inflammatory or bisphosphonate medication.

Co-culture models: Both direct and indirect co-culture was performed to test the effect of soluble and membrane bound factors. The co-culture studies were performed as one of these four different combinations. Combination 1: Normal SBOs *with* Normal ACCs, Combination 2: Normal SBOs *with* OA ACCs, Combination 3: OA SBOs *with* Normal ACCs, Combination 4: OA SBOs *with* OA ACCs. In the ***direct co-culture system*****,** ACCs ($5x10^4$ cells /well) and SBOs ($5x10^4$ cells/well) were mixed in a 15 ml falcon tube and plated out in 6 well culture plates and incubated in a serum free high glucose DMEM medium supplemented with 50 u/ml penicillin and 50 µg/ml streptomycin for 72 hr. After 72 hr the conditioned medium (CM) was harvested and centrifuged at a low speed of 500×g for 15 minutes and aliquoted into micro-centrifuge tubes and stored in -80º C until further analysis. As controls, normal or OA ACCs and SBOs, the number of cells were equal to the mixed culture, were cultured separately and the conditioned media was collected after 72 hr. This co-culture model is illustrated in ***fig 1A*****.**

In the ***in-direct co-culture system***, cells were co-cultured in a serum free high glucose DMEM medium, using cell culture inserts with a 0.4 μm porous polycarbonate membrane to separate ACCs ($7.5x10^4$ cells/well) and SBOs ($7.5x10^4$cells/well), allowing only soluble factor transfer across the membrane. After co-incubation for 72 hours in a serum free high glucose DMEM medium supplemented with 50 u/ml penicillin and 50 μg/ml streptomycin, total protein was isolated from ACCs and SBOs that were co-cultured with their respective combinations. All co-culture studies were repeated at least three times on different occasions each time from the cells coming from three different donors. The diagrammatic illustration of indirect co-culture models used in this study is illustrated in ***fig 1B***.

RNA Extraction and Quantitative RT-PCR (qRT-PCR): Total RNA was isolated with TRIZOL reagent, 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

Detection of MAPK pathway: Western blotting using antibodies against phosphorylated p38, ERK1/2 and JNK were first used to detect MAPK signal activation in the indirectly co-cultured *vs.* non co-cultured SBOs and ACCs. The MAPK mediated cellular interactions were further evaluated in the indirect co-cultures by the use of MAPK specific inhibitors (anti-p38: SB203580; anti-ERK1/2:

PD98059; anti-JNK: SP600175). Briefly, the concentrated stock solution of each inhibitor was dissolved in DMSO, and the co-cultures were incubated with or without the MAPK inhibitors. The final concentration of DMSO never exceeded 0.1% (v/v) and the same amount of DMSO vehicle was added to the control cultures. Pilot experiments showed that the optimal 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 cell 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.

Zymography: The gelatinolytic activity of serum free CM from the co-cultures was assessed by separating the proteins on 10% SDS–PAGE gel containing 1mg/mL gelatine as a substrate. The gels were washed for 30 min with 2.5% Triton X100 and subsequently incubated at 37°C for 12–24 hr in an incubation buffer containing 50mM Tris-HCl (pH 7.6), 10mM CaCl2, and 50mM NaCl. The gels were stained with coomassie brilliant blue and then destained to visualize white colored bands against the blue coloured background. Further verification was sought by incubating the gel in 10mM EDTA (an MMP inhibitor).

Enzyme-linked immunosorbent assay (ELISA): The content of MMP-2 and MMP-9 secreted proteins was determined in the conditioned medium of co-cultured and non co-cultured cells using an ELISA kit. Samples and standards (100 μl) were incubated in 96-well plates precoated with specific antibody overnight in 4ºC. After several washings, 100μl biotinylated antibody was added and incubated for 1 hr at room temperature. The MMP secreted proteins were detected by a HRP- conjugated streptavidin solution. After washing, the amount of conjugate bound to each well was determined by addition of tetramethylbenzidine substrate. The reaction was quenched by adding a stop solution and optical density was measured immediately using a plate reader (Bio-Rad) at 450 nm. The concentration of the total MMP protein in each sample was extrapolated from a standard curve.

Western blot: The total cell lysate from co-cultured and non co-cultured ACCs and SBOs was harvested (total protein lysates were prepared by lysing the cells in a lysis buffer containing 1M Tris HCl (pH 8), 5M NaCl, 20% Triton X100, 0.5M EDTA and a protease inhibitor cocktail (Roche, Castle Hill, Australia)) and then clarified by centrifugation and the protein concentration was 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 were transferred to a nitrocellulose membrane, and blocked in a 0.1% Tris-Tween buffer containing 5% non-fat milk. The membranes were incubated with primary antibodies overnight at 4ºC. After washing the membranes three times in TBS-Tween buffer they were incubated with a goat anti-mouse / rabbit IgG-horseradish peroxidase conjugated antibody at 1:2000 dilution for 1hr. 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).

Data analysis: Results were presented as a mean ± SEM. Repeated measures ANOVA with post hoc tests was used to determine the statistical significance and $p \leq 0.05$ was considered to be a significant difference.

RESULTS

Chondrocyte phenotype: The primary ACCs grown in monolayer culture undergo a process of dedifferentiation within a few passages and is characterized by a loss of type II collagen (COL2) expression and an upregulation of type I collagen (COL1) expression. To ensure the phenotypic integrity of the ACCs used in this study the cells were assessed for the expression of the cartilage-specific genes aggrecan (AGG) and COL2. The chondrogenic phenotype of ACCs was confirmed by the robust expression of both COL2 and AGG in all the ACCs collected from patients for this study ***(fig 1C)***.

Effect of direct co-culture on the expression of MMP-2 and MMP-9: To explore whether interactions between SBOs and ACCs isolated from normal and OA tissue samples shows the differential acivation of MMP-2 and MMP-9, a direct co-culture model was set up as aforementioned. After 72 hr in culture, conditioned media was collected from co-cultured and non co-cultured cells, and the presence of the bioactive proteases was assessed by zymography and ELISA.

Zymography: Zymography analysis of conditioned media showed an increase in the MMP-2 (72 KDa) proteolytic activity in OA ACCs compared to normal ACCs alone (compare lane 1 ***vs.*** lane 3). Similarly, OA SBOs showed increased MMP-2 activity compared to normal SBOs (compare lane 2 ***vs.*** lane 4), indicative of the pathological role of this MMPs in relation to OA pathogenesis. The levels of MMP-2 and MMP-9 remained unchanged in the co-cultures of normal SBOs and ACCs compared to their corresponding non co-cultured cells (compare lane 1&2 ***vs.*** lane 5). By contrast, co-culturing OA SBOs with OA ACCs resulted in the hyper-activation of MMP-2 proteolytic activity compared to OA SBOs and OA ACCs cultured on their own (compare lane 3&4 ***vs.*** lane 8). Also, when OA ACCs were co-cultured with normal SBOs there was an increase in MMP-2 levels compared to normal SBOs and OA ACCs alone (compare lane 2&3 ***vs.*** lane 6). Similarly, when OA SBOs were co-cultured with normal ACCs an increase in the activity of MMP-2 was observed when compared to the levels that seen in the individual cells (compare lane 1&4 ***vs.*** lane 7).

MMP-9 activity was not seen in either normal or OA ACCs and SBOs alone (compare lane 1&3 and lane 2&4). Interestingly, a band corresponding to pro MMP-9 (92 KDa) was induced when co-culturing OA SBOs with OA ACCs (compare lane 3&4 ***vs.*** lane 8). By contrast, co-culturing normal SBOs with normal ACCs did not show any activation of MMP-9 (compare lane 1&2 ***vs.*** lane 5). The enzyme activity was abolished by the addition of EDTA to the developing buffer, indicating that the induced enzyme belonged to the MMP family (data not shown). The zymography experiments performed on three different patient samples is shown in the ***fig 2A***.

ELISA: The co-culture of normal ACCs with normal SBOs did not show any significant difference of MMP-2 and MMP-9 release relative to normal ACCs and

normal SBOs on their own. By contrast, ELISA showed that total MMP-2 and MMP-9 levels secreted by the co-culture of OA SBOs with OA ACCs was significantly greater than the sum of MMP-2 from the respective cells on their own (193.57 ng/ml *vs.* 38.14 ng/ml for OA ACCs and 51.64 ng/ml for OA SBOs) as was the case with MMP-9 (1507.04 pg/ml *vs.* 432.02 pg/ml for OA ACCs and 354.15 pg/ml for OA SBOs). Furthermore, the results showed that co-culture of OA ACCs with normal SBOs also led to increased MMP-2 expression (102.23 ng/ml *vs.* 38.14 ng/ml for OA ACCs and 19.57 ng/ml for normal SBOs), but not MMP-9 expression (481.10 pg/ml *vs.* 432.02 pg/ml for OA ACCs and 196.79 pg/ml for normal SBOs), compared to sum of mono cultured cells. However, the co-culture of OA SBOs with normal ACCs significantly increased both MMP-2 (120.49 ng/ml *vs.* 51.64 ng/ml for OA SBOs and 16.34 ng/ml for normal ACCs) and MMP-9 (983.51 pg/ml *vs.* 354.25 pg/ml for OA SBOs and 225.52 pg/ml for normal ACCs) activity **(*fig 2B&C*)**.

Effect of indirect co-culture of SBOs and ACCs on the expression of MMP-2 and MMP-9: To verify the relative contribution of each type of cell with respect to MMP-2 and MMP-9 expression, ACCs and SBOs were co-cultured indirectly in their respective combinations for 72 hours using a 0.4 µm porous membrane to separate the cells. The aim of this experimental design was to provide an opportunity to determine the effects of secreted factors between SBOs and ACCs on the MMP expression of the individual cell types.

Effect of normal and OA SBOs on ACCs with respect to MMP-2 and MMP-9 production*:** When normal SBOs were co-cultured with ACCs (both normal and OA), the protein levels of MMP-2 and MMP-9 are not significantly effected in ACCs. By contrast, the protein levels of MMP-2 and MMP-9 were significantly elevated in ACCs (both normal and OA) when co-cultured with OA SBOs, thereby confirming that this effect could be attributed to the SBOs accordingly to their OA disease severity ***(fig 3A).

***Effect of normal and OA ACCs on SBOs with respect to MMP-2 and MMP-9 production*:** The results showed that OA ACCs, but not normal ACCs, had significantly increased expression of MMP-2 proteins in co-cultured SBOs (both

normal and OA). There was, however, no significant difference in MMP-9 expression in co-cultured SBOs *vs.* non co-cultured SBOs ***(fig 3B)*.**

Phosphorylation status of MAPKs in co-cultures of ACCs and SBOs: Further experiments focused on elucidation of molecular mechanisms involved in the interaction of ACCs and SBOs. Previous studies suggest that there is a close association between MAPK signalling pathways and the regulation of MMP expression [27]. Therefore, in this study we focused our attention on the potential role of MAPK pathways in the upregulation of MMP-2 and MMP-9 as the result of interactions between SBOs and ACCs.

Effect of normal and OA SBOs on ACC MAPK phosphorylation: Basal activation of ERK1/2 and JNK was greater in OA ACCs compared to normal ACCs, indicating that upregulation of these pathways are biologically relevant to OA pathogenesis. When ACCs (both normal and OA) were co-cultured with normal SBOs, the levels of phospho-ERK1/2 and phospho-JNK are not augmented. By contrast, the levels of phospho-ERK1/2 and phospho-JNK were significantly enhanced in both normal and OA ACCs when co-cultured with OA SBOs ***(fig 4A)*.**

Effect of normal and OA ACCs on SBOs MAPK phosphorylation: When the effects of normal and OA ACCs was assessed on SBOs (both normal and OA) it was found that the phosphorylation of ERK1/2 increased when SBOs were co-cultured with OA ACCs. By contrast, SBOs co-cultured with normal ACCs did not show any changes of phospho-ERK1/2 expression. JNK phosphorylation was significantly upregulated in OA SBOs alone compared to normal SBOs alone. However, there was no discernible difference in phospho-JNK expression between SBO cells grown in the presence of normal and OA ACCs ***(fig 4B)*.**

Addition of ERK1/2 and JNK inhibitors reversed the MMP-2 and MMP-9 activity that was induced in OA SBOs–ACCs co-cultures: Co-cultures were performed in the presence of p38 inhibitor (SB203580), ERK1/2 inhibitor (PD98059) and JNK inhibitor (SP600125), to verify if the phosphorylation of ERK1/2 and JNK was implicated in the stimulation of MMP-2 and MMP-9 expression during OA SBOs-ACCs interactions. The results obtained indicated that the inhibition of ERK1/2

and JNK phosphorylation robustly inhibited the OA SBOs-ACCs induced MMP-2 and MMP-9 expression, in the direct co-cultures as assessed by ELISA ***(fig 5A&B)*** and zymography ***(fig 5C)*.** Inhibition of p38 phosphorylation failed to abolish the MMP-2 and MMP-9 expression induced in the OA SBOs and ACCs co-cultures.

DISCUSSION

It has long been known that MMPs, in particular MMP-2 and MMP-9, play a crucial role in OA pathogenesis (5). Applying an *in vitro* co-culture model, we reported here for the first time that a bi-directional communication between OA bone cells and cartilage cells, appear to promote MMP-2 and MMP-9 expression, in the supernatant of OA SBOs and OA ACCs co-cultures, compared with the summation of those in the supernatant of OA SBOs cells and OA ACCs cells cultured alone. To further test the relative contribution of each type of cell with respect to MMP augmentation, SBOs and ACCs cells are cultured indirectly by separating them using a 0.4 µM inserts. Our results showed that OA SBOs could induce MMP-2 and MMP-9 expression in ACCs cells, and that OA ACCs, in turn could stimulate MMP-2 production in SBOs upon co-culture. These findings indicate that co-culture of SBOs and ACCs have profound reciprocal effects on MMP-2 and MMP-9 profiles, with the most dramatic differences seen in ACCs compared to a more modest effect in SBOs. In normal SBOs-ACCs co-cultures, such changes in MMPs expression was not noticed when compared to non co-cultured cells. This indicates that under normal conditions, SBOs and ACCs interact in such a way to supress MMP activation in order to maintain the joint homeostasis. Our study suggests that such paracrine restrictions are abolished during OA SBOs-ACCs interactions, leading to an reciprocal enhanced de-novo expression of MMP activity by both cell types thereby aggravating the degradative changes in the joint microenvironment.

The increased activity of MMP-2 and MMP-9 during OA SBOs-ACCs co-cultures provides evidence to central role of MMPs in the biology of OA and is relevant to the major phenotypic characteristics observed in OA pateints: the excessive bone remodelling and cartilage degeneration that occurs during the late stages of the disease. Indeed, several studies have showed a strong expression of MMP-2 and

MMP-9 in OA compared to normal cartilage and subchondral bone, proof of relevance of these proteases to OA patho-physiology (5, 8). It is known that the excessive MMP-2 and MMP-9 activities shift the turnover balance in cartilage which precipitates its degradation (19). Cartilage collagen fibril degeneration initially involves the cleavage within the triple helix predominantly by the gelatinases A (MMP-2) and B (MMP-9) (20). Given that both MMP-2 and MMP-9 are substrate specific for two of the major constituents of articular cartilage, it is reasonable to suspect that these enzymes are responsible for the cartilage degeneration typical of OA. The results of our co-culture experiments suggest that the interaction with OA SBOs could be one factor responsible for these augmented MMP-2 and -9 activities in OA cartilage cells.

In addition, it is interesting to note that, even co-culture of OA ACCs with SBOs significantly increased MMP-2 activity in both normal and OA SBOs. A sequential evaluation of subchondral bone changes in an OA animal model suggests a predominance of bone formation in the more advanced late stage of the disease (21), whereas the remodeling in the early phase favors bone resorption (22). Since, MMP-2 is one of the principal protease capable of degrading the bone matrix, the production of MMP-2 by SBOs, as a result of their interaction with OA ACCs, points to a central mechanism contributing to the elevated bone remodeling leading to a sclerotic bone. Furthermore, several studies reported significantly greater levels of the osteogenic markers in OA SBOs when compared to SBOs isolated from the healthy patients (23). It was reported that MMP-2 is developmentally regulated during *in vitro* osteoblast differentiation *in vitro* (24) and is also regulated *in vitro* by factors implicated in controlling bone tissue turnover (25). Therefore, we cannot exclude the possibility that this protease is involved in enhanced osteoblast activity a typical feature of OA bone. In contrast to levels of MMP-2 which were increased in SBOs upon co-culture with OA ACCs, the levels of MMP-9 were not further elevated upon co-culture with either normal or OA ACCs, despite the fact that the levels of MMP-9 are greater in OA SBOs compared to normal SBOs alone. One explanation for this is that MMP-9 up regulation is independent of the interactions with OA ACCs; it may perhaps be regulated by autocrine factors from the SBOs themselves.

The mechanisms governing the up regulation of MMPs as the result of OA SBOs and ACCs interactions are so far not known. We therefore focussed on the elucidation of the signalling pathways that are modulated by the co-cultures. It is known that the promoter regions of inducible MMP genes are regulated by MAPKs, depending on the nature of the extracellular stimuli (26). Our results revealed that interaction of OA SBOs with ACCs led to increased phosphorylation of the ERK1/2 pathway, as well as JNK signalling in ACCs. In turn, the contact of OA ACCs with SBOs led to increased ERK1/2 phosphorylation. Together these results suggest that cross-talk between OA SBOs and OA ACCs results in bi-directional signal activation, and which signify that these pathways may be intimately involved in regulating abnormal MMP-2 and MMP-9 levels in the co-culture of OA SBOs and ACCs. Indeed, by pharmacologically suppressing the activity of ERK1/2 and JNK activity we demonstrated a complete attenuation of co-culture induced MMP-2 and MMP-9 production in culture supernatants and this is strongly suggestive of a patho-physiologic role of these pathways in the interaction between OA SBOs-ACCs. These results are consistent with recent reports which suggest that targeting of MAPKs in particular ERK1/2 and JNK, may be a potent approach to reduce MMP expression in a variety of cells (27). In fact, MAPKs have been shown to be activated in OA cartilage and there is evidence that ERK1/2 and JNK play a key role in cartilage destruction (28). In this study we have also observed that the ERK1/2 and JNK pathways were expressed in significantly higher levels in OA ACCs and SBOs compared to normal ACCs and SBOs, evidence of the relevance of these pathways to the OA patho-physiology.

It still remains unclear what are the putative soluble and transcription regulators of the ERK1/2 and JNK signalling pathways in the OA SBOs-ACCs co-cultures when compared to normal SBOs-ACCs co-cultures. The MAPK-MMP pathways appears to be regulated transcriptionally by classic mediators such as TNF-α (29), VGEF (30), TGFβ3 (31) and interleukins (32), all of which in turn activate a pleotrophic cascade of signaling pathways. It is therefore possible that one or multiple factors that are described above or some unknown factors are induced in the OA SBOs-ACCs co-cultures, thereby giving rise to a positive feedback to ERK1/2 and JNK pathways, which leads to the MMP mediated degenerative changes in the OA joint. We are

currently conducting a proteomic study to identify the soluble markers that may be responsible the modulation of MAPK in the interaction between ACCs and SBOs in OA progression.

Conclusion: Our *in vitro* study is the first to provide direct insight in to the mechanisms underlying the vicious cycle between subchondral bone and cartilage in OA development. Signals from OA SBOs stimulated MMP-2 and MMP-9 expression in ACCs. In turn, OA ACCs stimulated the MMP-2 activity in SBOs. This bi-directional interaction was mediated by the phosphorylation of ERK1/2 and JNK pathways. Therefore, therapeutic strategies aimed to combat this interaction with pharmacological ERK1/2 and JNK inhibitors may be likely to be effective in reducing OA associated joint damage.

FIGURES AND LEGENDS

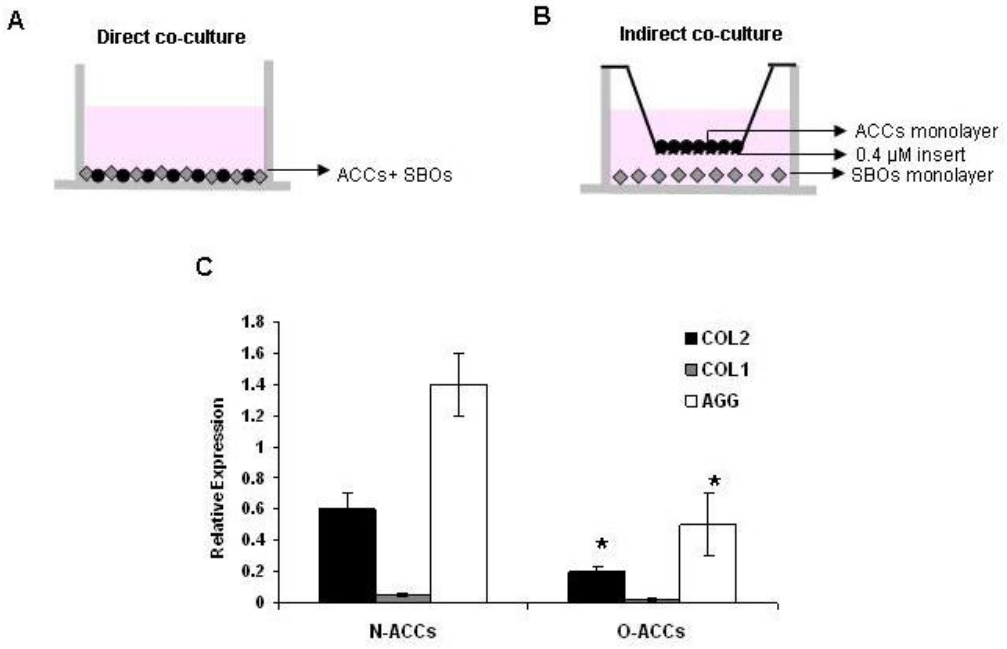

Figure 1: Schematic representation of co-culture models. **(A)** In the direct co-cultures ACCs and SBOs were mixed in 1:1 ratio as described in the Methodology and co-cultured for 72 hr. **(B)** In the indirect co-cultures, a cell culture insert with a 0.4 µm membrane was used. The SBOs were cultured in the inserts and ACCs in the 6 well plates for 72 hours. This method allowed only soluble factor transfer between cell populations, eliminating direct cell-to-cell contact. **(C)** ACCs phenotypic characterization was asessed by the gene expression of Type II collagen (COL2), Type 1 collagen (COL1) and Aggrecan (AGG) in freshly isolated normal (n=3) or OA ACCs (n=4). The relative gene expression values are normalized to 18srRNA and shown as mean ± SEM. * Represents a significant difference between normal vs. OA ACCs. (N-ACCs: Normal Articular Cartilage Chondrocytes, O-ACCs: Osteoarthritic Articular Cartilage Chondrocytes).

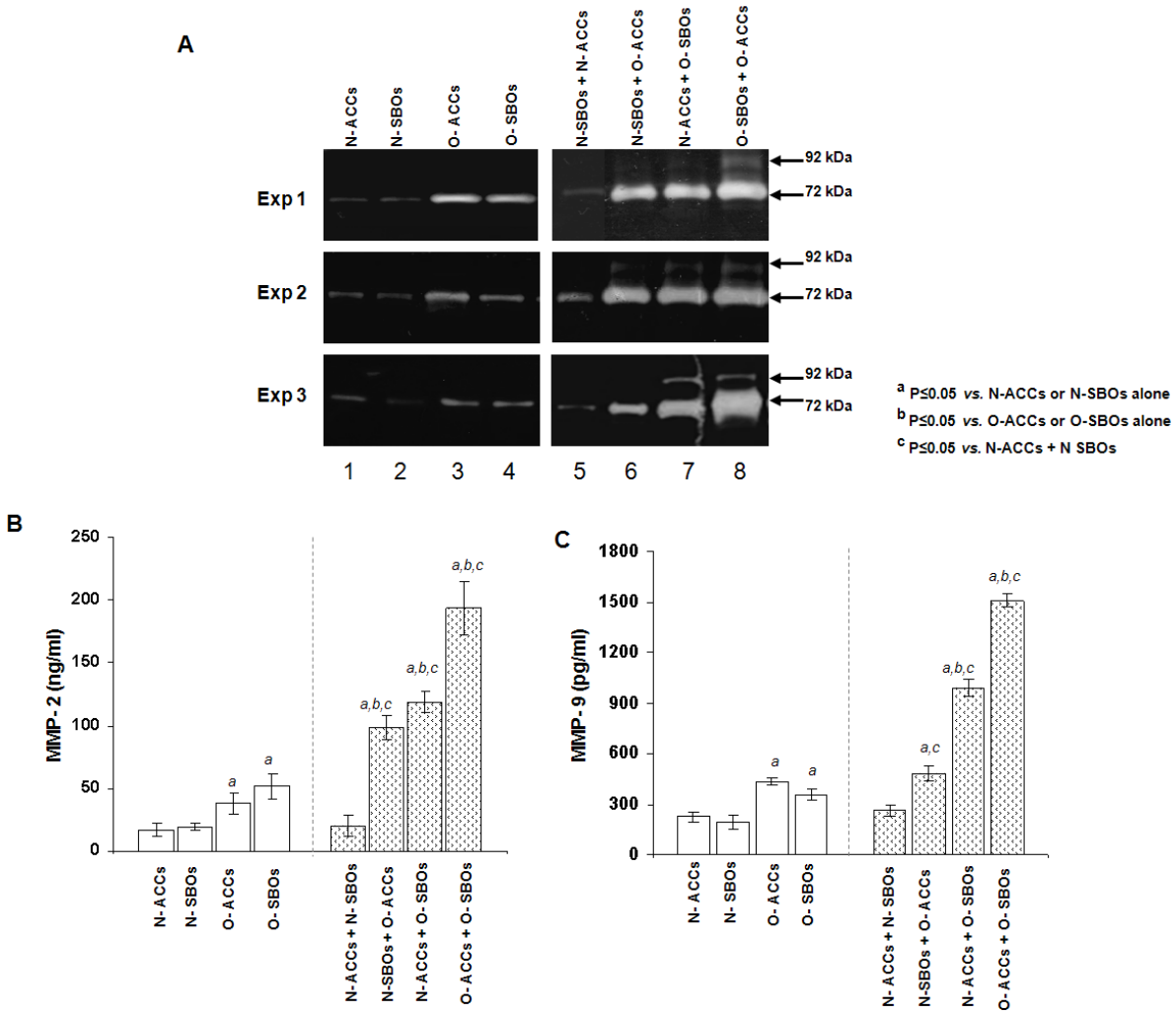

Figure 2: Stimulation of MMP-2 and MMP-9 activities in the direct co-cultures as assessed by zymography and ELISA in non co-cultured *vs.* co-cultured cell conditioned media. **(A)** Zymographic detection of MMP-2 (72 KDa) and MMP-9 (92 KDa) gelatinase activity released by non co-cultured or co-cultured SBOs and ACCs. A representative gel from two duplicate experiments performed on three different normal and OA patient samples is shown. The white bands were decomposed gelatin by gelatinase (Photos: Gels are wrapped in the polythene cover, scanned and proceesed with Microsoft power point). **(B&C)** Quantitative measurement of **(B)** MMP-2 and **(C)** MMP-9 levels in conditioned media of non co-cultured and co-cultured SBOs and ACCs as detected by ELISA assay. (N-ACCs: normal articular cartilage chondrocytes, O-ACCs: osteoarthritic articular cartilage chondrocytes, N-SBOs: normal subchondral bone osteoblasts, O-SBOs: osteoarthritic subchondral bone osteoblasts). The values are mean ± SEM (n=3), statistical differences are noted by letters where $P<0.05$ was considered significant.

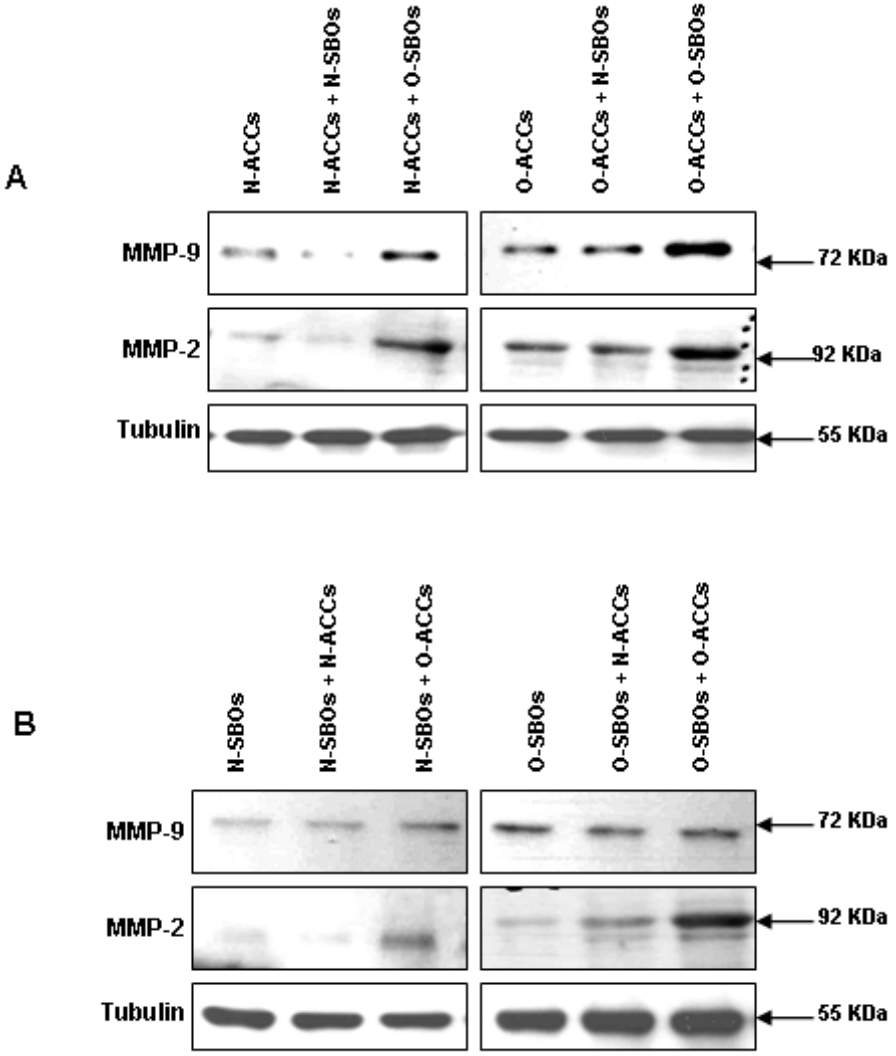

Figure 3: Indirect co-cultures: SBOs and ACCs were co-cultured for 72 hr by separating them using 0.4 µm cell culture inserts, in different combinations as described in methodology. As controls SBOs and ACCs were cultured alone. **(A)** Normal (N) and OA (O) SBOs mediated MMP-2 and MMP-9 cellular protein expression detected by western blotting technique in co-cultured ACCs. **(B)** Normal (N) and OA (O) ACCs mediated MMP-2 and MMP-9 cellular protein expression detected by western blotting technique in co-cultured SBOs. Tubulin is shown as a loading control. Images are representative of experiments performed on three different patients with comparable results.

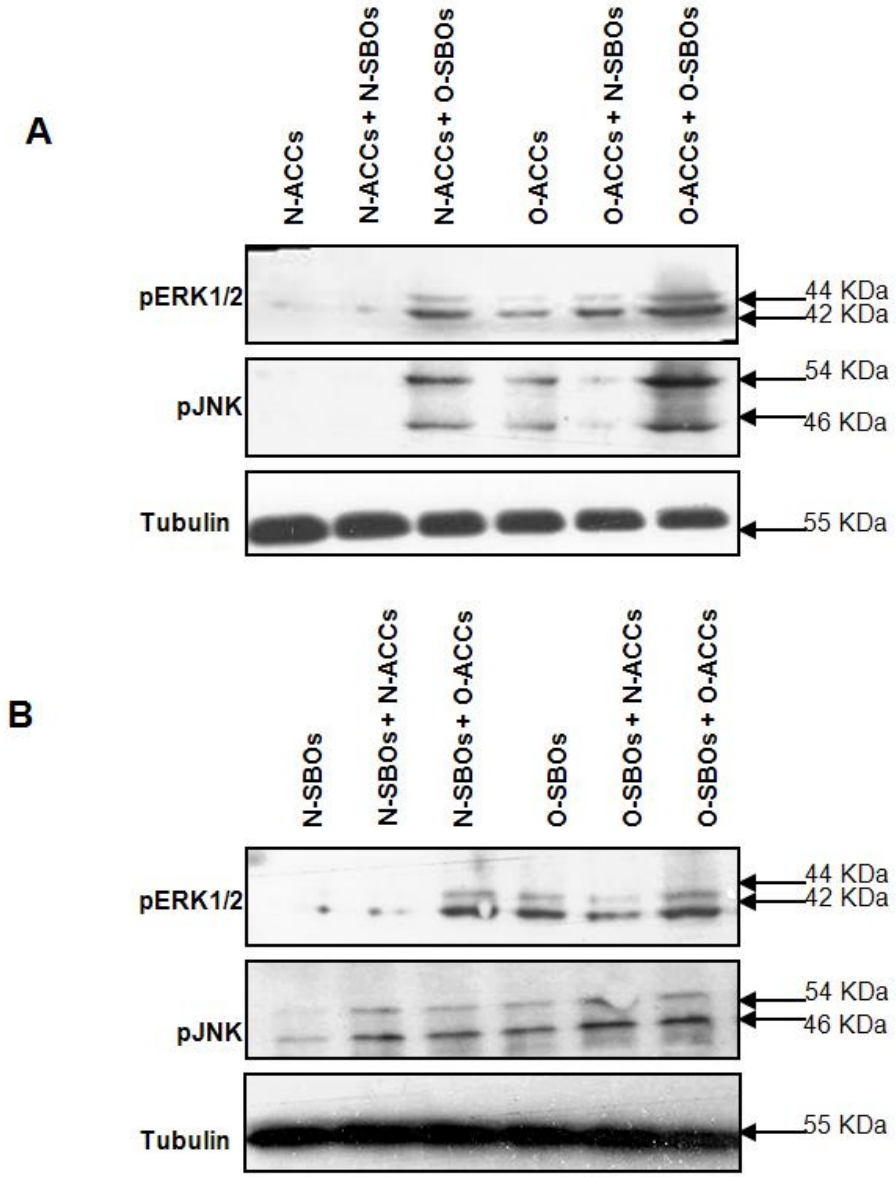

Figure 4: ERK1/2 and JNK phosphorylation status in the indirectly co-cultured ACCs and SBOs: SBOs and ACCs were co-cultured in-directly for 72 hours by separating with a 0.4 μM cell culture insert, in different combinations as described in methodology. **(A)** Normal (N) and OA (O) SBOs mediated ERK1/2 and JNK signalling changes in co-cultured ACCs. **(B)** Normal (N) and OA (O) ACCs mediated ERK1/2 and JNK signalling changes in co-cultured SBOs. The bands for tubulin showed as a loading control. Images are representative of experiments performed on three different patients with comparable results.

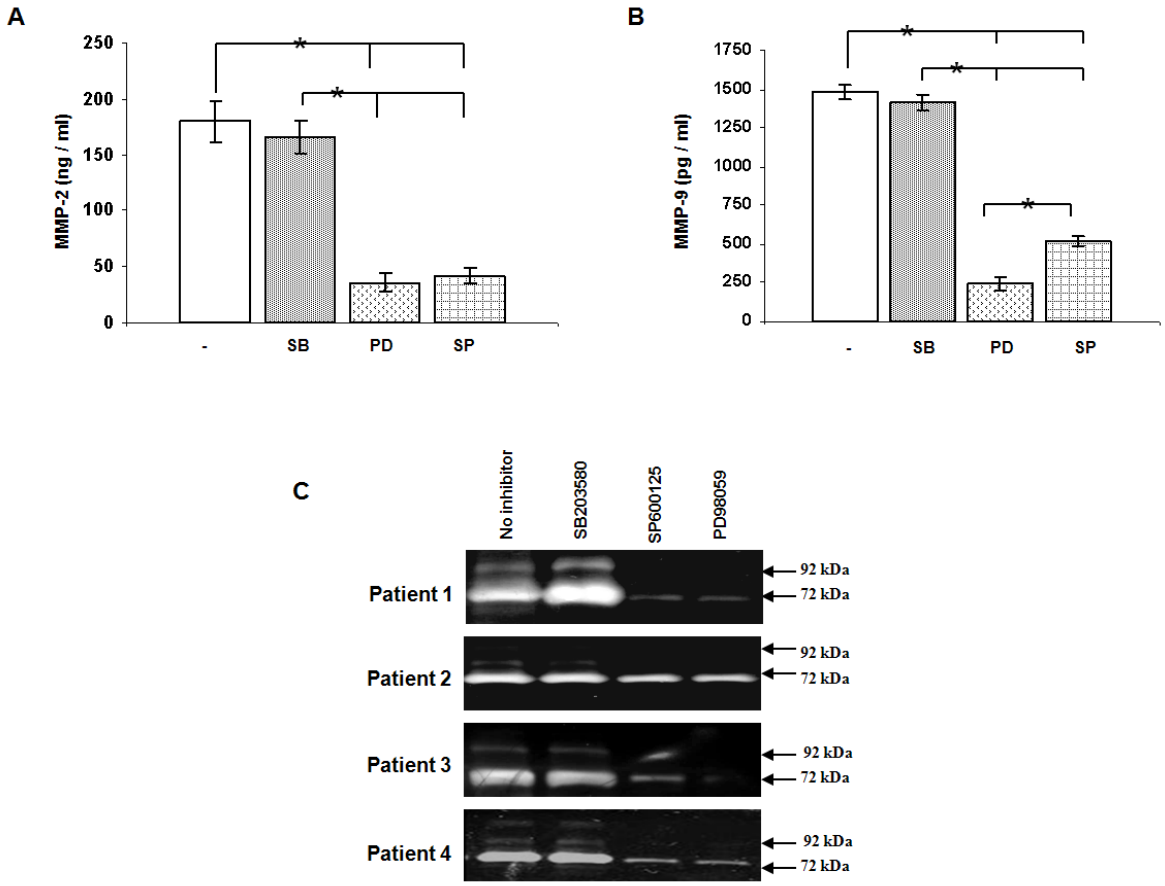

Figure 5: The effect of p38, ERK1/2 and JNK pathway inhibition on the levels of MMP-2 and MMP-9 levels in OA SBOs-ACCs co-cultures. OA ACCs-SBOs were co-cultured directly for 72 hours, as described in the methodology, in the presence or absence of p38 inhibitor SB203580 (SB), ERK1/2 inhibitor PD98059 (PD) and JNK pathway inhibitor SP600125 (SP). Conditioned medium was collected and analysed by ELISA and zymography. **(A&B)** Quantitative levels of MMP-2 **(A)** and MMP-9 **(B)** in OA SBOs-ACCs co-cultures, assayed by using ELISA, in the presence or absence of various inhibitors. The results are shown as mean±SEM (n=3). * Represents a significant difference ($p<0.05$). **(C)** MMP-2 (72 KDa) and MMP-9 (92 KDa) activity in OA SBOs-ACCs co-cultures, assayed by using zymography, in the presence or absence of various inhibitors. Note that the OA SBOs-ACCs co-culture induced MMP-2 and MMP-9 activity was decreased by PD98059 and SP600125. Images of experiments performed on four different pateints were shown.

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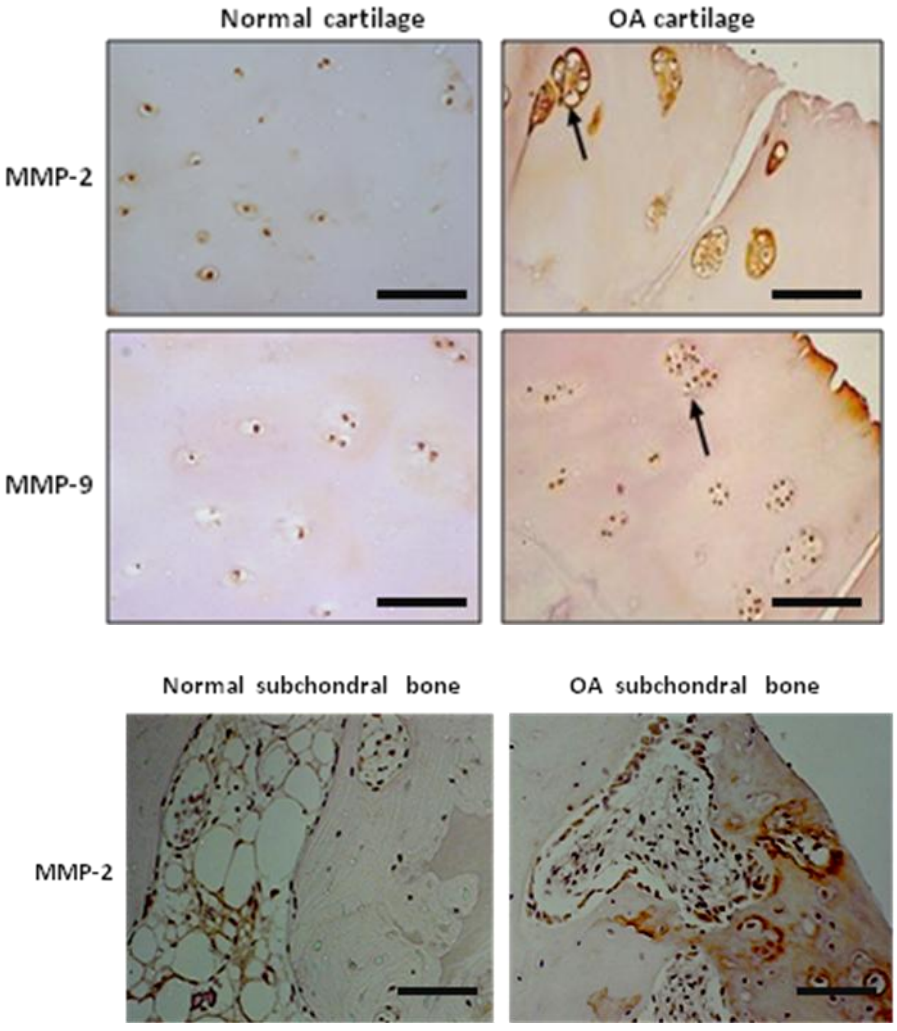
CHAPTER 6: SUPPLEMENTARY DATA

A



B

Clinical relevance of MMP-2 and MMP-9 in OA cartilage and subchondral bone: Our co-culture experiments revealed that OA SBOs significantly induced MMP-2 and MMP-9 expression in ACCs. In turn, OA ACCs has significantly induced the MMP-2 expression in SBOs. To test the clinical relevance of these proteases to OA pathogenesis, we performed immunostaining for MMP-2 and MMP-9 in normal and OA articular cartilage and subchondral bone. In OA cartilage tissue sections both MMP-2 and MMP-9 are expressed strongly, virtually covering all of the cells in the remaining cartilage. However the normal cartilage showed only a mild expression **(A).** Similarly, increased expression of MMP-2 was also noticed in the OA subchondral bone compared to normal subchondral bone **(B).** Original magnifications 20X.

Chapter 7

Conclusions & Perspectives

Osteoarthritis (OA) is the most common form of musculoskeletal disorder and is a major cause of disability in the elderly, affecting approximately 60% of men and 75% of woman above the age of 65 (National Health Priority Action Council, May 2004). The aetiology of OA is not fully understood but it is most likely multi-factorial, including metabolic, biomechanical and genetic factors. By its latter stages the condition is irreversible, chiefly because it has already affected the joint tissues some time before its symptoms, joint pain and discomfort, become apparent. Considerable focus has been given to the management of this disease - both surgical and non-surgical. Most of these therapies have sought to alleviate the symptoms of the disease and not to prevent disease progression and subsequent cartilage degeneration. A more detailed understanding of the cell and molecular factors involved in this condition is needed in order to develop therapies capable of identifying the disease at an earlier stage and prevent the cartilage from being degraded in the first place.

OA initially was considered as a disease of the cartilage, a condition mainly characterized by total loss of the cartilage cushion between the bones of the joints (Tanaka *et al.* 1998). However, the current understanding of OA is that changes are not simply restricted to cartilage, but that adjacent subchondral bone tissues also undergoes abnormal changes, characterized mainly by subchondral sclerosis and increased subchondral bone metabolism (Lajeunesse *et al.* 1999). *OA is therefore increasingly recognised as a whole-of-joint condition and that treating bone and cartilage as separate entities is a false division since they are intrinsically inter-related both biomechanically and developmentally* (Aspden *et al*. 2001). An ideal disease treatment modality should therefore target the structural changes to both of these tissues. In order to achieve such treatment outcomes, the specific factors responsible for these structural changes, must be identified and targeted. *In this project we have investigated whether an alteration of the communication between subchondral bone cells and cartilage cells might be responsible for the development of OA associated cartilage and subchondral bone structural changes in the joint.*

At the time that this project was first conceived, very few attempts had been made to understand the inter-relationship between bone and cartilage, although an apparent co-existence between these two components is present at a macroscopic observation scale.

This project sought to advance the knowledge and understanding of cross-talk of subchondral bone and articular cartilage, both during normal physiological joint conditions and in OA joint conditions, with respect to the mutual regulation of the others phenotype and also to reveal the potential molecular mechanisms involved during this process at a cellular level. *The ultimate aim is to identify the potential molecular pathways that interfere with the normal OA subchondral bone osteoblast (SBO) - articular cartilage chondrocyte (ACC) axis, as a potential treatment target and strategy to stop OA progression.*

Firstly, in **Chapter 3** of this thesis, we highlighted some of the discrepancies that exist in the literature with respect to the hypertrophic phenotypic changes in OA cartilage. In this chapter we discuss some studies having reported that the OA cartilage chondrocytes shows signs of post natal hypertrophic changes (Pullig *et al.* 2000; Pullig *et al.* 2000). On the other hand, some studies have shown no proofs for generalized hypertrophic changes in OA cartilage chondrocytes (Brew *et al.* 2008). In order to reconcile these differences in the literature, we immuno-localized a broad array of hypertrophic and mineralization markers in osteochondral tissues categorised and scored according to disease severity. This approach allowed us to account for variables such as age, gender and life style of the sample population. *The findings from this study indicate that arthritic cartilage, but not normal cartilage, showed a strong expression of hypertrophy and mineralization markers, confirming that these phenotype changes are an integral part of OA pathogenesis.* An interesting finding from this study was, we observed the presence of cartilage islands deeply embedded within the subchondral bone matrix, which hints to the possibility that cartilage is being replaced by the bone during OA disease process.

The general purpose of **Chapters 4-6** was to evaluate how cell-cell interactions within the OA joint-microenvironment contribute to the progression of OA. Based on clinical observations of the state of the articular joint of OA patients, we developed different co-culture models to resemble, as closely as possible, the actual *in vivo* situation. Using these co-culture models we examined the interaction between SBOs and ACCs by creating an artificial *in vitro* joint-microenvironment.

In **Chapter 4**, we showed for the first time that OA ACCs play an integral role in altering the SBOs phenotype. Briefly, OA ACCs appeared to enhance SBO differentiation compared to normal ACCs. Notably, the interaction of SBOs with OA ACCs activated the signalling cascade of ERK1/2. Further, the inhibition of ERK1/2 phosphorylation reversed the OA ACCs induced abnormal mineralization in SBOs. *These in vitro data provide strong clues that the enhanced subchondral bone activity, manifest in vivo OA subchondral bone, may be due to altered interaction with OA ACCs, which is mediated via the activation of the ERK1/2 signalling.* The observation that the ERK1/2 phosphorylation was hyper-activated in OA SBOs compared to normal SBOs suggests an implicit clinical significance of these results.

In **Chapter 5**, we investigated the reciprocal interactions of normal SBOs and OA SBOs on ACCs phenotype. The data showed that OA SBOs increased hypertrophic gene expression and matrix calcification in ACCs, both by means of direct and indirect cell-cell interactions. The most interesting finding, nonetheless, was that the OA SBOs suppressed p38 phosphorylation and induced ERK1/2 signal phosphorylation in co-cultured ACCs. We further demonstrated that the, inhibition of ERK1/2 was accompanied by an increase of p38 phosphorylation, which, in effect, rescued the ACCs from undergoing hypertrophic differentiation, a phenotype which has been reported to be detrimental for the integrity and function of the articular cartilage. *This is the first study, which provides some insight into the molecular mechanisms by which the OA SBOs induced the altered phenotypic properties of ACCs.*

The preceding studies, in **Chapters 4** and **5,** demonstrated that the interaction of ACCs and SBOs under OA conditions mutually altered the differentiation capabilities of the other, by forming a feedback cycle. Since it is known that the altered differentiation capacities of a cell intensify the degradative enzymes (Wu *et al.* 2002), we also tested the effect of OA ACCs and OA SBOs interactions on the expression and regulation of MMPs. We particularly focussed on MMP-2 and MMP-9 since our preliminarily work showed that these proteinases are significantly upregulated in both OA subchondral bone and OA articular cartilage compared to normal tissues. Also, other researchers have demonstrated the pathological role of these two proteases in

joint destruction (Burrage *et al.* 2006). Our co-culture studies showed that the interaction of OA SBOs and OA ACCs upregulated MMP secretion in both the cells, illustrating a mutual paracrine interaction mechanism. Briefly, OA SBOs increased MMP-2 and -9 in ACCs, whereas OA ACCs promoted MMP-2 expression in SBOs. It is known that augmented secretion of MMPs is associated with increased cartilage degradation and abnormal bone remodelling aggravating OA related changes. These observations provided further strong evidence that the OA changes are a part of altered cross-talk between SBOs and ACCs. The promoter regions of inducible MMP genes are regulated by MAPKs depending on the nature of extracellular stimuli (Chakraborti *et al.* 2003). Therefore, we also investigated the role of MAPK signalling pathways with respect to MMP upregulation. Our results showed that by inhibiting the ERK1/2 and JNK pathways there was a reduction of MMP-2 and MMP-9 expression in the OA SBOs - OA ACCs co-cultures. *Thus, the results from this study for the first time showed that the altered bi-directional interaction of SBOs and ACCs in OA could trigger the degradative pathways, which may lead to joint destruction.*

Taken together, the observations from this work suggest that normal SBOs and ACCs interact differently compared to that of OA SBOs and ACCs. Importantly, we have demonstrated that the transformed OA ACCs and SBOs can induce altered phenotypic changes in normal cells, which highlights the novelty of the obtained results. Currently the only treatment of OA is given when the disease is at the end stage, leaving clinicians, ultimately, with only joint replacement surgery to treat the debilitating symptoms of OA. *Based on the results in the present study, strategies directed at disrupting the altered cross-talk between SBOs and ACCs may define new approaches for therapeutic interventions to stop the progression and development of full blown OA, possibly via altering the MAPK signalling pathways.*

The major interactions studied in this thesis are summarised schematically in **figure 1**.

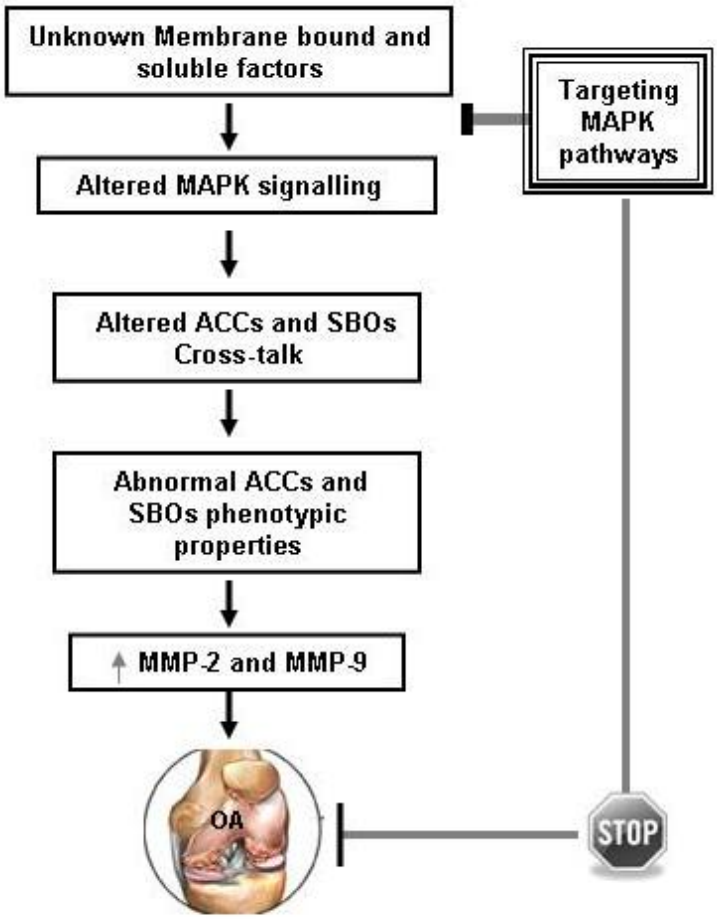


Fig 1: Schematic drawing illustrating the proposed mechanism by which SBOs and ACCs initiates a paracrine signalling cascade that results in altered phenotype and enhanced expression of MMPs, in OA joint in a signalling pathway mediated by MAPKs. This cross-talk between SBOs and ACCs is centrally regulated by MAPKs. Interference with MAPK signalling inhibitors might reverse altered OA SBOs-ACCs axis thereby slowing down the OA progression.

Limitations of the present study and some unanswered questions

The limitations of the particular research approach adopted in this study have already been considered within the individual chapters of this work. One overarching limitation, however, relates to the ability to infer biological significance of the

findings of the *in vitro* co-culture model. We did perform a combination of different experimental approaches in order to validate the findings obtained *in vitro* and provide evidence for biological significance; however *in vitro* findings are not necessarily reflected to the *in vivo* scenario. Furthermore, it is unclear what putative soluble factors that are involved in normal SBOs-ACCs compared to OA SBOs-ACCs interactions. In addition, it is still not known why this interaction of SBOs-ACCs takes place in the OA joint not seemingly in the normal joint.

Future perspectives and clinical implications

In this thesis, we have demonstrated that the interaction of OA SBOs and OA ACCs significantly affected the natural properties of co-cultured cells, with the aid of an indirect co-culture model that allowed only the transfer of soluble factors. These results suggest a role of one or more soluble factors leading to altered cross-talk between SBOs and ACCs. Therefore, the proteins released in to extracellular space during the co-cultures are of great interest and may lead to the discovery of putative serum markers as an alternative method for disease manipulation. Identifying such putative "secretome" markers may also lead to the development of cheaper diagnostic and treatment options. Therefore, further studies are justified to identify such soluble factors, probably by using proteomic mapping of the conditioned medium of co-cultured *vs*. non co-cultured cells.

Also, additional studies should be designed to understand the root causes of these altered interactions in OA. It is of great interest to test, whether there is any potential link between mechanical properties and altered cross-talk of SBOs and ACCs in OA. This study further demonstrated that the inhibition, by chemical inhibitors, of the ERK1/2 pathway reversed the pathological changes in both of OA bone cells and cartilage cells. It is tempting to speculate, based on these *in vitro* results, on future application of MAPK-ERK1/2 inhibitors in clinical OA to minimize the major symptoms of OA, namely the abnormal subchondral bone activity and cartilage changes. However, many questions remain unanswered, since the scope of this work is limited to *in vitro* studies at present. The obvious way to remedy this uncertainty is to validate the effects of MAPK inhibitors in an OA animal model to further consider

the potential clinical application of these drugs in the treatment of OA. An animal study would also reveal what side-effects MAPK pathway inhibition has in *in vivo* conditions.

Clinical trails with MAPK inhibitors have been successfully used to treat several diseases. For example, MAPK inhibitors have been trialed on patients with rheumatoid arthritis (Foster *et al.* 2000; Meyer and Pap 2005). Moreover, in the field of oncology, powerful protein kinase inhibitors have already been used in numerous clinical trials with promising results and with surprisingly benign side-effects (Hendrickson *et al.* 2008). Our *in vitro* study results therefore fully justify the testing of MAPK inhibitors in an OA animal model to investigate its potential efficacy in relation to OA treatment.

Several OA animal models, already reported in the literature, could serve at useful *in vivo* models. These animal models generally fall into two OA categories: spontaneous and induced (surgical instability or genetic manipulation) (Bendele 2001; Bendele 2002). Animal models of naturally occurring OA in knee joints of guinea pigs and mice have been reported. The most widely used surgical instability models are medial meniscal tear and anterior cruciate ligament transaction in guinea pigs and rats, medial or lateral partial meniscectomy in rabbits, medial partial or total meniscectomy or anterior cruciate transection in dogs, all of which have proved to be useful models to study the development of OA (Brandt 2002). Any of these models have potential uses in the study of the molecular mechanisms associated with OA development by using histomorphometry and immunohistochemistry techniques to identify altered matrix molecules at different stages in disease progression (Brandt 2006). Therefore, the use of any of these models would potentially answer whether the treatment with MAPK inhibitors have successfully prevented the subchondral bone changes and cartilage changes compared to untreated animal models. However, necessary care must be taken in order to select the most appropriate animal model to serve the purpose of the study. Most importantly, however, is the consistency of results and this might see the need to perform experiments on various animal models ranging from smaller size to larger size models.

Concluding remarks

The studies presented in the thesis highlights the importance of bi-directional communication of SBOs and ACCs within the microenvironment of the OA-joint. The viciuos cycle between these cell types mutually activated and deactivated a pleotrophic cascade of MAPK signalling pathways. A systematic targeting of these molecular events by MAPK antagonists may help to control increased bone mineralization, cartilage hypertrophy and MMP expression and therefore provides a scientific rationale for the potential development of novel therapeutic strategies.

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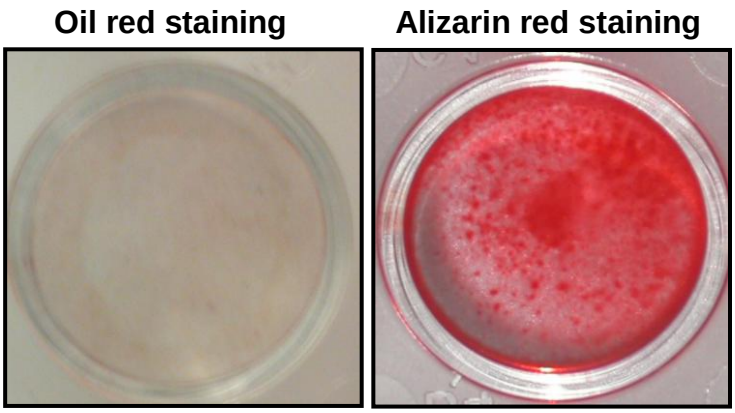
Supplementary data

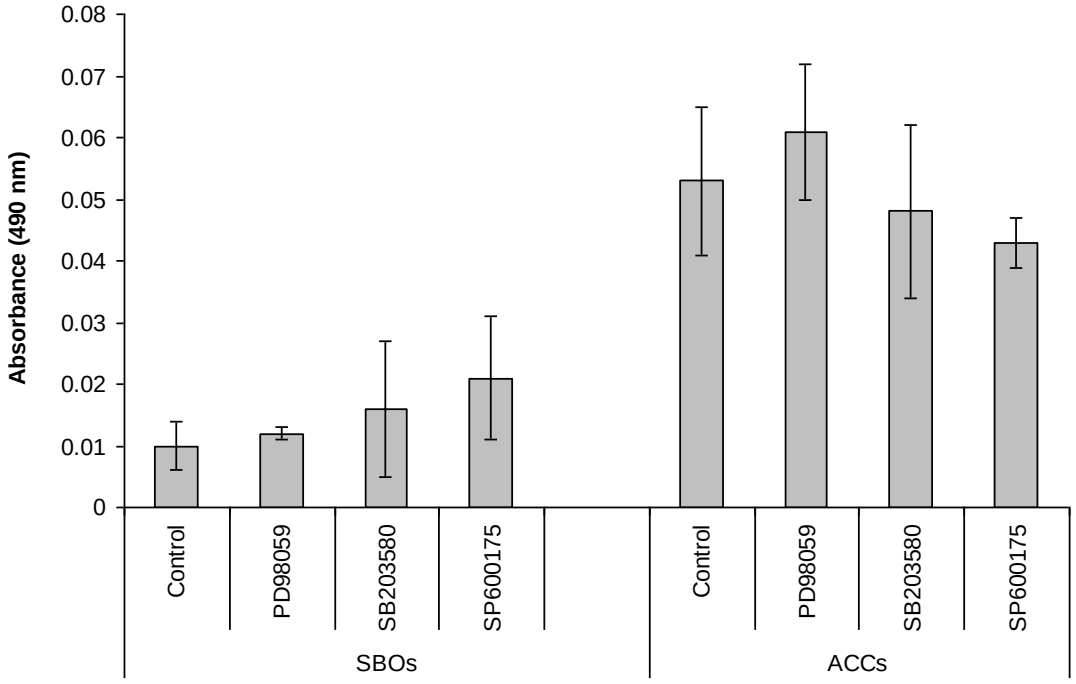
1. Characterization of subchondral bone osteoblasts

Subchondral bone osteoblasts were isolated from the bone tissues as described in the chapter 4, 5 and 6. Note the isolated osteoblasts showed a strong staining for Alizarin red and negative staining for Oil red after 4 weeks of osteogenic differentiation. This observation confirms the absence of adipogenic lineage specific cell contamination in the population of osteoblasts isolated from the bone tissues. The bone cell phenotype was further confirmed by the gene expression of bone specific markers as shown in the figure 2 of chapter 4.



2. LDH Assay to determine the cytotoxicity of MAPK inhibitors

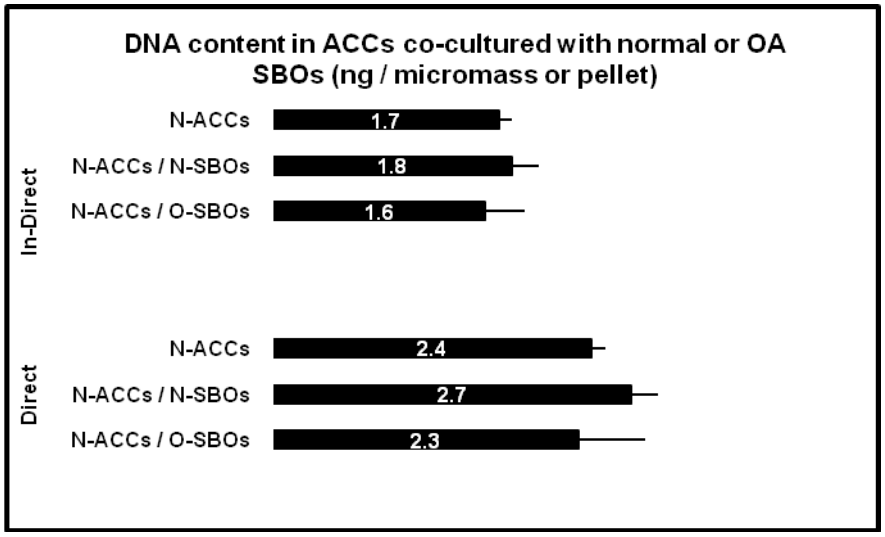
ACCs ($2x10^5$ cells) pellets (n=3) and SBOs (75000 cells) (n=3) were cultured in the presence (10 µM PD98059, 5 µM SB203580 and 10 µM SP600175) or absence of various inhibitors for 48 hrs. Cytotoxicity of cells after inhibitor treatment was measured by the amount of lactate dehydrogenase (LDH) leakage into the medium using LDH based *invitro* toxicology assay kit (Sigma, Missouri). Briefly, 200 µl of cell supernatant was transferred to 96-well microplate and 100 µl of LDH assay mix was added. The plate was covered with aluminium foil and incubated for 20-30 min. The reaction was stopped by adding 30 µl of 1N HCl and the absorbance was measured at a wavelength of 490 nm. Data are shown as mean ± standard deviation. It was observed that there is no difference in the LDH release in the inhibitor treated *vs.* control cells.



3. Cell proliferation in the co-cultured *vs.* non co-cultured SBOs and ACCs

Direct and indirect co-culture of SBOs and ACCs were performed as described in the chapter 4 and 5. No significant differences were observed between co-cultured vs. non co-cultured ACCs and SBOs. These results indicate that the process of interactions between chondrocytes and osteoblasts might have taken place without any change in cell number.

Cell proliferation in ACCs co-cultured with normal and OA SBOs (methodology refer chapter 5)



Cell proliferation in SBOs co-cultured with normal and OA ACCs (methodology refer chapter 4)