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The Role of Activated Protein C in Bone, Arthritis, and Fracture Healing

Kaitlin Shen

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Medicine

University of Sydney

Ph.D 2016

DECLARATION

The work described in this thesis was performed by the candidate, except where due acknowledgement has been made. Institutional ethics committee approval was sought and obtained prior to the commencement of all animal experiments and human tissue collection and procession.

This work was undertaken at the Sutton Arthritis Laboratory, Kolling Institute of Medical Research, Royal North Shore Hospital and the Kids Research Institute, The Children's Hospital at Westmead. I declare that no part of this work has been submitted previously for the purpose of obtaining a degree or a diploma in any other universities.

In Chapters 3 and 4, animal surgeries were performed by Kathy Mikulec and Lauren Peacock with assistance from myself, Tegan Cheng, and Nikita Deo. In Chapter 3 studies of APC on MC3T3-E1 and MG-63 cells using MUSE® cell count and viability kit were completed by Ciara Murphy. In Chapter 6, paraffin embedding was carried out by Sue Smith. Subchondral bone tissues were sectioned and stained with the help of Ms. Agnes Chan.

Editing of this thesis was completed with the help of Prof Jackson, Dr Schindeler, and Dr Bullock.

……………………………..

Kaitlin Shen

NOTICE OF DRUG WITHDRAWAL

The drug in our studies, Activated Protein C (APC) or Xigris, was sold and clinically used for the treatment of sepsis. However, in October 2011, the producing company Eli Lilly, in consultation with the Therapeutic Goods Administration, voluntarily withdrew this drug from the Australian market. This was prompted by the lack of drug efficacy seen in the PROWESS-SHOCK clinical trial rather than any specific safety concerns.

For further information please visit:

http://www.tga.gov.au/safety/alerts-medicine-xigris-111026.htm

ABSTRACT

Activated protein C (APC) is an endogenous anticoagulant that also possesses a wide range of other actions including: stimulation of proliferation; inhibition of apoptosis; induction of migration in a range of cell types; and suppression of inflammation. APC is effective as a systemic treatment in models of arthritis, severe sepsis, pancreatitis, spinal cord/brain injury, and stroke. APC delivered locally also has proven efficacy as a wound healing agent in patients with chronic venous wounds, diabetic wounds, and recalcitrant orthopaedic skin ulcers. These properties of APC are primarily modulated through its receptors, endothelial protein C receptor (EPCR) and protease-activated receptors (PAR)1/2, and subsequent activation of downstream proteins including extracellular signal-regulated kinases (ERK)-1/2, protein kinase B (PKB/Akt), p38, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB).

In this study, APC was investigated for its potential application in bone repair and arthritic bone conditions, including rheumatoid arthritis (RA) and osteoarthritis (OA), through its actions on osteoblasts. The effects of APC on osteoblasts and the underlying cellular mechanisms are presented in Chapter 3 using human MG-63 and murine MC3T3-E1 osteoblast-like cells. Cell viability was assessed by MTT assay, the trypan blue exclusion method, and MUSE® cell count/viability assay. Receptor expression and signalling molecules were measured by immunostaining, PCR, and western blotting. Treatment with APC significantly increased MG-63 and MC3T3-E1 cell viability, concordantly with increased phosphorylation of ERK, Akt, and p38. EPCR, PAR1, and PAR2 were expressed by MG-63 cells, and accordingly, PAR1 antagonists were found to ameliorate the effects of APC. These results were consistent with two reports published whilst the work for this thesis was undertaken. These reports showed that APC can induce osteoblast proliferation through ERK-

1/2, and osteoblast differentiation by up-regulating type I collagen, calcium deposition, and alkaline phosphatase activity.

In Chapter 4, the *in vivo* effects of APC on bone are presented, using a recombinant human bone morphogenetic protein (rhBMP)-2-induced ectopic bone formation model. Absorbable collagen sponges were infused with rhBMP-2 in the presence or absence of APC and implanted into the hind limbs of C57BL/6J mice. Bone formation was assessed at 2 and 3 weeks by X-ray and micro-computed tomography (μCT). Osteoclast numbers were quantified by tartrate-resistant acid phosphatase (TRAP) staining. Significantly increased bone and tissue volume were found after APC treatment, without any alteration to bone volume/tissue volume ratio. This was associated with a significant increase in TRAP+ cells within the ectopic bone tissue.

The results of APC-stimulated osteoblast viability in conjunction with its *in vivo* effect on BMP-2 indicated that APC may prove useful for improving bone repair. Further study of APC using a closed murine mid-tibial fracture model is shown in Chapter 5 to evaluate this hypothesis. APC, however, did not enhance osteoclast number, bone volume, or tissue volume in the hard callus specimens. Further studies are suggested to better evaluate the therapeutic potential of APC in bone.

The effects of APC on OA and RA human bone-derived cells (HBDCs) are presented in Chapter 6. The expression of protein C (PC), EPCR, and PARs were determined in OA and RA condyles and isolated subchondral HBDCs. The osteoblastic phenotype of the subchondral HBDCs was also examined. Then, the effects of APC and its receptor antagonists on HBDC viability and intracellular protein signalling were measured. Contrastingly to the results from Chapter 3, treatment with APC significantly decreased cell viability and ERK1/2 activation but

increased p27 levels in OA HBDCs. EPCR, PAR1, and PAR2 were all found to be involved in APC-mediated suppression of OA cell viability. APC, however, had no effect on RA HBDC viability.

The effect of APC on OA and RA HBDCs was not limited to changes in cell viability. In Chapter 6, APC was further investigated for its effects on inflammation. Collagen-degrading matrix metalloproteinase (MMP)-2 and -9 activity in OA and RA HBDCs were assessed using gelatin zymography and the cytokines TNF-α and IL-6 were quantified by ELISA. Following APC treatment, TNF-α-induced production of IL-6 was significantly reduced in RA but not OA HBDCs. Furthermore, APC treatment significantly increased levels of activated MMP-2 in both OA and RA HBDCs.

Collectively, these results demonstrate the diverse actions of APC in normal and arthritic bone biology. Stimulation of MG-63 viability and enhancement of ectopic bone formation suggests that APC has potential, at least in combination with BMP-2, for bone repair. In arthritic cells, the down-regulation of OA bone cell viability by APC may be beneficial by moderating turnover in subchondral bone. Furthermore, the suppression of IL-6 production and activation of MMP-2 suggests that APC ameliorates inflammation in arthritis. The implications for these actions are discussed in Chapter 7.

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The work embodied in this project has been a life changing experience and was a result of five intensive years of perseverance. None of this would be possible without the help of people who have contributed their time, intellect, support, and advice to the completion of this thesis.

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PEER REVIEWED PUBLICATIONS

Shen K, Murphy C, Chan B, Kolind M, Cheng T, Mikulec K, Peacock L, Xue M, Park S-Y, Little DG, Jackson C, Schindeler A. Activated protein C (APC) can increase bone anabolism via a Protease-Activated Receptor (PAR)1/2 dependent mechanism. *Journal of Orthopedic Research*, 32, 1549-1556, 2014.

Xue M, McKelvey K, **Shen K**, Minhas N, March L, Park S-Y, Jackson C. Endogenous MMP-9 and not MMP-2 promotes rheumatoid synovial fibroblast survival, inflammation and cartilage degradation. *Rheumatology,* 53, 2270-2279, 2014.

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PRESENTATIONS AT SCIENTIFIC CONFERENCES

1. Kolling Institute, Scientific Research Meeting, 2010, Poster Presentation
2. American Society for Bone and Mineral Research Annual Meeting, San Diego, CA, USA, 2011, Poster Presentation
3. Advanced Lecture Course, 7th International Conference on Proteoglycans held in conjunction with the Matrix Biology Society of Australia and New Zealand, 2011, Poster Presentation and Oral Presentation
4. Kolling Institute, Scientific Research Meeting 2011, Oral Presentation
5. Conjoint 3rd Australasian Wound & Tissue Repair Society Meeting and the 9th Australasian Society for Dermatology Conference: Repair and Regeneration, Sydney, Australia 2012, Oral Presentation
6. Kolling Institute, Scientific Research Meeting 2012, Oral Presentation, *Winner of Young Investigator's Award*
7. ECTS, Lisbon, Portugal, 2013, Poster Presentation

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LIST OF ABBREVIATIONS

AIA	Antigen-induced arthritis
ACTB	Beta-actin gene
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APC/PC	Activated protein C/protein C
CIA	Collagen-induced arthritis
DMARD	Disease-modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
ERK1/2	Extracellular signal-regulated kinase ½
EtOH	Ethanol
FGF	Fibroblast growth factor
FV(II, III)	Factor V or Factor VII, or Factor VIII
FX	Factor X
GPCR	G-protein-coupled receptors
H&E	Haematoxylin and eosin
HBDC	Human bone-derived cells
IL	Interleukin
IGF	Insulin-like growth factor
MAPK	Mitogen-activated protein kinase

MEOH	Methanol
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MTT	3[4,5-dimethyl(thiazole-2-yl)-3,5-diphenytle]tetrazolium
NF-κB	Nuclear factor-kappa B
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
RhBMP-2	Recombinant human bone morphogenetic protein-2
RT-PCR	(Reverse transcriptase)-polymerase chain reaction
PAGE	Polymerase agarose gel electrophoresis
PAR	Protease-activated receptor
PDGF	Platelet-derived growth factor
PTH	Parathyroid hormone
PROC	Protein C gene (human)
P(P)i	Inorganic phosphate or inorganic pyrophosphate
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RUNX2/CBFA1	Runt-related transcription factor 2/Core-binding factor subunit alpha-1
TRAP	Tartrate-resistant acid phosphatase
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

CHAPTER 1:

INTRODUCTION

1 Literature Review

1.1 Bone Structure and Function

Bone is a highly specialised connective tissue that serves as mechanical support, provides locomotion, protects vital organs, enables haematopoiesis, and provides a metabolic reserve of ions for homeostasis [1-4]. Bone is a composite material, consisting of an organic matrix with 90% type I collagen fibres, a non-organic component made of spindle-shaped hydroxyapatite crystals ($3Ca_3(PO_4)_2(OH)_2$), and ground substance including proteoglycans [5]. This composite structure confers both strength and flexibility to bone and enables support and protection of the body [6, 7].

1.2 Bone Cells

Bone is modulated by three key cell types including osteoblasts, osteoclasts and osteocytes. Osteoblasts and osteoclasts are associated with bone formation and resorption respectively. Osteocytes have been shown to have a major role in bone, particularly at a regulatory level.

1.2.1 Osteoblasts

Osteoblasts are small mononucleated cells (Figure 1-1), responsible for the production and mineralisation of bone matrix constituents during bone formation and remodelling [8-10]. They originate from mesenchymal stem cells (MSCs) that differentiate under the influence of local signals that include growth factors, hormones, and cytokines. Osteoblasts also have the capability to regulate osteoclast formation, via cell to cell contact or cytokine secretion, through receptor activator of nuclear factor-κB ligand (RANKL), and macrophage-colony stimulating factor (M-CSF) [9, 11, 12].

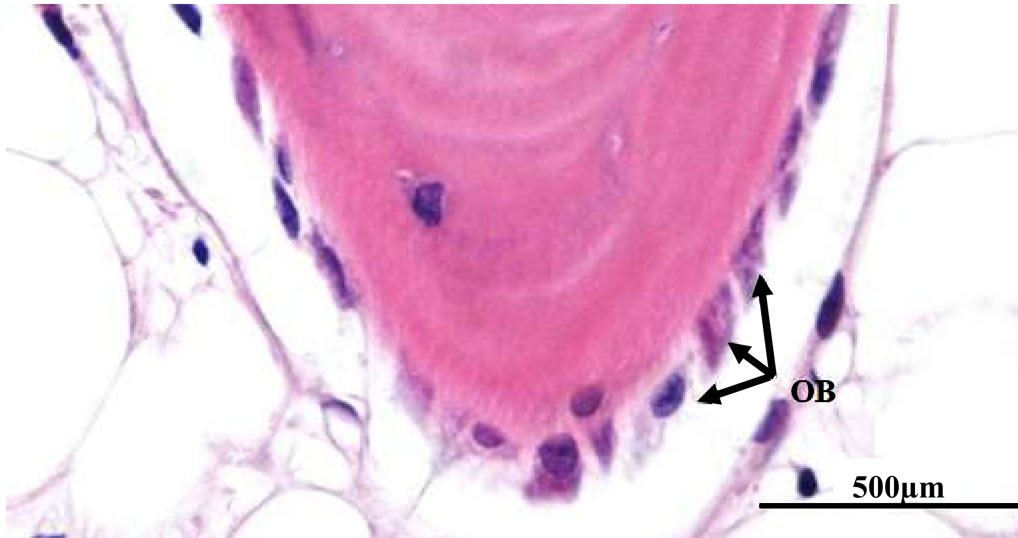

Figure 1-1 *Haematoxylin and eosin staining of osteoblasts in bone, with cell nuclei stained blue and bone matrix stained pink. This image is from the author's collection of human OA subchondral bone.*

Osteoblast Differentiation and Maturation

Prior to the commitment of MSCs into osteoprogenitors, they are pluripotent and possess the ability to differentiate into other cell lineages such as fibroblasts, chondrocytes, or adipocytes, each specified according to local and systemic signalling factors [13]. As MSCs undergo commitment, they further differentiate and mature into osteoblasts, a process consisting of metamorphosis from spindle-shaped cells into a cuboidal-shaped cell morphology [14].

The transformation from MSCs into osteoblasts depends on several local and systemic factors that act through autocrine or paracrine signalling pathways. These include fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), parathyroid hormone (PTH), and wingless (*WNT*) proteins. Such factors induce downstream intracellular signalling cascades including the extracellular signal-regulated kinase (ERK)1/2 pathway, which acts partly

through the stabilisation of transcription factors such as runt-related transcription factor 2/Core-binding factor subunit alpha-1 (*RUNX2/CBFA1)* [15-25].

This progressive development can be observed within *in vitro* bone nodule formation assays, and it has three distinct chronological stages: (1) the proliferation of osteoblasts, which depends on the synthesis of extracellular matrix (ECM), then (2) the accumulation and maturation of this ECM, which leads to the shutdown of proliferation, and subsequently (3) the progression of osteoblast differentiation and mineralisation [26-29].

Once differentiated, cuboidal osteoblasts play a central role in controlling bone formation by interacting with bone ECM, synthesizing osteoid that comprises of type I collagen, and expressing a high level of alkaline phosphatase (ALP) [30-34].

Type I Collagen

Type I collagen is a connective tissue protein found in tendons, muscle, and bone. It is the most abundant collagen of the human body, and together with hydroxyapatite, it confers much of the strength to bone [35]. It is also an early marker of osteoblast differentiation, whereby accumulation of this collagen in the ECM paves the way for maturation of osteoblasts and mineralisation of matrix [36]. Collagen is made up of three polypeptide chains (α-chains), which form a triple helical structure [5]. The accumulation of extracellular collagen interacts with α2β1 integrin on osteoblasts, conveying extracellular signal to the cell. This leads to the intracellular phosphorylation of ERK, a mitogen-activated protein kinase (MAPK) that is required for osteoblast proliferation and differentiation [31, 36-39].

Alkaline Phosphatase (ALP)

ALP is an enzyme that catalyses the removal of phosphate groups from substrate molecules such as proteins and nucleotides. It is classified into three main isoenzymes: intestinal, placental, and tissue-nonspecific. The latter is expressed throughout the body, but it is detected at its most abundant levels in liver, kidney, and bone. In bone, ALP hydrolyses inorganic pyrophosphate (PPi) that suppresses hydroxyapatite crystal formation, and yields free monophosphate ions (Pi) locally for incorporation into mineral matrix [40, 41]. ALP also serves as a maturation marker of osteoblast differentiation, its expression rises after the arrest of osteoblast proliferation, peaks just before mineralisation, and then declines with mineralisation of the ECM [26]. ALP is also mediated by ERK, where inhibition of ERK signalling results in a down-regulation of ALP expression by osteoblasts, and a subsequent reduction in the amount of matrix mineralisation [31].

Bone Morphogenetic Proteins (BMPs)

BMPs are multi-functional growth factors that comprise a large subgroup within the transforming growth factor (TGF)-β superfamily. BMPs are involved in the regulation of embryonic development and cellular functions such as bone induction [42-45]. BMP-2 signalling, in particular, plays critical roles in cartilage, heart, neural, and bone development, [42]. Mice lacking BMP-2 are non-viable [42].

In bone, the signalling action of BMP-2 is critical for the commitment of pluripotent MSCs towards osteoblastic lineage, via induction of ALP expression, stimulation of collagen synthesis, and enhancement of osteoblast differentiation [46-48]. BMP-2-induced differentiation of osteoblastic progenitor cells works through the up-regulation of type I collagen and subsequent ERK activation [23]. Inactivation of BMP-2 in the skeletal system

results in impaired fracture healing [49-51]. On the other hand, subcutaneous implantation of BMP-2 in rats results in ectopic cartilage and bone formation [52].

Preclinical and clinical studies have shown that BMP-2 can be utilised in various therapeutic interventions, such as critical-size bone defects, non-union fractures, spinal fusion, osteoporosis, and root canal surgery [42, 53-57].

1.2.2 Osteoclasts

Osteoclasts are large multi-nucleated cells of monocyte-macrophage lineage that degrade and resorb mineralised bone matrix (Figure 1-2) [58, 59]. Cytokines involved in the differentiation and activation of osteoclasts from its mononuclear phagocyte precursors: receptor activator of nuclear factor-κB ligand (RANKL), M-CSF, and IL-6 are all produced by osteoblasts [59-61]. The activation and differentiation of osteoclasts depends on the RANK/RANKL/osteoprotegerin (OPG) axis, where RANK on osteoclasts interacts with RANKL from osteoblasts to stimulate bone resorption [62]. OPG is a soluble decoy receptor for RANKL that inhibits osteoclastic bone resorption [63].

Once active, osteoclasts are polarised and form strong adhesion bonds to the bone surface. They create a ruffled border where the plasma membrane is highly infolded to increase resorption surface area [58, 64]. The cells then secrete H^+ through this border to create a locally acidic environment of resorption lacuna, called the Howship's lacuna, to digest the bone matrix [65]. They are found commonly with high levels of tartrate-resistant acid phosphatase (TRAP), and a foamy appearance due to the high concentration of vesicles and vacuoles required for bone resorption [59].

Abnormalities in osteoclast differentiation and resorption occur in inflammatory bone diseases including rheumatoid arthritis. In RA, excessive inflammation stimulates osteoclast activity and leads to disproportionate degradation of bone [66-69]. Osteoclasts are also extremely important in the early part of bone/fracture healing, where their resorption enables further progression of healing processes [2, 70].

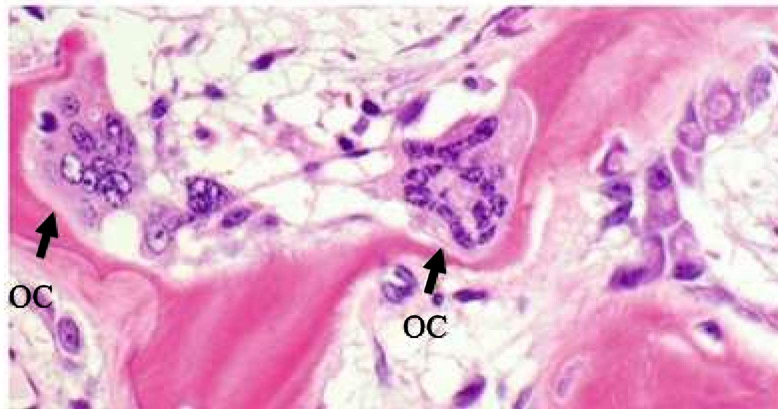

Figure 1-2 *Haematoxylin and eosin (H&E) staining of osteoclasts in normal human bone, with nuclei stained blue and bone matrix pink. Image from [71].*

1.2.3 Osteocytes

Osteocytes are terminally differentiated osteoblasts which have embedded themselves in the bone matrix [72]. During this process, they become encased in a lacuna and turn into stellate cells with thin dendritic processes [73]. These processes enable communication between osteocytes, from osteocytes to bone surfaces, and from osteocytes to bone lining cells [74]. Emerging evidence indicates that osteocytes are able to regulate bone structure in response to mechanical and hormonal stimuli; through their actions on both osteoblastic bone formation and osteoclastic bone resorption [74, 75]. Local apoptosis of osteocytes occurs in response to bone unloading, bone micro-damage, and oestrogen withdrawal [76, 77]. Apoptotic bodies from these cells release RANKL and stimulate osteoclastogenesis [78]. Conversely, the presence of viable osteocytes is necessary to prevent bone resorption and maintain bone mass

[72]. Osteocytes act in two ways on osteoblasts. They can positively regulate osteoblasts through nitric oxide and prostaglandins but also supress osteoblast activity through sclerostin [72, 75, 79].

1.3 Bone Remodelling

Bone maintains and adapts itself in response to mechanical challenges, micro-fractures, and plasma calcium homeostasis through continuous remodelling [80]. The bone remodelling process was originally described as focal repair of micro-damage by the interaction of osteoblasts and osteoclasts in a spatially and temporally restricted organisation known as a basic multi-cellular unit (BMU) [8, 11, 81, 82]. Activation of remodelling involves the detection of a remodelling signal such as mechanical strain or a hormone signal [83]. Osteoclast resorption of mineralised bone then follows, resulting in the degradation of organic bone matrix and dissolution of bone minerals [83]. Completion of the remodelling process is achieved by osteoblastic bone formation and subsequent mineralisation [84].

However, the bone remodelling process does not only involve the actions of osteoblasts and osteoclasts. It is a complex set of interactions between many cell contributors. For example, recent research demonstrates the importance of osteocytes in regulating skeletal mass [73, 75, 85]. The stimulation of osteoclastogenesis by osteocyte apoptosis suggests that osteocytes may be the initiators of remodelling in BMUs (Figure 1-3) [86]. Research also demonstrates the contribution of other cell types in bone remodelling, including endothelial cells, vascular cells, and immune cells [73, 86]. The interaction between these cells is also not representative of a linear algorithm as there are two-way communications between multiple cell types in bone [85].

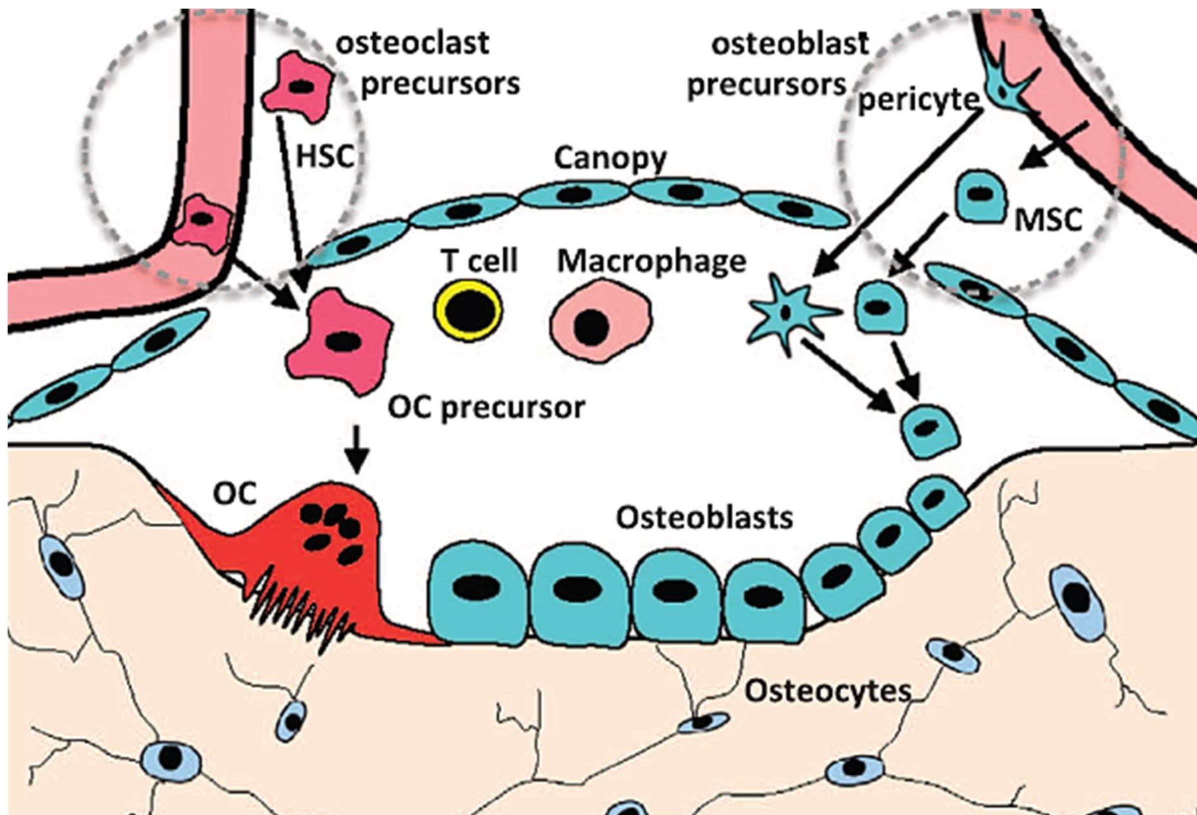

Figure 1-3 *Basic multi-cellular unit (BMU) in trabecular bone. A remodelling signal, which may come from osteocytes, recruits osteoclast precursors from the local vasculature and induces osteoclastogenesis and bone resorption. Subsequently, osteoblast progenitors and pericytes are recruited from the vasculature and synthesise new bone to replace resorbed tissue. T cells and macrophages can also access this local environment. Image from [86].*

1.4 Fracture Healing

1.4.1 Stages of Fracture Healing

Fracture/bone healing can be divided into four stages beginning with an initial injury that leads to haemostasis and inflammation, then the formation of soft callus, progression to hard callus formation and mineralisation, and finally remodelling to achieve the original bone contour (Figure 1-4) [70, 87-89].

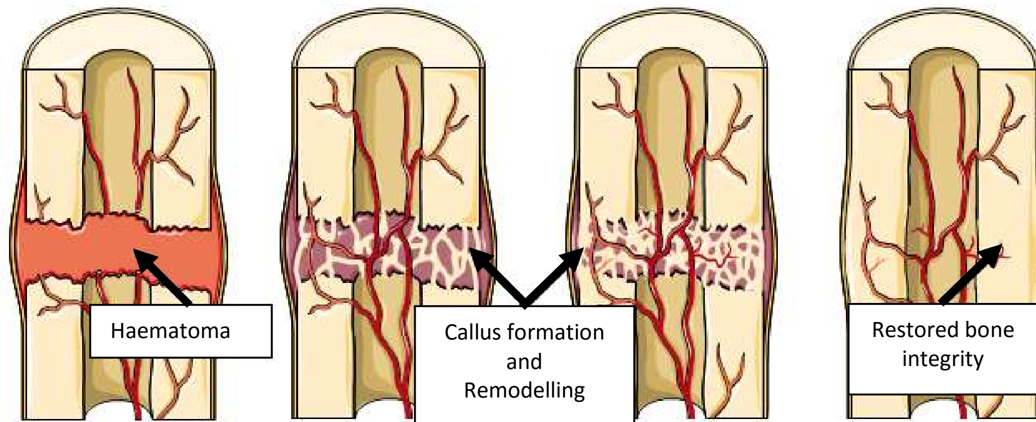

Figure 1-4 *Stages of fracture healing include injury and haematoma formation, followed by inflammation, callus formation, and remodelling/restoration of bone integrity. Images from Servier Medical Art at http://www.servier.com/Powerpoint-image-bank.*

Inflammatory Stage

In the inflammatory stage, tissue disruption results in interrupted vasculature and distortion of bone architecture [90]. Blood cells extravasate from vessels and form a haematoma at the fracture site. Inflammatory cells including macrophages, lymphocytes, and monocytes infiltrate the haematoma to remove bacteria, debris, and produce cytokines (Figure 1-4) [70, 91]. Growth factors, cytokines, and hormones from the acute inflammatory response recruit and proliferate local mesenchymal stem cells (Figure 1-5). This signalling cascade also promotes fibroblast survival and replication within the fracture space, forming new connective tissue containing small blood vessels. Known as granulation tissue, this forms a template for subsequent soft callus formation [70].

Reparative Stage - Soft Callus Formation

In the early reparative stage, migration and local differentiation of MSCs replace the granulation tissue with a cartilaginous template in a process called endochondral ossification [6, 92]. The cartilaginous template, or soft callus, supports the fracture and provides a base on

which the bony hard callus will later remodel over. At later stages the chondrocytes in the soft callus undergo hypertrophy and the extracellular matrix then becomes calcified cartilage [90].

Reparative Stage - Hard Callus Formation

After the formation of a soft callus plug, the mineralised cartilage is then degraded by osteoclasts. This allows neovascularisation, which is essential for the restoration of nutrients to the area. Neo-vascularisation also enables the migration of osteoblasts into the callus area to produce and mineralise osteoid matrix. These osteoblasts then quickly form irregular woven bone to enhance callus stability [93].

Remodelling Stage

The woven bone formed at the site of fracture gap is then remodelled by osteoclasts and osteoblasts into lamellar bone. Eventually, the medullary cavity is also reconstituted and the bone structure is restored to its original mechanical strength as it is further remodelled according to Wolff's law [94].

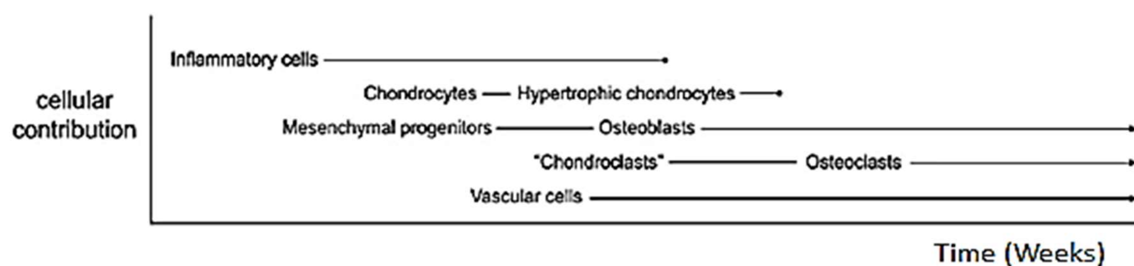

Figure 1-5 *Cellular contributions in fracture healing. Inflammatory cells first invade the site of injury and recruit mesenchymal progenitors. Chondrocytes form a cartilaginous template and undergo hypertrophy, these are then resorbed by chondroclasts. Hard callus is then formed by osteoblasts and osteoclasts and vasculature is restored. Bone is further remodelled its original strength. Figure modified from [70].*

1.4.2 Pathological Fracture Healing

The management of fractures, especially traumatic open fractures, are difficult to treat due to high rates of non-union, where permanent failure or cessation of healing occurs before fractures are bridged [95]. Non-union can necessitate recurrent surgical procedures and long in-hospital stays, which are challenging for both surgeons and patients [96, 97]. One of the main factors leading to non-union is a lack of requisite biological factors [95, 97].

Insufficient biological factors within the local environment can come from the disruption of local blood supply or the failure to create new blood supply into the fracture haematoma [98]. Blood vessels play an important role in the process of fracture healing, as they deliver all the necessary cells for repair, and the growth factors to stimulate new bone formation. The disruption of blood vessels disables the flux of cell recruitment and regenerative factors into the fracture haematoma, leading to atrophic non-union [99].

1.4.3 Current Treatments for Fracture Healing

Management of fractures in the acute trauma setting begins with stabilisation of patient vitals, control of haemorrhage, treatment with analgesia and antibiotics, and assessment of complications [100, 101]. The orthopaedic treatment of a fracture depends on whether the fracture is closed or open: the former describes fractures in which the skin is not broken and the latter occurs when a skin break communicates with the fracture [102].

Patients with closed fracture can undergo reduction, where the fracture ends are manipulated, apposed, and corrected into normal alignment [103]. Minimally displaced fractures can be reduced with closed manipulation and traction [103]. However, sometimes open or surgical reduction is the only option for regaining function. Factors including fracture stability, site of

injury, severity of injury, infection risk, pathology of injury, and failure of closed reduction are all considered when choosing open reduction [104]. After reduction, the fracture is then immobilised and protected until it consolidates [105]. Then patients will then undergo rehabilitation of the muscles and joints to restore function to the affected limb [105].

In an open fracture, patients are at increased risk of developing acute or chronic infections of the bone (osteomyelitis) [106-108]. Open fractures can be further classified according to the wound length, the level of contamination, the extent of soft tissue injury, and extent of bone injury [102, 104]. The mainstay of open fracture treatment includes debridement of wound edges and devitalised tissue, cleaning of contaminated tissue by irrigation, stabilisation of fracture by fixation, protection of soft tissue from further injury, and adequate soft tissue cover over exposed bone [102, 109].

Despite the differences in treatment of open or closed fractures, preservation of blood supply is essential to fracture management. In fracture healing, it is necessary for bone regeneration to restore vasculature in order to return full functionality to the tissue [110, 111]. In the initial haematoma formation, there is a high level of cytokine secretion to stimulate angiogenesis, resulting in a higher blood flow early in the fracture healing process [110, 112]. Hypertrophy and apoptosis of chondrocytes after soft callus formation also stimulates angiogenesis, and the absence of these signals results in delayed fracture progression [113, 114]. Angiogenesis enables the egress of osteoclast precursors from the vasculature to the site of fracture [115]. Osteoblast precursors also move into fracture sites at the same time as blood vessel invasion [116]. Angiogenesis not only delivers cells but also oxygen to enhance cell survival. Ischemic fractures result in increased apoptosis and delayed fracture healing [117-119]. Thus, immediate

surgical exploration is required for a devascularised limb, as ischemia of the tissue is a major risk factor for non-union [102].

Mechanical stability is also required for fracture healing. Fracture immobilisation provides mechanical stability and includes splinting, plaster cast, traction, external fixation, and internal fixation [102, 109]. Traction is applied to the distal half of the fracture, exerting a pull along the long axis of the bone to prevent angulation of the fracture joint and support normal alignment [103]. However, traction requires immobilisation of the patient, therefore, increasing the likelihood of complications, and cannot be used for long periods of times [103]. Casting and splinting can be used prior to operations but impedes the monitoring of soft tissue swelling, the evaluation of vascular impairment, the examination of compartment pressures, and increases the risk of joint stiffening [104]. Plaster casting can also be used following closed reduction and in conjunction with internal fixation [103, 104]. Internal fixation is used in unstable fractures, fractures that require surgery, pathological fractures, multiple fractures, or when closed reduction has failed [120]. It involves the fixation of bone fragments using inter-fragmentary screws, metal plates, transfixing wires, or intramedullary rods/nails [103, 121]. Internal fixation holds a fracture securely so that weight-bearing can commence straight away, although this fixation risks the development of sepsis [103, 121]. External fixation applies pins and bars above and below the site of injury [121]. It provides stability without the need for a foreign object, such as plates and screws, within the injured tissue itself, and it is useful in the presence of soft tissue injury [121].

When natural bone repair mechanisms fail, treatments to restore biological factors and a conducive healing microenvironment are used. These include bone grafting, bone transport,

the addition of growth factors, tissue engineering approaches, and bisphosphonate use [122-124].

Bone grafting can restore vascularity of the bone, however, it requires a donor site thus leading to donor site morbidity. Other complications of this procedure include bleeding, infection, and pain [125, 126]. Bone transport or distraction osteogenesis is a surgical process that induces bone regeneration through the gradual separation of fracture ends to enable new bone formation within the gap. The process is technically demanding and requires a lengthy treatment time [127].

Growth factors naturally produced by osteoblasts such as platelet-derived growth factors (PDGFs), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and transforming growth factors (TGF)-β, comprise an important component of the fracture healing process, and are investigated clinically for their bone repair and regenerative properties [8, 127]. PDGF promotes the osteogenesis of MSCs, however, it also inhibits osteoblast differentiation when secreted by osteoclasts, and its therapeutic utility still requires further investigation [128]. Temporal regulation of FGF-1 and 2 are essential to the process of bone healing [129-133]. Both FGFs accelerate the healing process in larger animal trials, yet the full role of FGFs is still unclear [134]. IGFs are released by osteoclastic digestion of the bone matrix during remodelling attract [135, 136]. It attracts osteoblasts to the remodelling surface and plays a role in the longitudinal growth of bones, however, the clinical effects of IGF on bone are limited by its potent stimulation of osteoclastogenesis [135, 136]. TGF-β exerts a biphasic effect upon bone formation depending on local concentrations - low levels stimulate, whereas high levels inhibit osteoblast proliferation [137, 138]. The expression of TGF-β is present in

the early stages of the fracture process, but it is subsequently down-regulated in the mid to late stages [137, 138].

Clinical application of these growth factors still require further investigation; their limitations demonstrate the complex nature of bone repair and highlight the need for an alternative therapy. Even BMPs, while clinically effective in promoting new bone growth, have significant complications including inflammation, induction of bone resorption, overgrowth of bone, oedema, wound problems, infections, and carcinogenicity [139-142].

Tissue engineering utilises a combination of cells and biomaterial scaffolds to repair or replace tissues. Modern tissue engineering approaches aim to produce scaffolds seeded with living cells to impart greater integration and functionality. Ceramics are the most commonly used biomaterials, due to their strength and structural similarity to bone, and they are currently applied clinically as bone fillers [143]. However, recent literature has suggested that to mimic the complex environment of bone by improving bioactivity and osteointegration, the ideal bone graft needs to not only possess mechanical strength and structural similarity, but also contain a combination of osteogenic factors, angiogenic factors, and MSCs [144-149]. The success of tissue engineering approaches not only relies on clinical applicability, but also needs to overcome clinical issues including efficacy, safety, and cost before its use can become practical [150].

Bisphosphonates are a class of drugs primarily used to inhibit osteoclastic resorption in diseases including osteoporosis, Paget's disease of the bone, and bone metastasis [151]. Bisphosphonates bind onto the bone surface, preferentially on areas undergoing active resorption [152]. Bisphosphonates released following the resorption of bone act to: inhibit

osteoclast attachment to bone surfaces, inhibit osteoclastic resorption, promote osteoclast apoptosis, and decrease numbers of osteoclast progenitor [153-156]. Newer bisphosphonates including alendronate, pamidronate, and zoledronate have greater potency owing to the inclusion of nitrogen in their structure [157].

Bisphosphonates have been trialled, both in animal models and clinically, for their use in bone healing. Results from animal models have been mixed, with studies reporting no effect, delayed fracture healing, or even enhanced fracture healing [158-162]. In patients, most bisphosphonate studies have been conducted in the context of osteoporosis and strong evidence from these studies demonstrates the protective effect of bisphosphonates in preventing osteoporotic fractures [162-167]. However, the use of bisphosphonates as treatments in human bone repair is not well studied [162]. One study assessed bisphosphonate treatment in osteoarthritic patients undergoing high tibial osteotomy and demonstrated that one infusion of zoledronic acid improves pin fixation in osteotomy, although no difference in time to healing was seen between treatment and control [168]. A recent review appraised the used of bisphosphonates in conjunction with anabolic agents, including BMPs, to enhance bone repair and reported mostly positive results [169]. The use of bisphosphonates, however, is associated with serious adverse effects including the suppression of bone turnover, increased incidence of atypical fractures, and bisphosphonate-related osteonecrosis of the jaw [170-176].

Overall, the complications and low success rates highlight the inadequacy of current treatments for traumatic fractures.

1.5 Arthritis

1.5.1 Rheumatoid Arthritis (RA)

Rheumatoid arthritis is a debilitating chronic inflammatory autoimmune disease that affects approximately 2% of Australians [177]. It causes pain, disability, and impaired quality of life in patients. RA primarily affects the joints but can also involve other organs including the eyes, lungs, skin, and heart [178].

1.5.2 RA Disease Pathology

RA pathology involves inflammation and an aberrant immune system. A primary feature of RA is the excessive production of inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-17 by CD4+ T-cells, macrophages, and synovial cells [66, 179-181].

The normal joint synovium is a one to two cell layer lining with thin-walled blood vessels. Early changes in RA lead to hyperplasia of the synovium, vessel ingrowth, recruitment and activation of endothelial cells, infiltration of T- and B- lymphocytes, and cytokine production [182, 183]. This leads to the formation of a pannus, which then infiltrates into the adjacent cartilage and bone, eroding it away with high levels of degrading enzymes including matrix metalloproteinases (MMPs) and osteoclasts activators [67, 184, 185]. Excessive inflammation from cytokines and enzymes cause joint pain, swelling, and damage to joint tissue, resulting in irreversible loss of shape and alignment in the joint (Figure 1-6) [177].

Animal models have been developed to better understand the pathogenesis of RA, yet no animal model fully recapitulates RA pathogenesis in humans [186]. RA models including the adjuvant model, collagen-induced arthritis (CIA), antigen-induced arthritis (AIA), spontaneous

inflammatory arthritis (K/BxN), and streptococcal cell-wall-induced arthritis (SCW) use various stimuli such as bacteria, viruses, and auto-antigens to initiate the arthritic process [187-189]. This demonstrates the multi-factorial nature of RA.

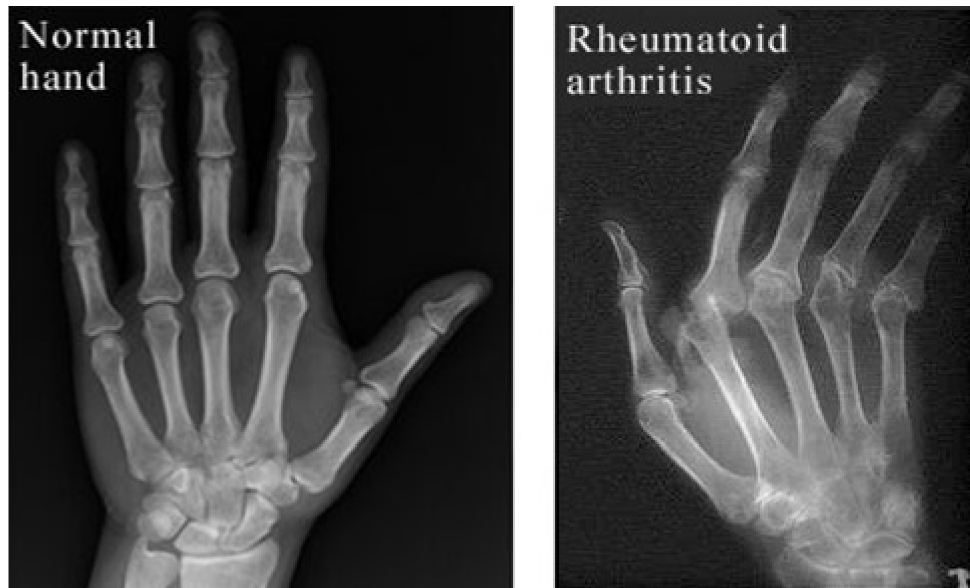

Figure 1-6 *Radiography of normal and RA hands. The normal hand shows good joint space between metacarpophalangeal joints (left) while the RA hand demonstrates severe bone erosion, ulnar deviation, loss of joint space and subluxation (right). Images from http://www.uofmhealth.org/health-library/zm6061 and http://www.glucosamine-arthritis.org/arthritis/RA-Hands, respectively*

Despite the multi-factorial nature of RA, animal models have highlighted one key cytokine in its pathogenesis: TNF-α. Injection of TNF-α in mice exacerbates CIA and inhibition of TNF-α with antibodies ameliorates CIA [190]. Furthermore, transgenic TNF-α mice spontaneously develop a severe erosive arthritis and treatment of these mice with TNF-α antibody completely prevents arthritis [191, 192]. These models have significantly advanced the understanding of

RA pathogenesis and demonstrated the importance of TNF-α in the progression of RA, thus enabling the design of specific targets against TNF-α as therapeutic treatments [193].

1.5.3 The Role of Osteoblasts in RA

There are three types of bone loss in RA: (1) focal bone loss at the joint margins, (2) peri-articular osteopenia, and (3) generalised skeletal osteoporosis [194, 195]. Osteoclasts are the principal cells responsible for bone loss in RA but osteoblasts also contribute to the problem. Abnormalities in osteoblasts occur at focal erosion sites in RA, where defective osteoblasts produce less bone than normal [194, 196, 197]. This is partly due to inhibition of osteoblast differentiation by cytokines such as TNF-α [196, 197]. In conjunction, the mineralisation of the newly formed bone at sites of inflammation is reduced, suggesting that impairment of normal osteoblast activity also plays a role [195, 198].

1.5.4 Current RA Treatments

The most common treatments used by RA patients are primarily: complementary/alternative medicines (e.g. vitamins, minerals, herbal medicines), non-steroidal anti-inflammatory drugs (NSAIDs) which relieve pain and dampen inflammation, analgesics for pain, and disease-modifying anti-rheumatic drugs (DMARDs), including the biological drugs (Figure 1-7) [199]. Unfortunately, due to the multi-factorial nature of RA, one treatment is unlikely to fit all patients.

Currently, the first line of treatment for RA is a DMARD called methotrexate. However, advances in the research of RA pathology has enabled the development of biological DMARDs (bDMARDs) that can target specific inflammatory cytokines. These include anti-TNF-α drugs

(infliximab, etanercept and adalimumab), IL-1 receptor antagonist (anakinra), B-cell response blocker (rituximab), or T cell co-stimulation blocker (abatacept) [179, 200-202].

There are several limitations to bDMARD use in patients, including increased infection risk, high costs, and the inability to reverse joint damage. The increased risk of tuberculosis reactivation and susceptibility to viral infection are the most common safety concerns from bDMARD immunosuppression [203-205]. These treatments are also classified as high-cost medications with each treatment costing around $20,000 per patient per year, which is substantially more expensive than conventional DMARDs [206]. Furthermore, bDMARDs cannot reverse the damage already done on the joint, thus early intervention with biologics is essential for the prevention of joint erosion [179]. Final stage RA patients who fail medical therapy including bDMARDs may need to undergo joint replacements to restore mobility.

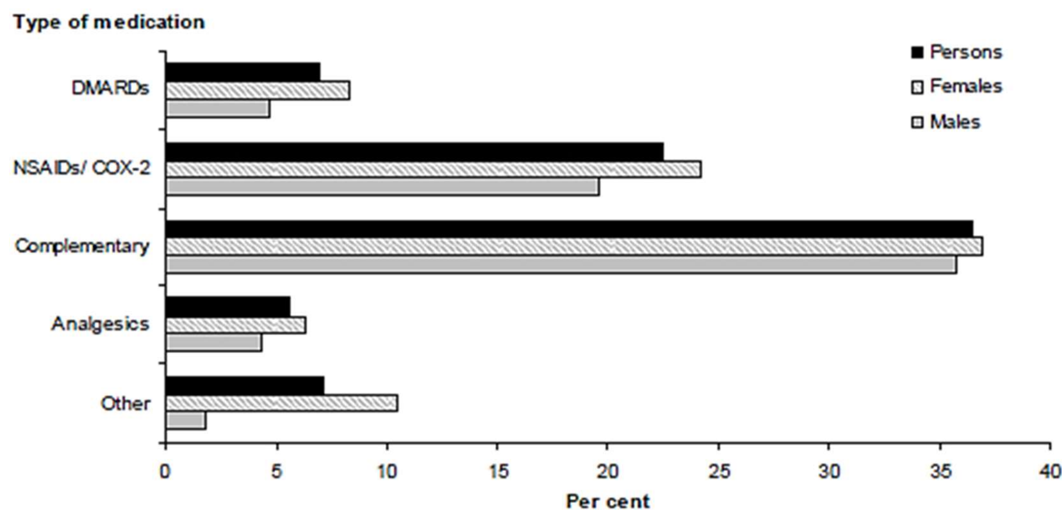

Figure 1-7 *Types of medications used in RA, by gender from 2004-05. RA is primarily medicated by complementary medicines followed by NSAIDs. Figure from [199].*

1.5.5 Osteoarthritis (OA)

Osteoarthritis is a progressive joint disease triggered by abnormal intra-articular stress. It was traditionally viewed as a cartilage-based disease with matrix fibrillation and proteoglycan loss, but increasing evidence shows that the whole joint is affected, with thickening of the synovium, degradation of the meniscus, and abnormalities of the subchondral bone [207-210].

1.5.6 OA Disease Pathology

OA is a joint condition that primarily affects the hands, spine, and weight-bearing joints, such as the hips, knees, and ankles [211]. OA symptoms include movement limitation, crepitus, tenderness, pain, and joint stiffness [211]. OA remains a mystery to scientists as its aetiology is largely unknown in patients but its risk factors include a combination of obesity, joint mechanics, age, and previous injury [208]. Several studies in patients with OA have found obesity to be a major modifiable risk factor for OA [212-214]. Spontaneous OA in guinea pigs also shows ageing to be a factor in the progression of OA [215]. Mechanical destabilisation and injury also contribute to OA as OA animal models can be induced by surgical destabilisation [215].

In the normal knee joint, the femur and tibia are held together by ligaments, lined with a synovial capsule, with attaching musculature to provide mechanical stability. The femur and tibia are covered with load-bearing articular cartilage that holds water using proteoglycans, providing shock absorbance [216]. The knee joint also includes two menisci to distribute load and protect the underlying hyaline cartilage.

In the OA joint, mechanical and biologic changes lead to uncoupling of extracellular matrix degradation and synthesis by chondrocytes [216, 217]. This results in loss or damage to the

articular cartilage. Emerging evidence from animal models indicates that there is also crosstalk between cartilage and bone within the OA joint [208-210, 218]. Models in guinea pigs and dogs show that subchondral bone changes can exacerbate cartilage degradation, and in some cases precede changes in the cartilage, highlighting the importance of subchondral bone in osteoarthritis [219, 220]. Furthermore, a monkey model of OA demonstrates that changes in the bone-cartilage interface correlate positively with OA severity [221]. In patients, changes in the bone-cartilage subunit include cartilage degradation, sclerosis of subchondral bone, decreased mechanical strength of bone, areas of hypomineralisation, increased bone turnover, and osteophyte formation (Figure 1-8) [210, 222-224].

It was thought that the major difference between OA and RA lies in the inflammation of the joint in RA, which is not evident in OA. However, more recent research indicates that the pathophysiology of OA involves inflammatory factors, especially in the early stages of the disease, including TNF-α, IL-1, IL-6 and the catabolic enzyme MMP-9 [225-229].

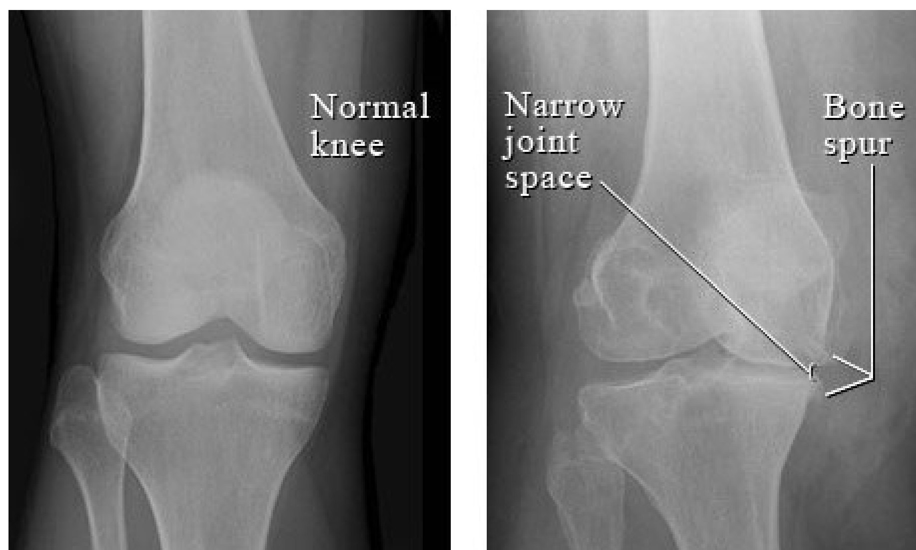

Figure 1-8 *Radiography of normal and OA knee. OA knee (right) shows narrowing of joint space and bone spur (osteophyte) formation as compared to normal (left). Image from http://www.webmd.com/osteoarthritis/x-ray-of-osteoarthritis-of-the-knee.*

1.5.7 The Role of Osteoblasts in OA

OA bone is characterised by changes in both macroscopic bone and also aberrant signalling in osteoblasts. Macroscopically, OA joints have extensive remodelling, leading to osteophyte formation at the margins of articular cartilage and sclerosis of the subchondral bone [230]. Although there is increased bone formation, the mineralisation of this bone is incomplete [210]. At a cellular level, OA osteoblasts exhibit aberrant Wnt signalling pathway activity, abnormal expression of OPG and RANKL, and elevated type I collagen [195]. They also express elevated levels of alkaline phosphatase, type I collagen, and osteocalcin and exhibit altered response to vitamin D [231-233]. These OA osteoblasts are uncoupled from osteoclasts, further contributing to distorted bone formation and remodelling [231, 233, 234].

1.5.8 Current OA Treatments

Current treatments for OA improve symptoms but do not delay disease progression. Instead, they primarily involve non-pharmacological managements including exercise and weight loss, physiotherapy, simple analgesics such as paracetamol, and NSAIDs [217, 235]. The use of complementary medicines, including glucosamine and omega-3, for easing pain and stiffness remains controversial as there is insufficient evidence for their efficacy in improving OA [236-240]. Failure to benefit from previous interventions, severity of symptoms, and functional limitations are the main reasons for joint replacement [241]. There are currently no effective evidence-based treatment options for OA. Further investigation into molecular/pathogenic mechanisms in OA may reveal novel targets for treatment.

1.6 Activated Protein C (APC)

1.6.1 Protein C (PC) Structure and Activation

Protein C (PC) is a vitamin K-dependent zymogen that, once activated, becomes a physiological anticoagulant. It is a 62kDa protein (419 amino acids) with a light (21kDa) and a heavy chain (41kDa) linked by a disulfide bond. PC is encoded by the *PROC* gene on chromosome 2q13-14 (nine exons) [242-244]. The light chain begins with a γ-carboxyglutamatic acid (Gla), which enables PC binding onto phospholipids to enhance its anticoagulant activity. The Gla domain also facilitates PC binding to its predominant receptor, endothelial PC receptor (EPCR), to exert its cytoprotective functions [242]. Further along the light chain are two epidermal-growth-factor-like domains and a small activation peptide (Figure 1-9) [242, 245]. The heavy chain consists of an active serine protease domain which is important for coagulation factor degradation [246].

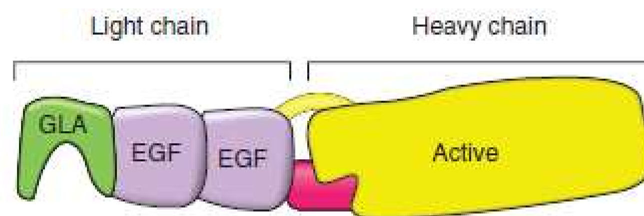

Figure 1-9 *PC structure including the position of glutamine-rich (Gla), epidermal growth factor, and active serine protease domains on PC. Adapted from [247].*

PC is activated when thrombin is bound to a membrane-bound receptor, thrombomodulin. Thrombin then cleavages the activation peptide in PC. This results in the release of an Arg-Ile peptide between residues 14 and 15 of the heavy chain. PC subsequently undergoes a conformation change into its active form (Figure 1-10).

PC circulates the body at 70 nM (≈4 μg/mL) with a circulatory half-life of ~8 hours. Its activated form is present at much lower circulating concentrations of 40 pM (≈ 2.3 ng/mL) with a half-life of 20 minutes [248].

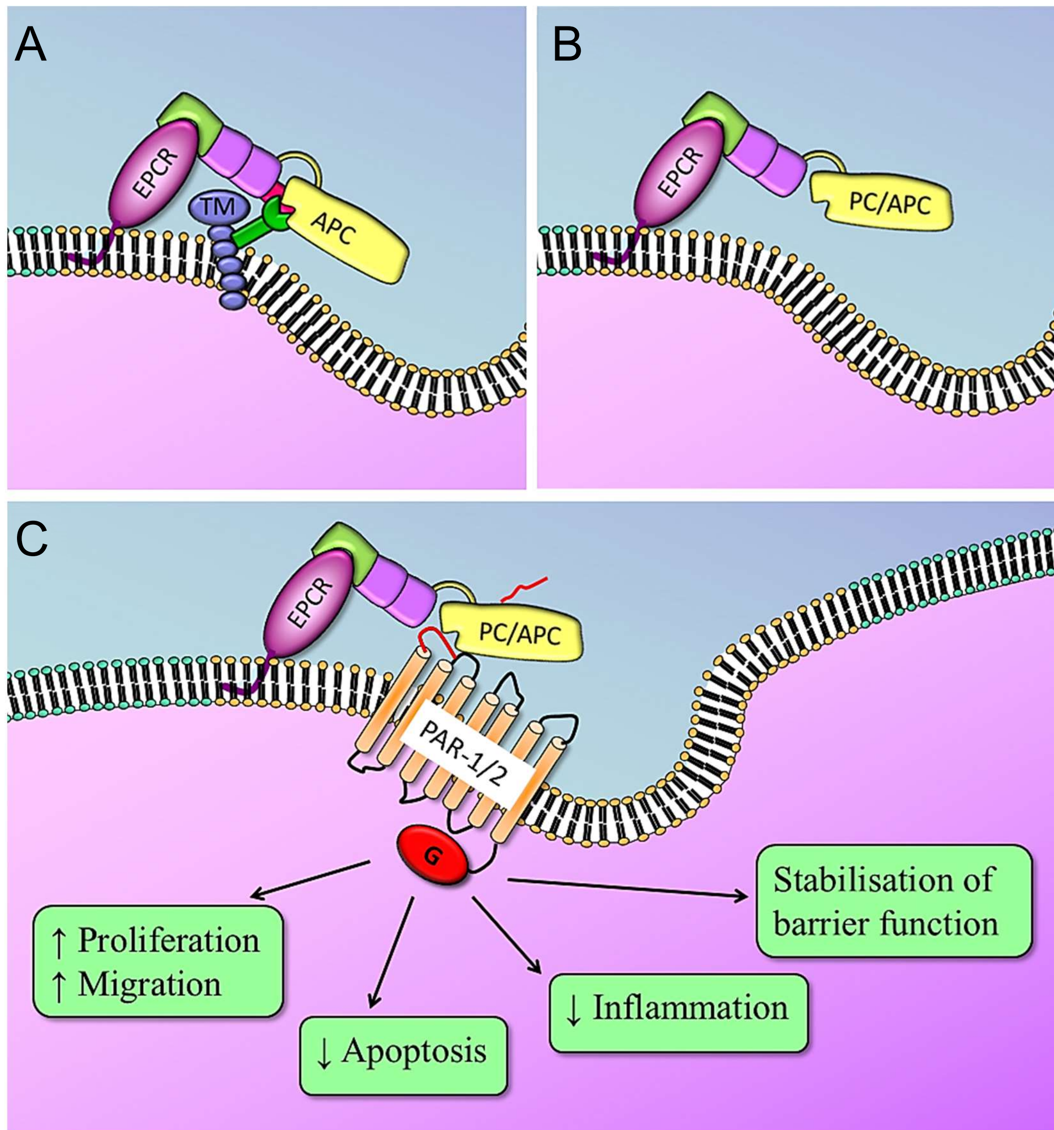

Figure 1-10 *PC activation by thrombin bound to thrombomodulin. PC binds to EPCR on the surface of lipid rafts (orange). This enables thrombin (dark green), bound to thrombomodulin (TM, blue), to cleave the activation peptide (pink, A). Subsequent conformation change of APC (B) enables it to cleave and activate PAR1/2 (red) in order to phosphorylate its intracellular G protein and exert its cytoprotective actions (C).*

1.6.2 Anticoagulant Functions of APC

APC proteolyses coagulation factors Va (FVa) and VIIIa (FVIIIa) in the presence of co-factor Protein S to inhibit the generation of thrombin in the coagulation cascade thus preventing clotting [249, 250]. APC is also profibrinolytic as it can bind to the plasminogen activator inhibitor and enable the conversion of plasminogen to plasmin, which then leads to fibrin degradation and anticoagulation [251]. Severe presentation of PC deficiency can result in the life-threatening condition of *purpura fulminans* characterised by skin necrosis and disseminated intravascular coagulation (DIC) [252]. Milder presentations of PC deficiency results in thrombophilia and is associated with an increased risk of venous thromboembolism (VTE) [253, 254]. APC treatment can induce bleeding in patients with severe sepsis [255]. However, new constructs of PC engineered without anticoagulant activity demonstrate no bleeding or bruising in patients in a recent Phase I trial [256, 257].

1.6.3 Cytoprotective Activities of APC

APC has been more recently been investigated for its cytoprotective functions, which can be modulated independently from its anticoagulant effects [242]. These include (but are not limited to) the promotion of cell proliferation & viability, prevention of apoptosis, suppression of inflammation, and stabilisation of the endothelial barrier.

APC stimulates cell growth and migration by increasing DNA synthesis, proliferation, viability, and decreasing apoptosis in muscle cells, brain endothelial cells, human keratinocytes, neural stem/progenitor cells, and podocytes, both *in vitro* and *in vivo* [258-264].

APC stimulates angiogenesis in endothelial cell tube formation assays, rabbit corneal assays, and chick embryo chorioallantoic membrane (CAM) assays [265]. APC's actions lead to the

stimulation of vascular endothelial growth factor (VEGF) and angiopoietins [265-267]. Recently, it has also been found that APC can utilise ang1/Tie2 to promote endothelial barrier stabilisation [267].

A recent paper by Kurata and colleagues, published during the first year of this PhD, demonstrates for the first time that PC is present in fracture haematomas [268]. They show that the application of APC, but not PC, enhanced DNA synthesis and stimulated pERK1/2 in normal human osteoblasts [268]. EPCR is expressed on these cells and mediates the effects of APC [268]. Notably, APC does not require its protease activity or PAR1 for these actions [268]. During this PhD, another paper by Lee and colleagues showed that APC can enhance viability and markers of osteoblast differentiation, including type I collagen, alkaline phosphatase, and calcium deposition in human osteoblast-like MG-63 cells [269]. This paper also investigated the complex interaction between APC and three bisphosphonates: alendronate, pamidronate, and zoledronate [269]. APC increased collagen production in the presence of all three bisphosphonates but only stimulated alkaline phosphatase activity and calcium deposition in combination with alendronate [269]. In contrast, APC protected against cell death and caspase-3 activation in zoledronate and pamidronate-treated cells but enhanced cell death in the presence of alendronate [269]. EPCR was required for the protective effect of APC in conjunction with zoledronate, but not its detrimental effects with alendronate [269].

1.6.4 Anti-inflammatory Activities of APC

The anti-inflammatory activity of APC involves: the suppression of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α; the counteraction of LPS-mediated inflammation; the down-regulation of nuclear factor-kappa B (NF-κB) in monocytes, keratinocytes and endothelial cells; and the inhibition of leukocyte migration [184, 270-280]. Elevated APC levels in RA

patient synovium and synovial fluid correlates with the anti-inflammatory matrix metalloproteinase (MMP)-2 [281]. APC activates MMP-2 in keratinocytes, RA monocytes, RA synovial fibroblasts, normal and OA chondrocytes [184, 282-284]. APC also inhibits MMP-9 production by RA monocytes, RA synovial fibroblasts, human brain endothelial cells *in vitro* and in animal models of brain haemorrhage, LPS-induced endotoxic shock *in vivo* [184, 285, 286]

1.7 APC in Disease

The cytoprotective properties of APC makes it a versatile and attractive agent for therapeutic investigation in a variety of diseases including wound healing, Alzheimer's disease, spinal cord injury, endotoxemia, ischemic reperfusion, renal failure, acute pancreatitis, and arthritis, however its role in severe sepsis is controversial [184, 265, 281, 282, 287-295]. Though APC was previously used for sepsis treatment, it has since been withdrawn from the market due to a lack of efficacy [287, 288, 296, 297].

APC preservation and stimulation of muscle cells, improvement of microcirculation, reduction of tissue hypoxia, reduction of apoptosis, and decrease in inflammatory leukocyte recruitment improves outcomes in endotoxemia models [298-301]. APC also inhibits cardiomyocyte apoptosis and protects against injury in rat ischemia-reperfusion models, by modulation of TNF-α, IL-6, and NF-kB pathways [301-303].

APC induction of proliferation on neural progenitors, neural stem cells, and neovascularisation is essential for the survival and improved functional outcomes in post-ischemic mice brains [264]. APC protects against ischemia/reperfusion injury in rat spinal cords by the reduction of

micro-infarcts, preservation of grey matter, attenuation in TNF-α levels, and reduction in neutrophil activation [304].

The stimulation of angiogenesis and cytoprotective effects of APC on keratinocytes also promotes cutaneous wound healing in animal models [265, 292]. In rat full-thickness punch biopsy wounds, APC enhances vasculature and decreases neutrophils throughout healing [265]. APC also improves cranially based dorsal cutaneous ischemic flap survival in rats by elevating pro-angiogenic and anti-inflammatory gene expression, enhancing muscle cell viability, increasing vessel infiltration, and inhibiting inflammatory cells [305].

Recently, PC was estimated to be 36 nM (≈2.2 μg/mL) in bone fracture haematomas, which is almost half the concentration of that which circulates the body [268]. A possible explanation for the difference between serum and fracture haematoma levels is that PC is depleted when local cells consume it to generate APC for cytoprotection [306]. APC levels have not been determined in bone [307].

1.8 APC in Clinical Trials

APC has been widely used in clinical trials, the most well-known of which was the PROWESS sepsis trial that led to the subsequent FDA approval in 2002 for commercial recombinant human APC (rhAPC, Xigris) [287, 296, 308]. It has since then been voluntarily recalled from the market by parent company Eli-Lilly due to lack of efficacy found in PROWESS-SHOCK trial and Cochrane review [255, 288]. However, APC is still being trialled as a potential therapeutic agent for other indications including cutaneous wound healing in chronic ulcers, diabetic wounds, and orthopaedic wounds [290, 291, 309].

Six diabetic patients with lower leg chronic ulcers (for >6 months) were treated with 400 μg APC for 6 weeks in a small randomised, double-blind, placebo-controlled pilot study. The patients demonstrated a significant improvement in healing in comparison to no change in saline-treated control patients [291]. Fracture healing shares many similarities with soft-tissue and cutaneous wound healing [92]. Repair of soft tissues is also an intrinsic component of managing orthopaedic injury [92]. The acceleration of wound healing by APC supports a role for it in orthopaedic medicine.

In a recent publication from our laboratory, topical APC treatment was trialled in patients with enduring orthopaedic wounds [309]. Treatment in these patients was made difficult by post-operative infection and concurrent metalware fixation [309]. APC treatment led to improved healing of soft tissue injuries, granulation tissue formation, re-epithelisation of surrounding tissue, and in some cases spontaneous resolution of chronic underlying osteomyelitis [309]. The local inflammatory reaction in response to osteomyelitis can results in necrosis of entrapped bone and formation of a sequestrum, a piece of separated necrotic bone. Viable organisms can persist in the sequestrum for years, which makes the management of osteomyelitis extremely challenging. Whether APC treatment can prevent the prolonged existence of a sequestrum is unknown.

The noteworthy and curative effects seen with APC treatment illustrate the efficacy of APC as a healing agent. At the time of initiation of this project, no studies had specifically investigated the effect of APC on fracture healing.

1.9 APC Receptor Candidates in Bone

1.9.1 Endothelial Protein C Receptor (EPCR)

EPCR was first discovered on endothelium but since then has been detected on vascular smooth muscle cells, eosinophils, neutrophils, monocytes, keratinocytes, hippocampal neurons, cardiomyocytes, placental trophoblasts, and more recently osteoblasts [259, 268, 279, 303, 310-314]. Mutations in EPCR or its promoter region by insertion and deletion can lead to increased odds for late foetal loss during pregnancy, recurrent miscarriages, and venous thromboembolism [315-324].

The Gla domain of PC and APC bind to EPCR. Recent evidence indicates that other vitamin K-dependent proteins including factor VII (FVII) and activated FVII (FVIIa) can also bind to EPCR, as Gla domains are highly conserved between vitamin-K-dependent proteins [325, 326]. EPCR is a non-signalling receptor located in the caveolin-rich membrane domains called lipid rafts, in close proximity to thrombomodulin [327]. PC produced by the liver, then circulates in the plasma and binds locally onto EPCR on the surface of cells where it becomes activated [245]. The occupancy of EPCR by PC/APC also recruits PARs to lipid rafts to enable the subsequent proteolytic cleavage and activation of these PARs [328].

1.9.2 Protease-Activated Receptor (PAR)1

Protease-activated receptors (PARs) are a heptahelical family of G protein-coupled receptors (GPCRs) that are irreversibly activated after the extracellular proteolytic cleavage of their NH_2-terminus tail [329]. Cleavage by serine proteases including APC and thrombin exposes a new NH_2-terminus that acts as a tethered ligand, binds intramolecularly to the receptor, and activates their cognate G-proteins [330, 331]. PAR signalling, due to its irreversible nature, is terminated by internalisation and degradation of the receptor.

Polymorphisms in PAR1 are associated with a decreased risk of developing thrombosis, an increased risk of coronary heart disease, protection against breast cancer recurrence, and depending on the location of the single-nucleotide polymorphisms (SNPs), differential risk for myocardial infarction [332-336].

APC is cytoprotective in various cells and it was later discovered that these actions required the activation of PAR1 [245]. However at that time, the only known downstream effects of PAR1 included promotion of inflammation and thrombosis which contradicted the protective effects of APC [337, 338]. Subsequent studies have demonstrated that APC signalling through PAR1 on lipid rafts is cytoprotective because PAR1 couples to anti-inflammatory G_i proteins when cleaved by APC, whereas it couples to pro-inflammatory $G_{12/13}$ proteins when cleaved by thrombin [327, 328, 339]. Additionally, APC cleavage of PAR1 occurs at a different site to thrombin and generates a unique agonist for PAR1 to induce cytoprotective effects [340].

Since the discovery of APC-mediated PAR1 signalling, APC has been demonstrated to act widely through this receptor, including its protection against staurosporine-induced endothelial apoptosis; its anti-inflammatory, anti-thrombotic, and neuroprotective effects in a mouse focal ischemic stroke model; and its anti-apoptotic effects in ischemic brain endothelium [261, 262, 271]. Through PAR1, APC also enhances endothelial barrier protection; mediates wound healing phenotype in keratinocytes and tenocytes; and protects against diabetic nephropathy by inhibition of endothelial and podocyte apoptosis [282, 341-343]. Intracellularly, PAR1 can bind to protein kinase C, protein kinase A, and its C-terminus tail also contains a serine/threonine residue that increases the affinity of the intracellular binding to β-arrestins, which can then lead to signalling through extracellular-signal regulated kinase (ERK) (Figure 1-10) [344].

1.9.3 Protease-Activated Receptor (PAR)2

PAR2, like PAR1, is a heptahelical G-proteincoupled receptor (GPCR) that requires a serine protease for activation, however, unlike PAR1, it cannot be cleaved by thrombin because PAR2 lacks a hirudin-like domain on its NH_2 tail which is essential for binding to thrombin [330]. Both PAR1 and PAR2 can be cleaved by trypsin, tissue factor, mast cell tryptase, and APC [345].

Polymorphisms in PAR2 are associated with an increased risk for atopy, higher serum IgE, higher total eosinophil count, changes in agonist sensitivity [346, 347]. PAR2 plays an inflammatory role in atopic dermatitis, pancreatitis, colitis, and autoimmune inflammation [348, 349]. PAR2 is up-regulated by TNF-α, IL-1 in endothelial cells and induces an inflammatory response including the stimulation of NF-kB [350, 351]. PAR2 can also be trans-activated by thrombin-cleaved PAR1 in invasive breast cancer [352].

APC acts through PAR2 to induce p38 and reduce ERK1/2 phosphorylation in wound healing [292]. APC acts through both PAR1 and PAR2 to induce vascular protection in sepsis, yet the functional activity of APC-activated PAR2 in other diseases and systems is still undetermined [353, 354].

1.10 Aims and Hypotheses

1.10.1 Hypotheses

APC is an anti-inflammatory, pro-angiogenic agent with cytoprotective effects, and one that has demonstrated efficacy in wound healing. The functional effect of APC on bone formation, fracture healing, and arthritic bone are yet to be explored. The work presented in this thesis explores the specific roles for recombinant human (rh)APC in the aforementioned conditions.

Based on the literature, we hypothesised that:

(1) APC promotes osteoblast viability through its receptors EPCR, PAR1, and PAR2 and downstream phosphorylation of ERK1/2;

(2) APC augments bone formation through the EPCR/PAR axis and by stimulation of osteoblast activity;

(3) APC improves fracture repair in a mouse model through increased bone formation; and

(4) APC regulates viability and down-regulates inflammatory cytokines in osteoblasts derived from OA and RA bone, which is facilitated by the EPCR/PAR axis and ERK1/2 intracellularly.

1.10.2 Aims

Based on our hypotheses, four main aims were formulated to:

1. confirm and identify the effect and mechanisms of APC on the viability of MG-63 osteoblast-like cells (Chapter 3).
2. determine the effect of APC on bone formation *in vivo* using a BMP-2-induced ectopic bone formation model (Chapter 4).
3. examine whether APC can impact on fracture healing using a closed-fracture model (Chapter 5); and
4. establish whether APC alters viability or inflammation of OA and RA osteoblast-like cells (Chapter 6).

Specific aims are detailed in each chapter.

CHAPTER 2:

GENERAL METHODOLOGY

2 General Methodology

2.1 *In Vitro* Studies

MG-63 and MC3T3-E1 cells were used in this study as they are versatile, share osteoblastic features, and are commonly used to model osteoblast responses in bone healing. Human osteoblasts, referred to as human bone-derived cells (HBDCs), were also derived from the subchondral bone of OA and RA patients.

2.1.1 MG-63 Cell Culture

MG-63 cells (provided by Professor Rebecca Mason, Department of Physiology, University of Sydney, Sydney, NSW, Australia) are osteosarcoma cells that synthesise type I collagen, osteocalcin, bone sialoprotein, and decorin [355-357]. In comparison to osteoblasts, MG-63 cells have a higher rate of proliferation, lack contact inhibition, and grow in multi-layers. They also exhibit karyotypic alterations as compared to human foetal osteoblastic cells [358]. MG-63 cells have been commonly used to model osteoblast responses due to their versatility [357, 359]. Cells from passage 5-10 were cultured in reconstituted DMEM medium (D7777, Sigma-Aldrich, St Louis, MO, USA) supplemented with 3.7 grams/litre (g/L) sodium bicarbonate, 10% (v/v) heat-inactivated FBS (Gibco, Life Technologies, Carlsbad, CA, USA) and maintained in 75 cm^2 flasks at 37°C in humidified atmosphere of 5% CO_2 in air. Once confluent, cells were passaged into 24- or 96-well plates for subsequent experiments.

2.1.2 MC3T3-E1 Cell Culture

MC3T3-E1 mouse pre-osteoblast cells were cultured in α-MEM supplemented with 10% FBS, 1% L-glutamine, and 2% penicillin/streptomycin (Invitrogen). Cells were passaged and maintained as per MG-63 protocol. MC3T3-E1 are capable of collagen synthesis, osteoblast

differentiation, express osteoblast markers, and can be stimulated by vitamin D and ascorbic acid [36, 356].

2.1.3 Human Bone-Derived Cell (HBDC) Isolation and Culture

HBDCs were isolated by sequential collagenase digestion (Sigma-Aldrich, C2674, St Louis, MO, USA) of fresh bone fragments from the subchondral bone of OA (*n=4*) or RA (*n=3*) patients undergoing knee replacement surgery [360, 361]. Tissues were collected with patient written informed consent and approval from the Northern Sydney Local Health District Human Research Ethics Committee. All patients fulfilled the American College of Rheumatology criteria for RA or OA [362, 363]. Bone specimens were unavailable from normal joints.

Isolation of HBDCs was performed using an original protocol by J.A. Gallagher modified by Dr Benjamin Chan [364]. Under aseptic conditions, tissues were sterilised in 70% EtOH for 10 sec to decontaminate before 3 thorough washes in warm phosphate buffered saline (PBS) with 50 U/mL of penicillin and 50 µg/mL of streptomycin (Gibco 15070-063; Figure 2-1). Soft tissues and cartilage were removed by scraping with a scalpel blade and subchondral cancellous bone sections were harvested using bone cutters (Figure 2-1). Bone sections were further dissected to 1-2 mm diameter fragments using bone rongeurs to increase surface area for collagenase penetration and digestion (Figure 2-1).

Bone fragments were then washed in warm PBS and centrifuged at 1200 rpm for 10 min per wash to isolate and remove the lipid layer. Minced bone was then incubated in 1 mg/mL of collagenase type I (Sigma-Aldrich, C2674) with 0.05% trypsin (MP Biomedicals, 103139, Santa Ana, CA, USA) with 0.53 mM ethylenediaminetetraacetic acid (EDTA, Aldrich, 431788) in PBS for 20 min to liberate non-osteoblastic cells.

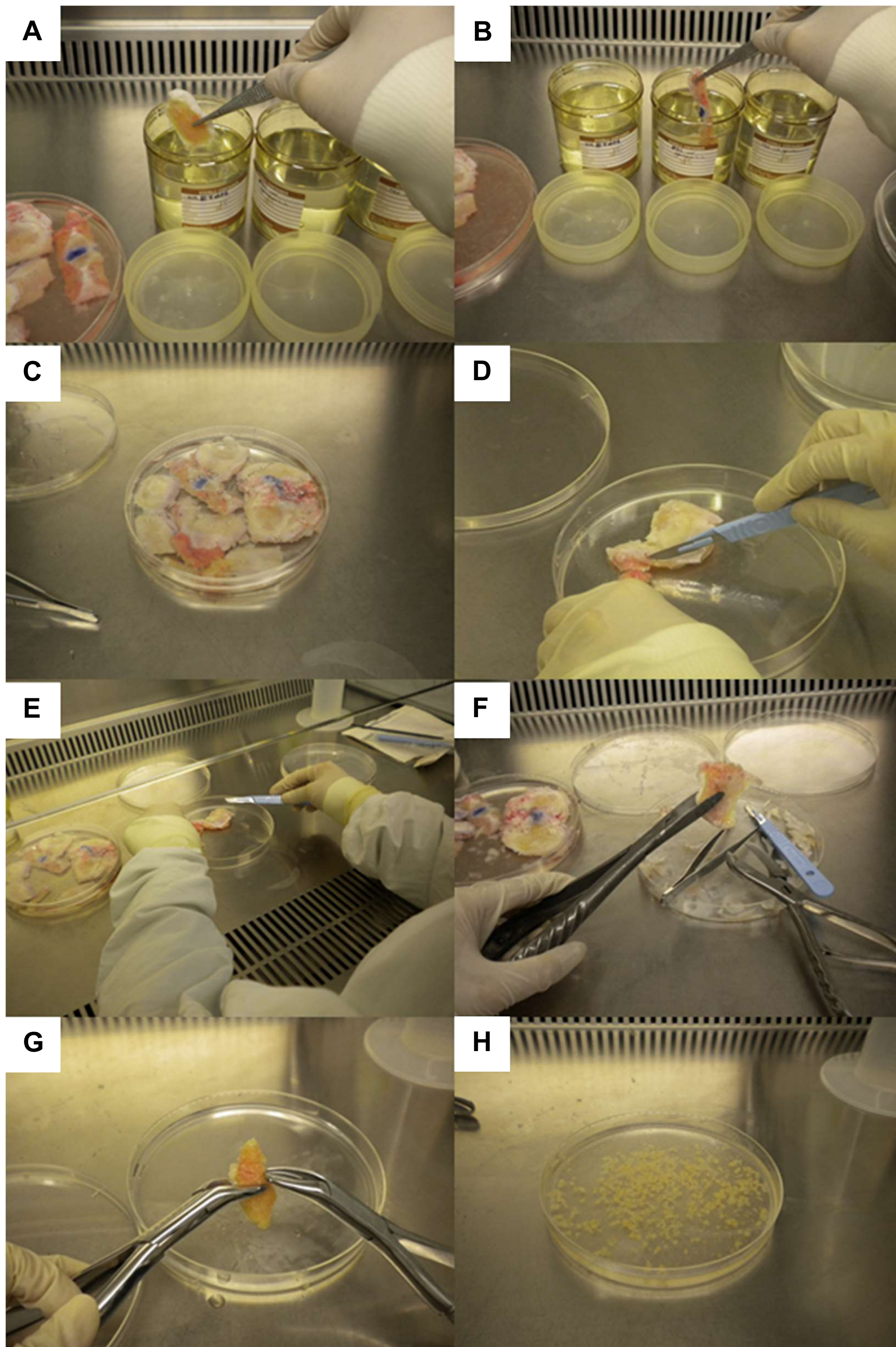

Figure 2-1 *Isolation of HBDCs from subchondral bone. Subchondral bone tissues were dipped into 70% ethanol (A), washed thrice in 37°C PBS (B, C). Cartilage and synovial tissue were removed (D, E) before small bone fragments were removed and washed in PBS (F-H).*

Minced bone was further digested in 1 mg/mL of collagenase type I without trypsin-EDTA for 3 h before the supernatant was collected and resuspended in 10% FBS α-MEM media (Gibco, 11900-024) with penicillin/streptomycin, and maintained in 75 cm^2 flasks.

2.1.4 Colony Forming Units (CFU-f)

Studies on mesenchymal stem cells (MSCs) have used CFU-fs to determine highly proliferative pluripotent mesenchymal cells which exhibit an uncommitted phenotype [365, 366]. This is an imperfect assay for defining MSCs, as these cells form adherent cultures which express high levels of CD90, CD73, CD105, are CD45 negative and can differentiate into bone, fat and cartilage *in vitro* [365, 367]. However, CFU-f assays were used as a simple and economical way to enrich for mesenchymal cells with osteoblastic potential.

High proliferative mesenchymal cells were characterised within each isolated HBDC population by CFU-f positive colonies. Passage 1 cells were plated in T25 flasks at a density of 200 cells per flask. Cells were left to duplicate and form colonies over 10 days with media replacements on days 3, 6, and 9. Flasks were then gently washed twice with ice-cold PBS and fixed in 100% MEOH (POCD, MA004, NSW, Australia) for 15 min. The flasks were allowed to air dry until MEOH had evaporated completely. Giemsa working solution was made up fresh from a mixture of 50 mL of water, 1.5 mL of 100% MEOH, and 2 mL of Giemsa stock consisting of 1 g Giemsa (Edward Gurr Ltd, 13900, London, UK), 66 mL of glycerol (Sigma, G5516), 66 mL of MEOH. Five millilitres of working Giemsa was added to each flask and was incubated at room temperature for 1 h. The flasks were washed twice with distilled water and left to air dry. Photographs were taken as required.

CFU-f positivity was quantified by counting colonies of greater than 100 cells with defined morphologies. Stromal cells were either rapidly proliferating or slowly proliferating populations (Figure 2-2).

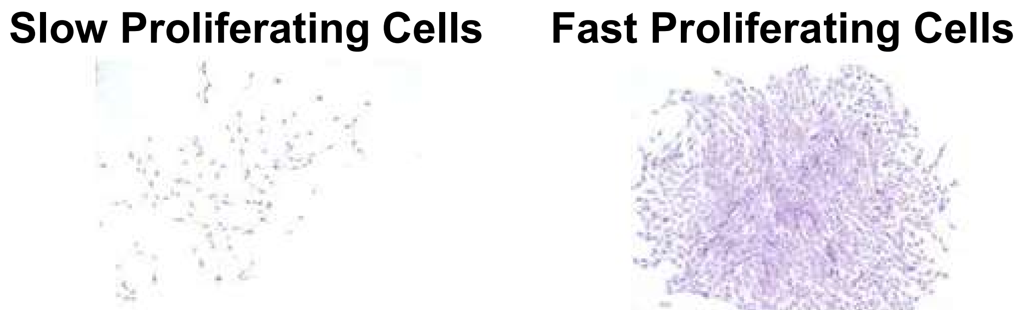

Figure 2-2 *CFU-f colonies. Slow proliferating mesenchymal stromal cells and fast proliferating stromal cells as stained by Giemsa staining.*

2.1.5 Alkaline Phosphatase Staining

To determine the osteoblast marker alkaline phosphatase, HBDCs were plated in 24-well plate at a density of $2.5x10^5$ cells per well in α-MEM, and left to equilibrate overnight. HBDCs were washed twice with ice-cold PBS and fixed in cold 10% neutral formalin buffer (10% PBS, 10% formalin in distilled water) for 15 min before rinsing and 15 min equilibration in distilled water. Naphthol stock (2×) solution was made from 0.01g naphthol AS MX-PO_4 (Sigma-Aldrich, N5000), 400 µL N,N-Dimethylformamide (Fluka, Sigma-Aldrich, 40255), 50 mL of 0.2M Tris-HCL pH 8.3, and aliquoted and frozen at -20°C for storage. Fresh naphthol substrate was filtered from 5 mL of stock solution and combined with 5 mL of distilled water and 6 mg of Red Violet LB salt (Sigma-Aldrich, F3381) then incubated in wells at room temperature for 45 min. Wells were rinsed thrice with distilled water to terminate the stain, then air dried, and photographed.

2.1.6 Immunocytochemistry

For immunocytochemistry of PC/APC and its receptors, cells were grown at a density of 10,000 per well in 8-well glass chamber slides. Media was discarded and cells were washed twice in PBS before fixation in 10% formalin for 30 min at room temperature as previously described [260, 368]. Cells were further washed thrice in PBS for 5 min/wash to remove formalin traces. Endogenous peroxidase activity was quenched by incubating slides for 15 min in 3% H_2O_2 in PBS. Cells were then washed in distilled water and PBS before permeabilisation. PBS containing 2% BSA and 0.05% Tween 20 was added for 1 h as a blocking agent. Cells were probed overnight with 2 µg/mL of anti-human antibodies including rabbit anti-PC and anti-PAR1 antibodies, goat anti-EPCR antibody, and mouse anti-PAR2 antibodies (Table 2-1).

Table 2-1 *Human (h) or mouse (m) antibodies used in immunostaining.*

Antibody	Isotype	Final Conc	Catalogue #	Company	Location
hPC/APC	Rabbit IgG	2 µg/mL	P4680	Sigma-Aldrich	St Louis, MO
mEPCR	Goat IgG	2 µg/mL	AF2749	R&D Systems	Minneapolis, MN
hEPCR	Goat IgG	2 µg/mL	AF2245	R&D Systems	Minneapolis, MN
h/mPAR1	Rabbit IgG	2 µg/mL	Sc-5605	Santa Cruz Biotechnology	Santa Cruz, CA
hPAR2	Mouse IgG	2 µg/mL	Sc-13504	Santa Cruz Biotechnology	Santa Cruz, CA
mPAR2	Goat IgG	2 µg/mL	Sc-8205	Santa Cruz Biotechnology	Santa Cruz, CA
Rabbit IgG	N/A	2 µg/mL	I-1000	Vector Labs	Burlingame, CA
Mouse IgG	N/A	2 µg/mL	MAB002	R&D Systems	Minneapolis, MN
Goat IgG	N/A	2 µg/mL	I5256	Sigma-Aldrich	St Louis, MO

After washing, cells were then stained using Dako LSAB+ (Dako, Glostrup, Denmark) system staining kit and visualised by 3,3'-Diaminobenzidine (DAB, Dako) following the manufacturer's instructions and counterstained with haematoxylin. IHC staining was optimised using serial dilutions of anti-PC, anti-PAR1, anti-EPCR, anti-PAR2 antibodies, and their respective negative controls. This ensured minimal background staining. Staining for human subchondral tissue, isolated HBDCs, and cell lines was carried out in a single batch.

2.1.7 Alizarin Red Staining

To determine the effect of treatments on matrix mineralisation, osteogenic media, i.e. α-MEM containing 10% FCS, 400 µM L-ascorbic acid (BDH, VWR, 44006, Radnor, PA, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich, G9891) was added to HBDC cultures. Fresh osteogenic culture media containing treatment reagents were replenished every two days over the course of 28 days. Supernatants were then discarded and cell monolayers were washed thrice in Ca^{2+}-free ice-cold PBS to remove free Ca^{2+} before fixation in 10% neutral formalin buffer (10% formalin in 10% PBS) for 15 min. The monolayers were equilibrated in distilled water before staining with 1% Alizarin Red S solution (A5533, Sigma-Aldrich) at pH 4.2 for 15 min. Finally, the stain was washed with distilled water. Plates were scanned (LiDE110, Canon, Tokyo, Japan) and processed in Microsoft Picture Manager (Microsoft, Redmond, WA, USA).

2.1.8 Viability Assays

APC's potential to affect viability was determined by 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl Tetrazolium Bromide (MTT, Table 2-2) cell viability assay and trypan blue dye exclusion. For MTT assays, cells were plated at a density of 5000 cells per well on 96-well plates and left to adhere for 4 h. Cells were switched to DMEM with 2% FCS overnight and

treated with rhAPC, EPCR blocking antibody RCR-252, EPCR non-blocking antibody RCR-92, PAR1 antagonists SCH7979, PAR2 antagonist ENMD-1068, thrombin and dimethyl sulfoxide (DMSO) vehicle control over 48 h (Table 2-2). MTT solution (10 µL) was added to each well to achieve a final concentration of 0.1 mg/mL and incubated for 4 h to allow for the conversion of MTT into formazan by live mitochondrial dehydrogenases. Supernatant media was drawn off at the end of an experiment and 100 µL DMSO was used to solubilise the formazan crystals. The number of viable cells was evaluated on a plate reader (BioRad, Hercules, CA, USA) at 570 nm and a background of 630 nm.

Table 2-2 *Reagents for cell culture. Rh = recombinant human, U = units.*

Reagent	Final Concentration	Catalogue #	Company	Location
MTT	0.1 mg/mL	M5655	Sigma-Aldrich	St Louis, MO
DMSO	2.5-10 µM	2225	Ajax Finechem	NSW, Australia
rhAPC (Xigris)	0-10 µg/mL	N/A	Eli Lilly	Indianapolis, IN
RCR-92	10 µg/mL	Gifts from Professor Fukudome, Department of Immunology, Saga Medical School, Nabeshima, Saga, Japan		
RCR-252	10 µg/mL			
PAR1 At	2.5-10 µM	SCH79797	Axon MedChem	Groningen, The Netherlands
PAR1 At	10 µM	ATAP-2	Santa Cruz Biotechnology	Santa Cruz, CA
PAR2 At	10 µM	ENMD-1068	Enzo Lifesciences	Farmingdale, NY
PAR1 Ag	10 µM	SP3108b	Abgent	San Diego, CA
PAR2 Ag	10 µM	530109	Calbiochem,	Billerica, MA
Thrombin	0.1 U/mL	T6884	Sigma-Aldrich	St Louis, MO

Cells counts were also performed by trypan blue exclusion assay as previously described [282]. Cells were cultured at 15,000 per well and left to adhere on a 24-well plate, then serum-reduced to 2%, and treated similarly to cells as described above for the MTT assay, over 48 h. Culture supernatants were then discarded and cells washed twice with PBS to remove media remnants, and then trypsinised with 0.025 trypsin. The enzyme was deactivated by adding fresh media with 10% FCS in a 1:2 ratio. Cells were then diluted 1:1 in trypan blue exclusion dye to differentiate live and dead cells. Live cells were counted on a haemocytometer with four replicates per well.

Cellular viability was also confirmed by MUSE® cell count and viability kit (Merck Millipore, Germany) according to the manufacturer's instructions. Briefly, cells were resuspended in MUSE count and viability reagent at a concentration of 1×10^5 cells/mL, Samples were analysed on a MUSE® Cell Analyzer. All samples were assayed in triplicate and the results are n=6.

2.1.9 PCR

Qualitative PCR was carried out on 1 µg of mRNA extracted from cells to determine the gene expression of *EPCR, PAR1,* and *PAR2* controlled against *β-actin*. Cells were cultured to confluence on 24-well plates and supernatants were discarded. Monolayers were washed twice to remove contaminating phenols and then cells extracted for RNA using RNAzol (RN 190, Molecular Research Inc, Cincinnati, OH, USA) according to the manufacturer's instructions. First strand cDNA was synthesised using Bioline cDNA synthesis kit according to the manufacturer's instructions (BIO-65026, Bioline, London, UK). cDNA was amplified in the presence of primer sequences (Table 2-3) and Immomix (Bioline), with a program cycle of 94°C for 4 min followed by 40 cycles of 94°C for 20 sec, 55-56°C for 30 sec, and 72°C for 35 sec finished with 1 cycle of 72°C for 8 min. Amplicon products were visualised on a 1%

agarose gel and detected via fluorescence on an Image Quant LAS (29-006-05, GE Lifesciences, Cleveland, OH, USA). Primers were used in one reaction with a Primers sequences for *EPCR*, *PAR1,* and *PAR2* were validated by a single melt curve. Primers are outlined in Table 2-3 and described previously [293, 368].

Table 2-3 *Primer list for PCR.*

Molecule	Species Accession #	Sequence 5' to 3'	Temp (°C)	Product Size(bp)
β-actin	Homo sap. BC002409	F- CAG AGC CTC GCC TTT GCC GAT CC R- GGC CTC GTC GCC CAC ATA GGA	56	228
EPCR	Homo sap. NM006404	F- CTC CTT TCT TCT CCC ACA TCT GC R- ATC CCA AGT CTG ACA CAC CTG G	55	305
PAR1	Homo sap. NM0001992	F- GCC ATC GTT GTG TTC ATC CTG R-AGA CCC AAA CTG CCA ATC ACT G	55	150
PAR2	Homo sap. AY3336105	F- CCT GTG GGT CTT TCT TTT CCG R- TTT GCC TTC TTC CTG GAG TGC	56	279

2.1.10 ELISA Assays

To quantitate the levels of antigens in Table 2-4, sandwich ELISAs for IL-1β, IL-6, IL-17, and TNF-α were performed according to the manufacturer's instructions using cell supernatants. Cell lysates were separately pretreated with 50 μL of 1N HCl and 50 μL 1N NaOH for EPCR ELISA. Capture antibodies were coated on the microtitre plate surface to enable immobilisation of antigens. Excess capture antibody was aspirated and washed off in buffer (0.05% Tween 20 in PBS, pH7.2-7.4). Plates were blocked in reagent diluent (1% BSA in PBS, pH 47.2-7.4) as per manufacturer's instructions. Antigen samples were then added and incubated for 2 h at room temperature, before being aspirated and the plate washed again. Detection antibody diluted in reagent diluent was added for 2 h at room temperature. Plates were further aspirated/washed before conjugation to streptavidin-HRP for 20 min. Plates were further

aspirated/washed before substrate solution (1:1 mixture of Color Reagent A, H_2O_2 and Color Reagent B, tetramethylbenzidine; R&D Systems, DY999) was added to visualise the signal. Stop solution (2N H_2SO_4; R&D Systems, DY994) was added to terminate the experiments, and the optical density was measured at 450 nm on a background of 570 nm. Pierce™ BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to correct protein loading on ELISA assays.

Table 2-4 *ELISA kits.*

Antigen	**Assay Concentration Limit**	**Catalogue #**	**Company**	**Location**
EPCR	800 ng/mL	DY2245	R&D Systems	Minneapolis, MN
IL-17	1000 pg/mL	DY317	R&D Systems	Minneapolis, MN
IL-1β	250 pg/mL	DY201	R&D Systems	Minneapolis, MN
IL-6	600 pg/mL	DY206	R&D Systems	Minneapolis, MN
TNF-α	1000 pg/mL	DY210	R&D Systems	Minneapolis, MN

2.1.11 Western Blotting

To quantify the expression of intracellular protein levels, whole cell lysates were extracted with lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 with phosphatase and protease inhibitors) and cleared by centrifugation. Lysates were then denatured at 90°C in SDS-polyacrylamide gel electrophoresis loading dye (75 mM Tris-HCl pH 6.8, 15% Glycerol, 1% SDS, 0.5 M β-mercaptoethanol, 0.1% Bromophenol blue) and separated on 10% SDS-PAGE before electrotransfer onto a PVDF membrane as previously described [248]. Novex Sharp (Invitrogen) pre-stained standards were loaded alongside samples for determining the relative molecular weights of the protein bands.

Membranes were subsequently blocked with 5% skim milk in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) and then incubated with primary antibodies listed in Table 2-5. After washing, membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at RT. After 3 washes with TBST, immunoreactivity was detected using the ECL detection system (Amersham Biosciences, Buckinghamshire, UK).

Table 2-5 *Primary antibodies for western blotting.*

Antibody	Isotype	Dilution	Catalogue #	Company	Location
p21	Rabbit IgG	1:1000	2947S	Cell signalling	Danvers, MA
p27	Rabbit IgG	1:1000	3686S	Cell signalling	Danvers, MA
pAkt	Rabbit IgG	1:1000	4060S	Cell signalling	Danvers, MA
Akt	Rabbit IgG	1:1000	9272S	Cell signalling	Danvers, MA
NF-kB	Mouse IgG	1:1000	MAB3026	Millipore	Billerica, MA
p38	Rabbit IgG	1:1000	Sc-535	Santa Cruz Biotechnology	Dallas, TX
p-p38	Rabbit IgG	1:1000	Sc-17852	Santa Cruz Biotechnology	Dallas, TX
ERK1/2	Rabbit IgG	1:1000	9102S	Cell signalling	Danvers, MA
pERK1/2	Rabbit IgG	1:1000	9101L	Cell signalling	Danvers, MA
β-actin	Mouse IgG	1:10 000	A5441	Sigma-Aldrich	St Louis, MO

Protein levels were normalised against β-actin and visualised on ImageQuant LAS 4000 (GE Healthcare Life Sciences, Buckinghamshire, UK) and semi-quantified using Multi Gauge software (FujiFilm, Tokyo, Japan). Graphs were compiled from 4 duplicate blots and changes were expressed as a percentage of control protein levels.

2.1.12 Zymography

To determine the regulation of MMP-2 and -9 in cells, gelatin substrate (1 mg/mL) was added to the standard acrylamide polymerisation preparation (as per western blotting protocol) to enable cleavage of gelatin by MMP-2 and -9. Culture medium supernatants were then collected, mixed with sample buffer (25 mM Tris pH 6.8, 2.5% SDS, 25% glycerol, 0.625% bromophenol blue) and separated on 10% SDS-PAGE under non-reducing conditions. A positive control consisting of MMP-2 and MMP-9 proteins were loaded alongside samples for determination of relative product masses. Sample proteins were normalised by BCA assay (Life Technologies) prior to loading.

After electrophoresis, gels were renatured in 0.25% Triton X-100 (BDH, 30632) with gentle agitation for 1 h at room temperature in developing buffer (50 mM Tris-B, 200 mM NaCl, 5 mM $CaCl_2.2H_2O$) overnight at 37°C. The gels were stained for 1 h in staining solution (0.2% Coomassie Blue R-250, 50% EtOH, 10% acetic acid, 40% distilled water) and destained for 1 h in 20% MEOH, 10% acetic acid, 70% distilled water before a final wash in tap water. Visualisation and semi-quantification were carried out as per western blotting protocol.

2.2 Studies of OA and RA Tissue

Tissues were collected and processed with patient written informed consent and approval from the Northern Sydney Local Health District Human Research Ethics Committee.

2.2.1 Histology of OA and RA Tissue

A total of 4 OA and 4 RA condyle samples were collected within 24 h of knee replacement surgery and fixed in 10% neutral formalin buffer for 24 h. Samples were then briefly rinsed in distilled water to remove remnant formalin before decalcification in 5% formalin and 10%

formic acid solution. Decalcification solution was changed daily between days 1-6 and remained unchanged for days 7-8. At the completion of decalcification, samples were placed into 70% EtOH for storage. Cross sections from femoral condyles of re-sectioned joints were extracted, rinsed briefly in distilled water, and paraffin embedded into cassettes by Sue Smith (Raymond Purves Laboratory, Kolling Institute) using a Shandon Excelsior ES (Thermo Fisher Scientific) and Leica EG 1150 H (Leica). The embedded tissues were then sectioned by Agnes Chan (Sutton Laboratory, Kolling Institute) or the author at 4.5 µM using a rotating microtome and then placed onto Superfrost Plus slides (HD Scientific Supplies Pty Ltd, Wetherill Park, NSW, Australia), and heated at 80°C to dewax for 1 h and 50°C overnight to adhere the tissue to the slides. The slides were then processed into H&E, Toluidine Blue, or immunohistochemistry. All samples fulfilled ethical criteria as set out in Section 2.2.1.

2.2.2 Haematoxylin and Eosin (H&E) Staining of OA and RA Tissue

Tissue sections of OA and RA subchondral bone were taken through two changes of 100% xylene, 3 min each to dewax the sections. Sections were then taken through decreasing gradient of EtOH from 100%, 100%, 95%, and 70% for 1 min each to remove xylene. Slides were washed under tap water for 1 min before they were stained with Mayer's haematoxylin for 5 min. Slides were then washed through tap water until excess haematoxylin was removed and sections were then dipped into Scott's blueing solution for 1 min before the excess solution was removed under running tap water. Slides were checked by microscopy to ensure the correct ratio of staining, then placed into eosin solution for 5 min, and washed briefly in running tap water to remove excess. The slides were dehydrated in 4 changes of 100% EtOH before they were cleared in 4 changes of xylene, then mounted in a non-aqueous resinous mountant, Euckitt and left to dry. Slides were scanned on ScanScope (Aperio, Vista, CA, USA) and processed on ImageScope software (Aperio).

2.2.3 Toluidine Staining of OA and RA Tissue

OA and RA subchondral bone were sectioned and stained with toluidine blue to assess cartilage degradation. Tissue sections were taken through 4 changes of xylene, each wash was 3 min, to deparaffinise the samples. Slides were then taken through decreasing gradients of EtOH from 100%, 100% to 95% for 1 min each to remove xylene and then placed into 70% EtOH for 15 min. Without rinsing, the sections were placed in toluidine blue O staining (C.I.52040) in 0.1M sodium acetate buffer pH 4.0 for 10 min. Slides were rinsed thoroughly in tap water, stained with 0.1% fast green FCF (C.I.42053) for 2 min and rinsed in running tap water. To prevent dilution of toluidine blue stain, sections were dehydrated in 3 changes of 100% propanol then cleared, mounted and scanned as per Section 2.2.2.

2.2.4 PC/APC, EPCR, PAR1, and PAR2 Stain of OA and RA Tissues

To stain for PC, EPCR, PAR1, and PAR2, tissue sections were deparaffinised, taken to EtOH, and rinsed in running tap water for 3 min before incubation in 3% H_2O_2 to quench endogenous peroxidase activity for 5 min. Slides were further rinsed in running tap water for 5 min before assembly into Sequenza^{TM} trays and coverslips (Thermo Fisher Scientific, 73310015 & 721100117). Slides were then washed in TBST (0.05M Tris, 0.15M NaCl, 0.05% Tween 20, pH 7.6), treated with serum-free protein block (Dako, X0909) for 10 min at RT, and then incubated overnight at 4°C with primary antibodies and a negative control. Reagents are listed in Table 2-1.

On the following day, slides were washed in TBST for 6 min at room temperature before incubation with biotinylated secondary antibody (LSAB+, Dako, K0690) for 30 min, followed by a TBST wash, incubation with streptavidin/HRP substrate for 30 min, and a final TBST wash. Slides were disassembled from the Sequenza^{TM} and placed in a humid chamber before

DAB+ (Dako, K3468) was added for 10 min to all sections. Slides were then placed into racks and washed in running tap water for 5 min to stop DAB reaction. Tissues were counterstained with haematoxylin for 2 min then dehydrated, cleared, mounted and scanned as per Section 2.2.2.

2.3 *In Vivo* Studies

2.3.1 PAR KO Mice Housing and Genotyping

To elucidate the *in vivo* involvement of rhAPC in WT, *Par1*$^{-/-}$ and *Par2*$^{-/-}$ mice, female 8-9 week old mice with homozygous deletion for *Par1* or *Par2* on a C57BL/6J background were bred at the Kearns Animal Facility at the Royal North Shore Hospital under ethics approval from Royal North Shore Hospital Animal Care and Ethics Committee (Protocol 1305-009A). PAR KO mice were age- and sex-matched with C57BL/6J wild types (WT). All mice were housed in groups of 5 with standard water and feed.

Par1$^{-/-}$ or *Par2*$^{-/-}$ mice were genotyped using tail tips taken from the offspring of knockout mice. Tails were digested and DNA extracted using PureLink^{TM} Genomic DNA kit (Invitrogen) according to the manufacturer's instructions. Mouse tail tips were digested in Purelink Genomic Digestion Buffer and proteinase K at 55°C overnight. Lysates were then centrifuged at maximum speed for 3 min to remove any particulates and transferred to new sterile microcentrifuge tubes. RNase A was added for 2 min to digest remnant RNA. PureLink^{TM} Genomic Lysis Buffer was then added with 100% ethanol and mixed by vortexing. The mixture was centrifuged at 10,000 g for 1 min on the PureLink^{TM} Spin Column. The collection tube was discarded, and the spin column was subsequently washed with Wash Buffer 1 and Wash Buffer 2 before the sample was eluted using the PureLink^{TM}, and then analysed for DNA quantity on NanoDrop (Thermo Fisher Scientific).

DNA was amplified by semi-quantitative reverse transcriptase (RT)-PCR using primers provided by Dr Miriam Jackson (Table 2-6). All four *Par1*$^{-/-}$ (Neo) and Internal standard (Instd) primers were used in one reaction with a program cycle of 94°C for 4 min followed by 12 cycles of 94°C for 20 sec, 60-64°C for 30 sec, and 72°C for 35 sec followed by 40 cycles of 94°C for 20 sec, 58°C for 30 sec, and 72°C for 35 sec, finished with 1 cycle of 72°C for 8 min. Wild-type (WT) and *Par1* primers were separately used in a program cycle with an annealing temperature of 60°C. All three *Par2* primers were added to one reaction with a program of 1 cycle at 94°C for 4 min followed by 40 cycles of 94°C for 30 sec, 68°C for 45 sec, and 72°C for 1 min, finished with 1 cycle of 72°C for 8 min. Amplicon products were visualised on a 2% (w/v) agarose gel stained with Gel Red and assessed on a LAS fluorescent imaging machine (GE Healthcare).

For subsequent *in vivo* studies, animals were transferred to Transgenic Animal Facility at Westmead hospital under animal ethics approval from Animal Care and Ethics Committee for the Children's Medical Research Institute and The Children's Hospital Westmead (CHW Animal Ethics, Protocol K294).

Table 2-6 *Primers for Par1*$^{-/-}$ *or Par2*$^{-/-}$ *mice genotyping, contributed by Dr Miriam Jackson.*

Primer	Species Accession Number	Sequence 5' to 3'	Temp (°C)	Product Size (bp)
Par1	Mus musc 20	Wt F- GAT TGT GTT CAT TGT CAG CCT TCC Wt R- ACG TGT AGC AGA CCG TGG AAA C Neo13 F- CTT GGG TGG AGA GGC TAT TC Neo14 R- AGG TGA GAT GAC AGG AGA TC IntS1 F- CAA ATG TTG CTT GTC TGG TG IntS2 R- GTC AGT CGA GTG CAC AGT TT	60 64 58 64 58	WT- 545 KO- 300 IntS-200
Par2	Mus musc. Monash	Par2 F- TAT CCG ACT CAT CAT CAC CGT GCT G WT R- AGC TGC ATG CTT GTG ATT GGT GCA G KO R- TGA GAC GTG CTA CTT CCA TTT GTC AC	68	WT- 500 KO- 350

2.3.2 Ectopic Bone Formation

To screen for the capacity of rhAPC to augment bone formation in combination with rhBMP-2, female WT type C57BL/6J mice (8 weeks) were assigned to groups of 5 with bilateral treatments of: 10 µg rhBMP-2 (Medtronic, Minneapolis, MN, USA); 10 µg rhBMP-2 + 10 µg rhAPC; or 10 µg rhBMP-2 + 25 µg rhAPC (Table 2-7). Anaesthesia was induced in mice by isoflurane gas inhalation, then an incision was made over the anterolateral aspect of each mouse femur. An absorbable collagen sponge (ACS, Medtronic) was cut to even sizes using a 3 mm punch biopsy. Recombinant protein treatments were dissolved in 10 µL of water for injection and added onto the scaffold. ACS were then implanted surgically into an intramuscular pocket, the wound was closed with suture (Vicryl, 5-0, Ethicon, Somerville, NJ, USA). All mice were given saline and 0.05 mg/mL of analgesic (Temgesic) post-operatively. Mice were monitored 2 and 24 h post-surgery and then twice a week. Likewise, female *Par1*$^{-/-}$ or *Par2*$^{-/-}$ mice on a C57BL/6J background (8-9 weeks) were also implanted with bilateral collagen scaffold were infused with 10 µg rhBMP-2 (Medtronic) with or without 25 µg rhAPC.

Ectopic bone formation experiments were carried out under approval from CHW Animal Ethics, Protocol K294. WT C57/B6 mice were sourced from the Animal Resources Centre (Perth).

Table 2-7 *Ectopic bone formation mouse groups. Rh stands for recombinant human.*

Strain	Sex	Age	Treatment	#mice	Procedure
C57BL/6J	F	8 weeks	rhBMP-2	5	Bilateral ectopic bone formation
C57BL/6J	F	8 weeks	rhBMP-2 + rhAPC 10 µg	5	Bilateral ectopic bone formation
C57BL/6J	F	8 weeks	rhBMP-2 + rhAPC 25 µg	5	Bilateral ectopic bone formation

Radiological Imaging & μCT

Ectopic bone formation was monitored radiographically using X-ray (Faxitron X-ray Corp, Wheeling, IL, USA) at 2 and 3 weeks. Nodules were analysed *ex vivo* using microCT (μCT) and histology. Bone nodules formed were dissected from mice 3 weeks after implantation (n=10 per treatment), fixed in 4% paraformaldehyde (PFA), and radiographed using X-ray (Faxitron). Nodules were then preserved in 70% ethanol for μCT analysis. Bone volume, tissue volume, and trabecular structure were measured using a Skyscan 1174 μCT machine as previously described [369]. Densities were calibrated to a global threshold of 0.3 g/mm^3 for bone tissue. Three-dimensional volumetric analysis and modelling were carried out on nodules through CTAn Software (Bruker, Billerica, MA, USA). Nodules midsections were reconstructed using 20 slices of scanned images to illustrate trabecular complexity.

2.3.3 Murine Closed Fracture Model

To investigate the effect of rhAPC on fracture healing, a closed murine fracture model was employed. For surgery, anaesthesia was induced with ketamine (35 mg/kg) and xylazine (5 mg/kg) via intraperitoneal injection, and maintained with isoflurane inhalation. Surgery was performed in female 8 week old C57BL/6J mice. A small incision was made slightly distal to the knee and an intramedullary rod (0.3 mm-diameter stainless steel insect pin) was surgically inserted into the medullary canal of the tibia, followed by the insertion of a second stabilisation pin. Closed mid-tibial fractures were then induced manually by three-point bending with a set of modified surgical staple removers. Incision site was then closed by sutures. Fractures were allowed to heal over 21 days as described previously [369]. Intramedullary pins impacted on ambulation. Injections were made from anterolateral and posteromedial axis into the site of injury, as guided by intact tibial bone surrounding the injury. Treatments were: 30 μL of saline (n=10); biweekly injection of rhAPC 25 μg in 30 μL of water for injection (n=10); or a single

bolus of rhAPC 50 µg in 30 µL of water for injection immediately post-surgery (n=10) (Table 2-8).

Fracture healing endpoints include day 3, day 10, and day 21. Fractures were radiographed by XR at 3 days, 7 days, 10 days, 14 days, and 21 days. Calluses at day 21 were analysed using µCT. After 3 weeks, tibiae and fibula were dissected from culled mice, fixed in 10% formalin and transported in 70% ethanol, similarly to bone pellets. A region of interest, including the callus but excluding the original cortical tibial bone, was applied to the fracture calluses. Then, bone volume, tissue volume, and trabecular structure were measured similarly to bone nodules. Densities were calibrated to a global threshold of 0.3 g/mm^3 for bone tissue. Three-dimensional volumetric analysis and modelling were carried out on nodules through CTAn Software (Bruker, Billerica, MA, USA). Fracture studies were carried out under approval from CHW Animal Ethics, Protocol K248. Exclusion criteria include infection of fractures or the slippage of intramedullary pins, which were both monitored by physically examining the mice and through regular XR.

Table 2-8 *Treatment groups in murine closed fracture model.*

Strain	Sex	Age	Treatment	# Mice	Endpoint
C57BL/6J	F	10 weeks	Saline	3	3 days
C57BL/6J	F	10 weeks	rhAPC 50 µg bolus	3	3 days
C57BL/6J	F	10 weeks	rhAPC 25 µg biweekly	3	3 days
C57BL/6J	F	10 weeks	Saline	3	10 days
C57BL/6J	F	10 weeks	rhAPC 50 µg bolus	3	10 days
C57BL/6J	F	10 weeks	rhAPC 25 µg biweekly	3	10 days
C57BL/6J	F	10 weeks	Saline	10	21 days
C57BL/6J	F	10 weeks	rhAPC 50 µg bolus	10	21 days
C57BL/6J	F	10 weeks	rhAPC 25 µg biweekly	10	21 days

2.3.4 Histology of Bone Nodules

Bone Nodules

Processing of bone nodules for histology was first carried out by fixation in 4% PFA overnight and then at room temperature for 4 h. Nodules were then stored in 70% alcohol for 24 h before incubation in 30% sucrose overnight in the cold room and then at room temperature for 4 h to enable cryoprocessing. Nodules were cut in half using a Struers minitom (Struers, Westlake, OH, USA) and then cryoembedded with cut side face down in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA USA). Cryosections were cut to 5 µM by cryostat (Leica, CM1900) onto cryofilm (Section Lab-Co Ltd, Tokyo, Japan). Sections were then adhered to glass slides using chitosan glue. Sections were stained for TRAP expression to evaluate osteoclast quantity and receptors EPCR, PAR1, and PAR2 to determine the receptor involvement in the bone formation process.

TRAP Staining

To determine osteoclast numbers, TRAP staining was conducted on nodules. Cryosections were rehydrated in PBS for 15 min at room temperature and then further incubated in 1 M Tris-HCl buffer (pH 9.4) for 5 min at room temperature before transfer into 1 M Na-Acetate buffer (pH 5.0 with 1% tartaric acid) for 10 min at room temperature. The tissues were then incubated for 4 min at 37°C in filtered TRAP staining solution (35 mg of tartaric acid + 40 mg of naphthol ASBI phosphate in 2 mL of dimethylformamide + 100 µL of new fuchsine and 100 µL of sodium nitrite). Cryosections were then counterstained with haematoxylin, dipped in lithium chloride and coverslipped in Aquatex mounting solution (Merck Millipore, Billerica, MA, USA). Osteoclast numbers were counted using Bioquant Software (BioQuant, University of Heidelberg, Heidelberg, Germany).

2.3.5 Immunohistochemistry (IHC)

Staining for EPCR, PAR1, and PAR2 was conducted using antibodies from Table 2-1. Cryosections were rehydrated in PBS for 2×10 min and permeabilised in buffer (PBS + 0.1% Triton X-100) for 20 min. Sections were then washed thrice in wash buffer (PBS + 0.05% Tween 20), and then blocked (PBS + 10% goat serum + 1% BSA + 0.05% Tween 20) for 1 h, before incubation overnight with primary goat anti-mouse EPCR antibody, rabbit anti-mouse PAR1, or goat anti-mouse PAR2 antibody in dilution buffer (PBS + 10% goat serum + 1% BSA + 0.1% Triton X-100). The slides were washed thrice in washing buffer the next day and endogenous peroxidase activity was quenched using 3% H_2O_2 in PBS. The slides were further equilibrated in wash buffer before incubation with secondary HRP goat antibody (E0449, Dako) or HRP rabbit antibody (BD Pharmingen, 554021) for 2 h. Three further washes were conducted before incubation with streptavidin-horseradish peroxidase (HRP) for 30 min. Labelling was visualised using DAB staining according to the manufacturer's instructions, and slides were counterstained and coverslipped as per the TRAP staining protocol. Slides were scanned as per Section 2.2.2.

2.4 Statistics

Statistical analyses and data graphing were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). All duplicated values were presented as mean ± standard error (SE). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by a non-parametric Student-Newman-Keuls post-hoc comparison between groups to identify significantly different means between treatment groups. *P* values less than 0.05 were considered statistically significant. Non-parametric tests have been used to assess ectopic bone formation and fracture union as these parameters do not follow a normal distribution. Fracture unions were statistically analysed using Fisher's exact test.

CHAPTER 3:

APC ENHANCES

MG-63 AND MC3T3-E1 VIABILITY

3 APC Enhances MG-63 and MC3T3-E1 Viability

3.1 Introduction

Bone formation is a tightly regulated anabolic process that begins with the commitment of osteoprogenitor cells, and then their subsequent differentiation into pre-osteoblasts. These cells will then further mature in the presence of local and systemic osteogenic factors [1, 8, 9, 29, 258, 370]. Actively proliferating osteoblasts progress into mature osteoblasts, increasing extracellular matrix maturation, and mineralisation of the matrix through calcium deposition [11, 26, 83]. Osteoblasts are integral to both the normal and pathological processes that occur in bone [195].

APC is a physiological anticoagulant that exerts a broad range of cytoprotective activities, including stimulation of cell proliferation, suppression of apoptosis, inhibition of inflammation, and protection of cells from damage [247, 342]. APC exerts profound effects upon the mitosis and differentiation of endothelial cells, vascular smooth muscle cells, keratinocytes, tenocytes, and neural stem/progenitor cells [258, 259, 282, 343, 371]. In endothelial and smooth muscle cells, APC's effects are mediated at least partly by stimulation of ERK1/2 activity [259, 371]. In conjunction with its stimulatory effects on cellular proliferation, APC also prevents apoptosis in endothelial and neuronal cells [258, 262, 372]. The effects in the latter are mediated through an Akt-dependent pathway [258]. These *in vitro* effects translate into *in vivo* protection by APC against various diseases. For example, the protective effect of APC on neuronal and endothelial cells is also apparent in neurotoxicity, traumatic brain injury, and stroke [264, 371, 373, 374]. Some of these effects are mediated through APC-induced suppression of NF-κB signalling and reduction of inflammation [375]. In mouse models of diabetic nephropathy and ulcerative colitis, APC also rescues endothelial cells and podocytes from injury [261, 282, 373, 376]. Both human and animal studies have

shown APC promotes wound healing through its effects upon both endothelial cells and keratinocytes, and this is at least partly the consequence of inhibiting the p38 signalling cascade [292].

Canonical APC signalling requires PC binding to EPCR and subsequent activation by thrombin cleavage [327, 328, 353]. Once activated, EPCR bound APC cleaves either PAR1 or PAR2, and stimulates intracellular G_i protein signalling through the aforementioned effectors, ERK1/2, Akt, p38, and NF-κB, to exert cytoprotective effects [247, 248, 292, 377]. APC signals through the PAR1 receptor in many cell types, including, neurons, podocytes, leukocytes, keratinocytes, cardiomyocytes and endothelial cells [260, 261, 264, 299, 342, 353, 372, 378, 379]. There is much less experimental evidence with regard to the actions of APC through PAR2, though it has been observed in both keratinocytes and endothelial cells [292, 353]. In contrast to APC, thrombin-mediated cleavage of PAR1 on endothelial cells activates either G_q or $G_{12/13}$ to exert inflammatory effects [327, 328, 339]. However, in osteoblasts, thrombin is known to signal via PAR1 to induce cell proliferation, and the release of both cytokines and growth factors; or independently of PAR1, to exert anti-apoptotic effects [24, 337, 380-387].

Following the commencement of this study, Kurata and colleagues demonstrated that APC can stimulate the proliferation of normal human osteoblasts [268]. Here we have been able to confirm this observation, and also provide additional insight into APC-mediated signalling and regulatory mechanisms within osteoblasts. In this chapter, we hypothesised that APC would act through EPCR and PARs to promote viability of both the MG-63 and MC3T3-E1 osteoblastic cell lines, as these receptors have been shown to be required for many of APC's known cytoprotective actions [260, 261, 264, 299, 342, 353, 372, 378, 379]. We further

hypothesised that APC exerts its effects through regulation of ERK1/2, Akt, p38, and NF-κB signalling activity, as these pathways have been shown to be regulated by APC in numerous other cell types [247, 248, 292, 377], and it is likely that the same would be true for the osteoblast.

3.2 Aims

The overall aim of this chapter was to examine the role of APC on osteoblast viability. Specifically, we aimed to:

1. examine the effects of APC and thrombin on osteoblast-like MG-63 and MC3T3-E1 cell viability;
2. determine whether or not APC receptors, including EPCR and PARs, were present on osteoblasts and implicated in APC's effects; and
3. elucidate APC signalling mediators, particularly ERK1/2, Akt, p38, and NF-κB activity.

3.3 Methods

3.3.1 MG-63 and MC3T3-E1 Culture

Human MG-63 osteoblast-like cells and murine pre-osteoblast MC3T3-E1 cells were cultured in DMEM and αMEM respectively, both supplemented with 10% FCS as per Sections 2.1.1 and 2.1.2 and maintained in T75 culture flasks until passage for experiments.

3.3.2 APC Receptor Expression in MG-63 Cells

EPCR, PAR1, and PAR2 receptor gene and protein expression in MG-63 cells were determined by RT-PCR and immunocytochemistry respectively. In brief, MG-63 were cultured in 24-well plates, the medium was aspirated and the cells were washed with pre-warmed sterile PBS. RNA

was then extracted from the cells using RNAzol (Molecular Research Inc.), according to the manufacturer's instructions. To determine the relative mRNA expression corresponding to the receptor primers listed in Table 2-3, RNA extraction was carried out. RNA was then reverse transcribed into cDNA, and then amplified by PCR, and the products resolved and visualised by agarose gel electrophoresis. Receptor gene levels were normalised to *β-actin* gene expression (Sections 2.1.9). PCR efficiencies were conducted for each set of receptor primers and confirmed to be between 90-100%.

For immunocytochemistry, sub-confluent MG-63 cells were cultured on 8-well chamber plates. Cell culture medium was aspirated, the cells were washed with PBS and then fixed in 10% formalin. Fixed cells were further washed with PBS, and then incubated overnight with EPCR, PAR1, and PAR2- specific primary antibodies as listed in Table 2-1. Respective secondary antibodies were conjugated to these MG-63 cells, before visualisation with DAB, and haematoxylin counterstain (Section 2.1.9).

3.3.3 APC and Receptor Involvement in Cell Lines

To determine the effect of APC and thrombin on MG-63 viability, the cells were grown as previously described, and MTT cell viability and trypan blue exclusion assays were performed at 24, 48, or 72 h time points. APC treatment of 0.1, 1, or 10 μg/mL was first assessed in MG-63 cells cultured in 10% FCS, and then in subsequent experiments, in 2% FCS-containing media. Thrombin, EPCR blocking antibody and non-blocking antibody, and PAR antagonists were used 30 min prior to APC addition. The effects of APC on PARs were confirmed in osteoblasts derived from *Par*$^{-/-}$ mice. PAR1 antagonist SCH79797 was originally reconstituted in DMSO, but was further diluted in PBS for experiments. SCH79797 was diluted to 0.1, 1, or 10 μM in and assessed against respective concentrations of vehicle DMSO to confirm the

independent effect of SCH79797 in MTT assays. MC3T3-E1 experiments were independently conducted by Dr Ciara Murphy using MUSE viability kit (Merck).

3.3.4 APC Treatment on Activation of Signalling Proteins ERK, Akt, NF-κB, & P38

Western blotting was performed to assess the effects of APC treatment upon ERK, Akt, p38, and NF-κB in MG-63 cells treated with APC for 0, 15, 30, or 60 min. Then APC's effect was assessed in the presence and absence of PAR1 and PAR2 antagonists at the optimised time of 60 min. Cell lysates were extracted with lysis buffer, separated by electrophoresis and electrotransferred onto a PVDF membrane as per Section 2.1.11. Detection of the proteins was conducted by incubation of the blot with the respective primary and secondary antibodies (Table 2-5) and then visualised by addition of ECL, which was detected on ImageQuant.

3.3.5 Statistics

Statistical analysis was conducted by one-way ANOVA and post-hoc Student-Newman-Keul as per Section 2.4. N is representative of the number of duplicates in each experiment.

3.4 Results

3.4.1 EPCR, PAR1, and PAR2 are Expressed in MG-63 Cells

To examine whether EPCR, PAR1, and PAR2 could facilitate APC's effect on MG-63 cells, we first examined the expression of these receptors. Immunocytochemistry staining and qualitative RT-PCR showed that EPCR, PAR1, and PAR2 were all expressed by MG-63 osteoblasts, at both the gene and protein levels (Figure 3-1). Although immunocytochemistry showed that cell cultures expressed all three receptors, staining of each receptor was heterogeneous on different MG-63 cells (Figure 3-1B).

3.4.2 APC Stimulates MG-63 Viability over 72 h

We first investigated the effect of APC on the viability of MG-63 cells grown in DMEM containing 10% FCS, but found there to be no effect following a 24 h treatment (Figure 3-2). We hypothesised that the presence of APC inhibitors in the serum may mask the effect from APC. Thus, in a subsequent experiment, we reduced the FCS concentration to 2% and then measured cell viability in response to APC treatment over a 72 h time period.

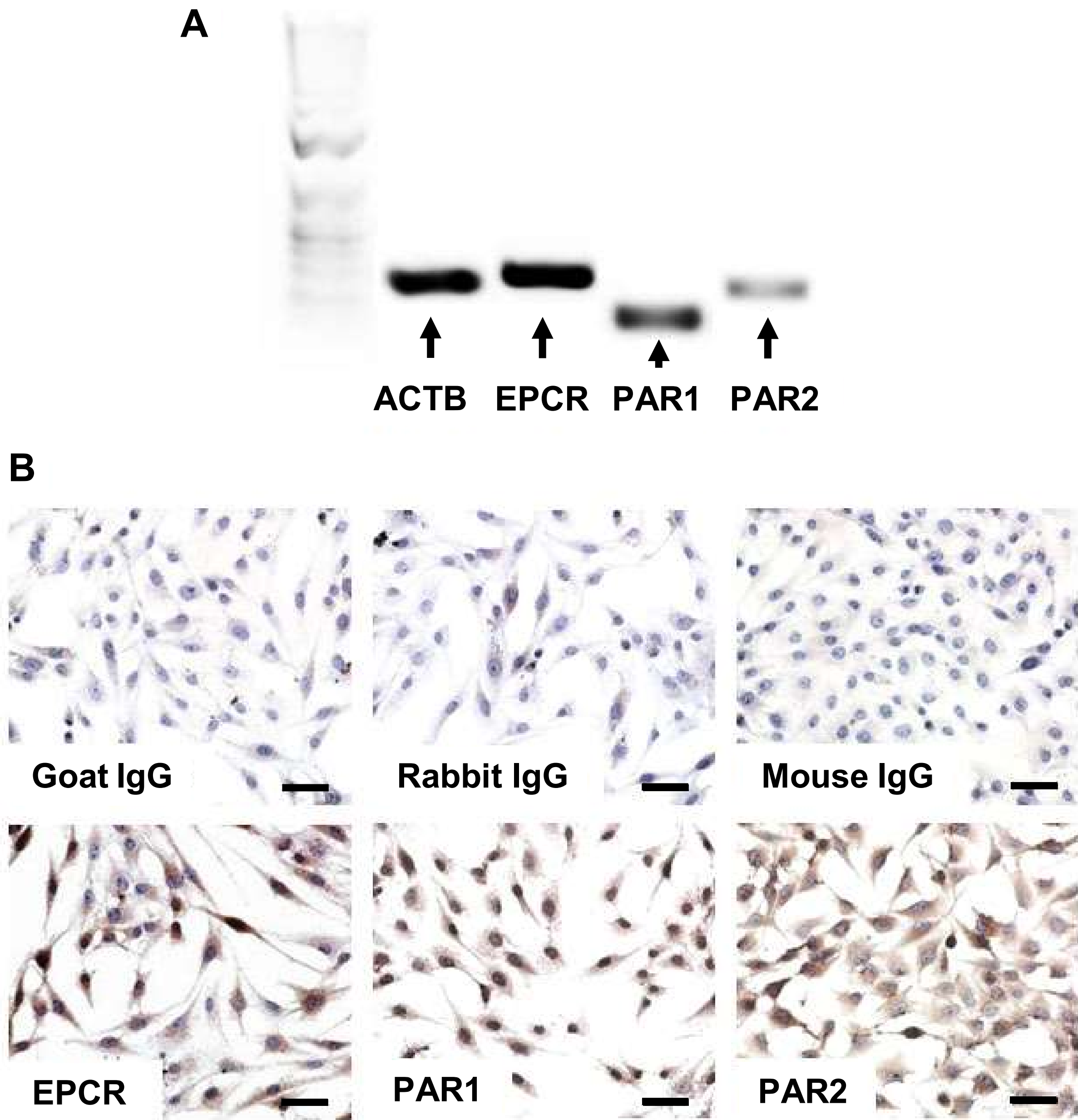

Figure 3-1 *EPCR, PAR1, and PAR2 expression in MG-63 cells. RT-PCR was carried out on mRNA derived from MG-63 cells to determine qualitative levels of β-actin (ACTB, control), EPCR, PAR1, and PAR2 (A). Immunocytochemistry was performed on EPCR, PAR1, and PAR2 on MG-63 cells (B) with respective negative control IgGs visualised above. Scale Bar = 50 µm.*

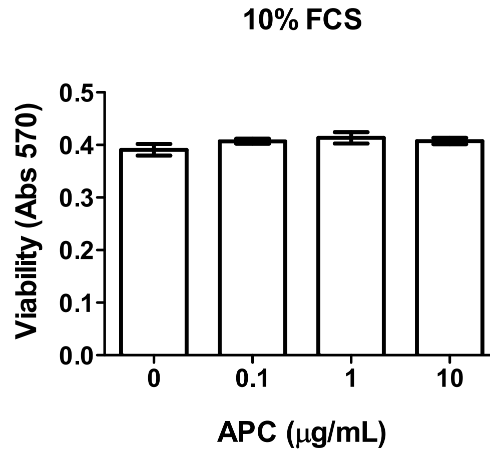

Figure 3-2 *The effect of APC treatment on MG-63 cells at 10% FCS. MG-63 cells were treated with 0.1, 1, or 10 μg/mL of APC over 24 h as assessed by MTT assay. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=4).*

Further treatment of MG-63 cells with APC at 10 μg/mL in serum-reduced media over 24 h showed a significant increase of 17% (P<0.05; Figure 3-3) in viability. However, treatment of MG-63 cells with APC at 0.1 or 1 μg/mL did not change viability over 24 h (6%, P=0.15 and 11%, P=0.054 respectively; Figure 3-3). At 48 h, there were subsequent increases of 15% or 12% at 1 or 10 μg/mL of APC treatment (P<0.05 for both; Figure 3-3). At 72 h, there was a continued increase in viability by 13% using 10 μg/mL of APC (P<0.05; Figure 3-3). Although the stimulation by APC was modest, the results were robust, with similar results found in further experiments (Figure 3-3, 3-5, 3-6). However, there was no significant difference between the 24, 48, or 72 h time points for any of the APC doses.

Cell counts with trypan blue exclusion dye yielded similar results to the MTT assays. In response to APC treatments at 0.1, 1, or 10 μg/mL, cell counts demonstrated no significant difference (3%, 17%, or 14% and P=0.67, P=0.06, P=0.07 respectively; Figure 3-3). Over 48

h and 72 h, an increase of 26% was seen from 10 µg/mL of APC treatment 48 h ($P<0.01$) and 25% at 72 h ($P<0.05$). There was no significant difference between APC treatments at 24 h but an increase of 16% or 14% was found between 0.1 vs. 1 µg/mL and 0.1 vs. 10 µg/mL of APC treatments at 48h ($P<0.05$ for both; Figure 3-3). Similarly, a difference of 24% was found between 0.1 vs 10 µg/mL of APC treatment at 72 h ($P<0.05$; Figure 3-3). These results suggest that the effect was partially dose-responsive.

Independent confirmation studies were carried out using the commercially available kit, MUSE® viability and cell count analyser, and this showed similar results. Murine MC3T3-E1 cells tolerated growth in low-serum media less well than human MG-63 cells, and thus were analysed at 48 h rather than 72 h. APC treatment of MG-63 cells by MUSE® showed significant increases at all treatment doses over 72 h (42%-56%, $P<0.05$ for all; Figure 3-4). APC treatment of cultured cells at 1 µg/mL led to significantly increased numbers of viable MC3T3-E1 cells (37%, $P<0.05$; Figure 3-4).

3.4.3 APC-Mediated MG-63 Viability is Unaffected by Thrombin

Thrombin signals through PAR1 in osteoblasts and is required for PC activation [245, 337, 380, 382-386]. Here, we investigated the effect of thrombin, both alone and in combination with APC, upon MG-63 viability by MTT assay. Treatment of cells with APC over 48 h demonstrated a similar increase to that shown in Section 3.4.2 (14% increase, $P<0.05$; Figure 3-5). Thrombin treatment alone at 1 U/mL was no different to control (8%, $P=0.09$; Figure 3-5). Combined treatment with thrombin and APC revealed no difference to APC alone (3%, $P=0.58$; Figure 3-5), but a significant increase when compared to control (10%, $P<0.05$; Figure 3-5). This suggested that APC, rather than thrombin, protected osteoblast viability.

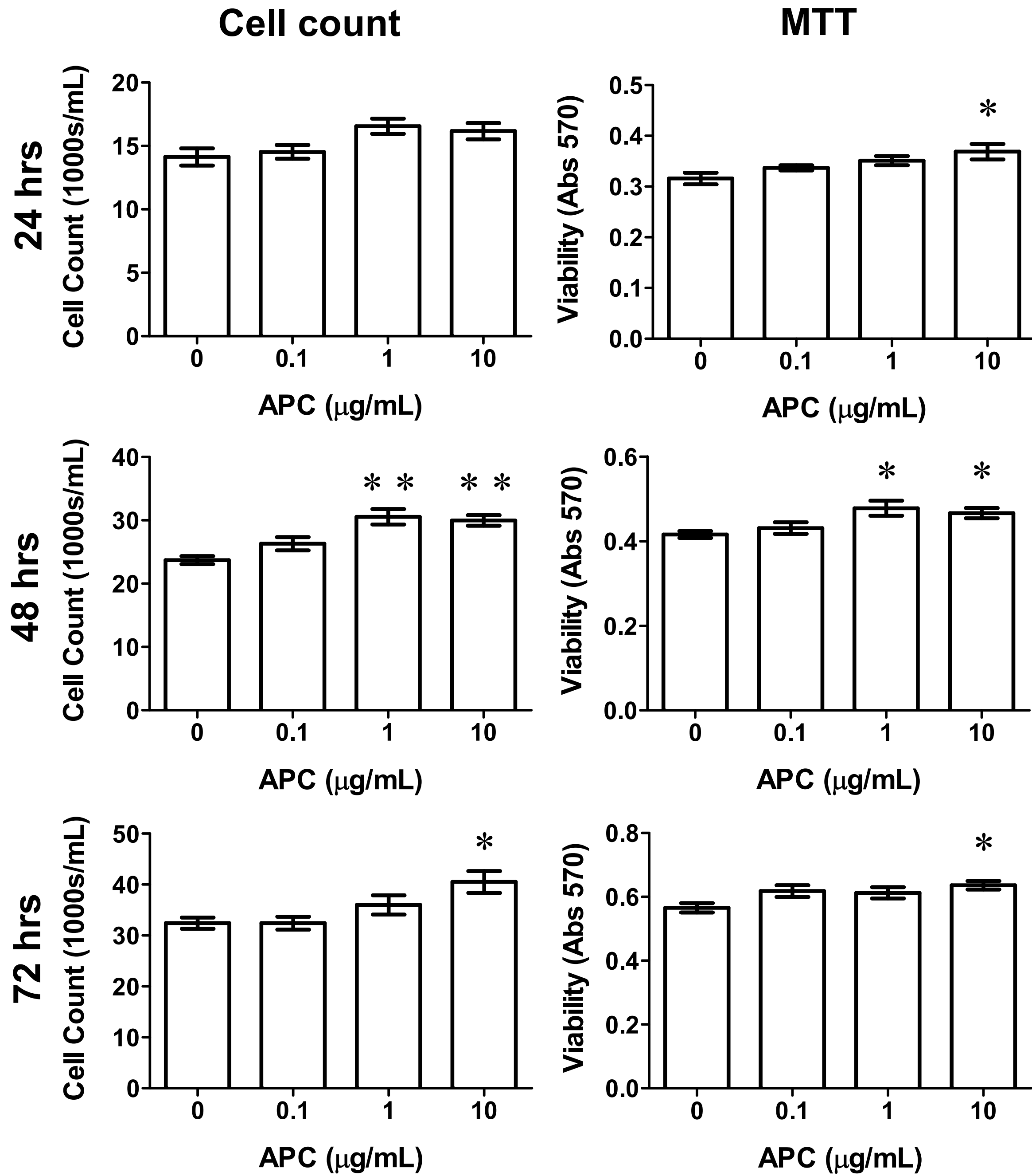

Figure 3-3 *The effect of APC on MG-63 cellular viability over 72 h, as assessed by MTT assay and cell counts. Serum-reduced (2%) cells were treated with 0.1, 1, or 10 μg/mL of APC, then assayed for cell viability by trypan blue exclusion dye and MTT at 24, 48, and 72 h. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=4). * Denotes P<0.05 and ** denotes P<0.01 between treatment and control.*

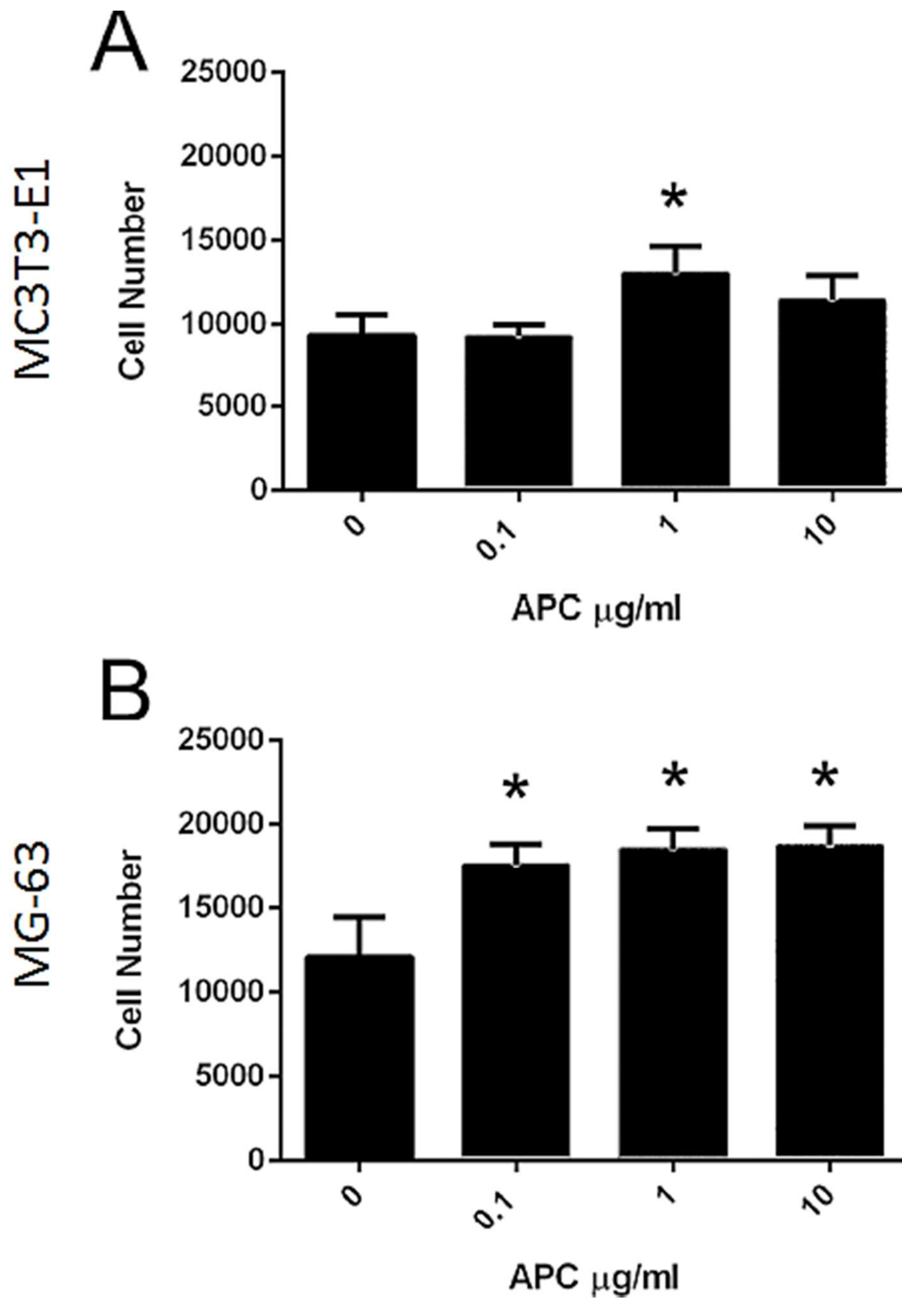

Figure 3-4 *The effect of APC on MG-63 and MC3T3-E1 cell numbers as assessed by MUSE® assay. Serum-reduced cells treated with 0.1, 1, or 10 µg/mL of APC were assayed for cell viability by automated cell analyser in MC3T3-E1 cells at 48 h post-treatment (A) and MG-63 cells at 72 h post treatment (B). Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=4). * Denotes P<0.05 versus untreated controls.*

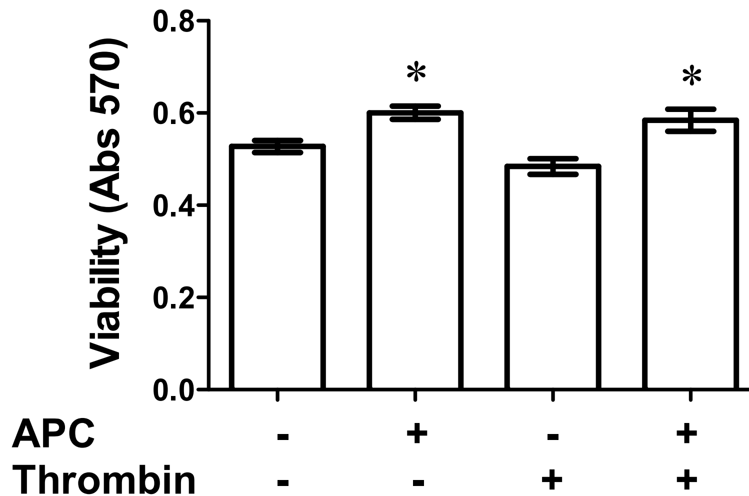

Figure 3-5 *The effect of thrombin treatment of MG-63 cells, as assessed by MTT assay. MG-63 cells were treated with 1 U/mL of thrombin, 10 µg/mL of APC or both, and assayed for cell viability by MTT. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ±S.E (n=4). * Denotes P<0.05 between treatment and control.*

3.4.4 APC-Mediated MG-63 Viability Requires PARs

As EPCR, PAR1, and PAR2 were expressed by MG-63 cells, we further examined the roles of these receptors in APC-mediated cell viability. EPCR blocking antibody (RCR-252) was added 30 min prior to APC treatment on MG-63 cells to block APC binding to EPCR on cell surfaces, controlled against EPCR non-blocking antibody (RCR-92), and viability was determined after 48 h (Figure 3-6A). In comparison to control, APC treatment induced a 21% increase ($P<0.05$) in viability, whereas cells pre-treated with RCR-92 or RCR-252 prior to APC treatment demonstrated no difference as compared to APC treatment alone (3%, $P=0.67$ or 5%, $P=0.31$, respectively). RCR-92 or RCR-252 treatment alone were not significantly different to control (5%, $P=0.44$ or 8%, $P=0.16$, respectively). This suggested that APC activity on MG-63 cells did not work through EPCR (Figure 3-6A).

PAR1 antagonist (SCH79797) and PAR2 antagonist (ENMD-1068) were used to block PAR1 and PAR2 signalling. SCH79797 or ENMD-1068 treatment 30 min prior to APC addition led to the abolishment of APC-mediated up-regulation of cell viability (Figure 3-6B). Interestingly, SCH79797 treatment substantially suppressed viability in comparison to control alone (57%, $P<0.001$). This marked suppression by SCH79797 was confirmed in a dose-dependent study, controlled against vehicle DMSO to excluded any effect from DMSO as a diluent (Figure 3-6C). SCH79797 treatment at 2.5, 5, or 10 μM dose-dependently decreased MG-63 viability over 48 h by 28%, 41%, or 49% ($P<0.01$ for all; Figure 3-6C) as compared to DMSO control, where viability was only significantly decreased by 19% at 10 μM ($P<0.05$), indicating that the effect was largely from endogenous inhibition of PAR1 activity by SCH79797, rather than DMSO.

ENMD-1068 treatment significantly increased MG-63 viability as compared to non-treated controls by (12%, $P<0.05$) and this difference was maintained after APC treatment (10%, $P<0.05$; Figure 3-6B). However, $Par2^{-/-}$ osteoblasts has similar viabilities as compared to WT osteoblasts (Figure 3-6D).

To test the specificity and efficacy of PAR agonists and antagonists, osteoblasts isolated from $Par1^{-/-}$ and $Par2^{-/-}$ mice were used [388]. Throughout this thesis, osteoblasts from $Par1^{-/-}$ or $Par2^{-/-}$ mice will be referred to as $Par1^{-/-}$ or $Par2^{-/-}$ osteoblasts. Mice genotypes were confirmed by semi-quantitative PCR. APC treatment of WT osteoblasts provided a significant increase in viability (24%, P<0.05), whereas treatment of $Par1^{-/-}$ or $Par2^{-/-}$ osteoblasts had no effect on viability (6%, P=0.5 or 10%, P=0.09, respectively; Figure 3-6D), confirming the effect found in MG-63 cells (Figure 3-6).

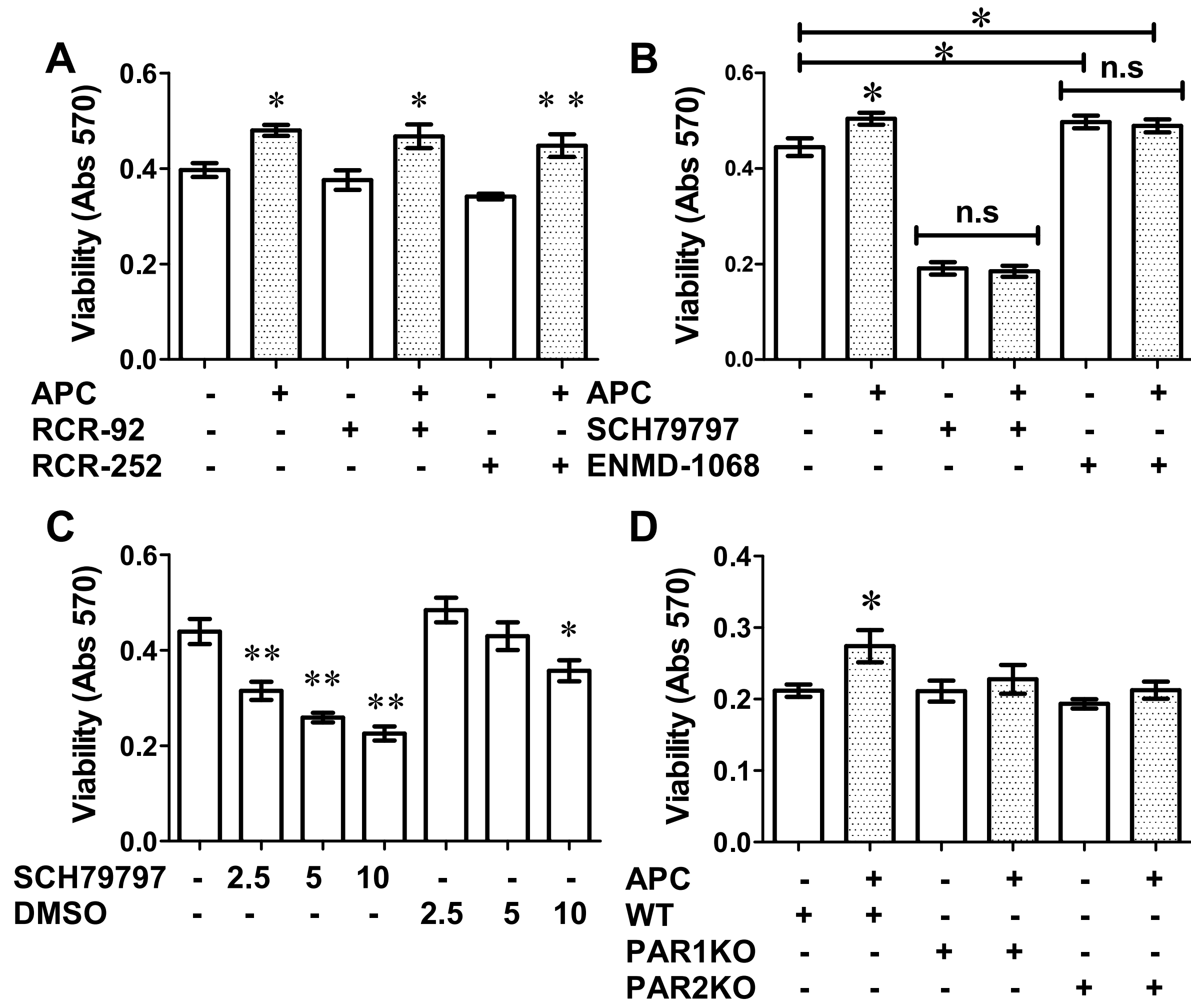

Figure 3-6 *EPCR, PAR1, and PAR2 involvement on APC-induced MG-63 viability, as assessed by MTT assays. APC (10 µg/mL) treatment followed blocking of EPCR by antibody RCR-252, controlled against a non-blocking antibody, RCR-92 (A), and blocking of PAR1 by SCH79797 or PAR2 by ENMD-1068 (B). These results were controlled against DMSO (C) or confirmed in WT, Par1$^{-/-}$, or Par2$^{-/-}$ murine osteoblasts (D). Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ±S.E (n=4). * Denotes P<0.05 and ** denotes P<0.01 between treatment and control.*

3.4.5 APC Induces pERK1/2, pAkt, and p-p38 in MG-63 Cells

We next determined the effect of APC, PAR1, and PAR2 on Akt, p38, ERK1/2, and NF-κB in MG-63 cells in serum-reduced media. Time courses were first established by APC treatment of MG-63 cells at 0, 15, 30, 60 min, or 24 h for each intracellular protein (Figure 3-7). Subsequent experiments were carried out at the optimal time of 60 min post APC treatment, in the presence of PAR receptor antagonists, SCH79797 or ENMD-1068.

APC treatment led to increases in phosphorylation of ERK1/2, Akt or p38 by 25%, 31%, or 35% ($P<0.05$, $P<0.05$, $P<0.01$ respectively; Figure 3-8) but did not significantly affect NF-κB (13%, $P=0.16$; Figure 3-8). SCH79797 addition did not affect pERK1/2, pAkt, NF-κB, or p-p38 (16%, 4%, 17%, 6%, and $P=0.07$, $P=0.22$, $P=0.44$, $P=0.59$ respectively; Figure 3-8). APC addition after SCH79797 blocking was not different to SCH79797 alone for any of the proteins. This suggests that APC acts via PAR1 to phosphorylate these intracellular proteins.

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ENMD-1068 treatment resulted in a 20% and 22% increase in pERK1/2 and pAkt as compared to control ($P<0.05$ for both) but had no effect on NF-κB or p-p38 (12% or 6%, $P=0.23$, $P=0.49$ respectively; Figure 3-8). APC treatment after ENMD-1068 led to a further 25% increase in pERK1/2 ($P<0.01$) and 18% increase in pAkt ($P<0.05$), but not NF-κB, or p-p38 (5%, $P=0.3$ and 25%, $P=0.49$ respectively) which, interestingly, suggested that APC acts through PAR2 to induce p-p38 but not pERK1/2 or pAkt at 60 min on MG-63 cells (Figure 3-8).

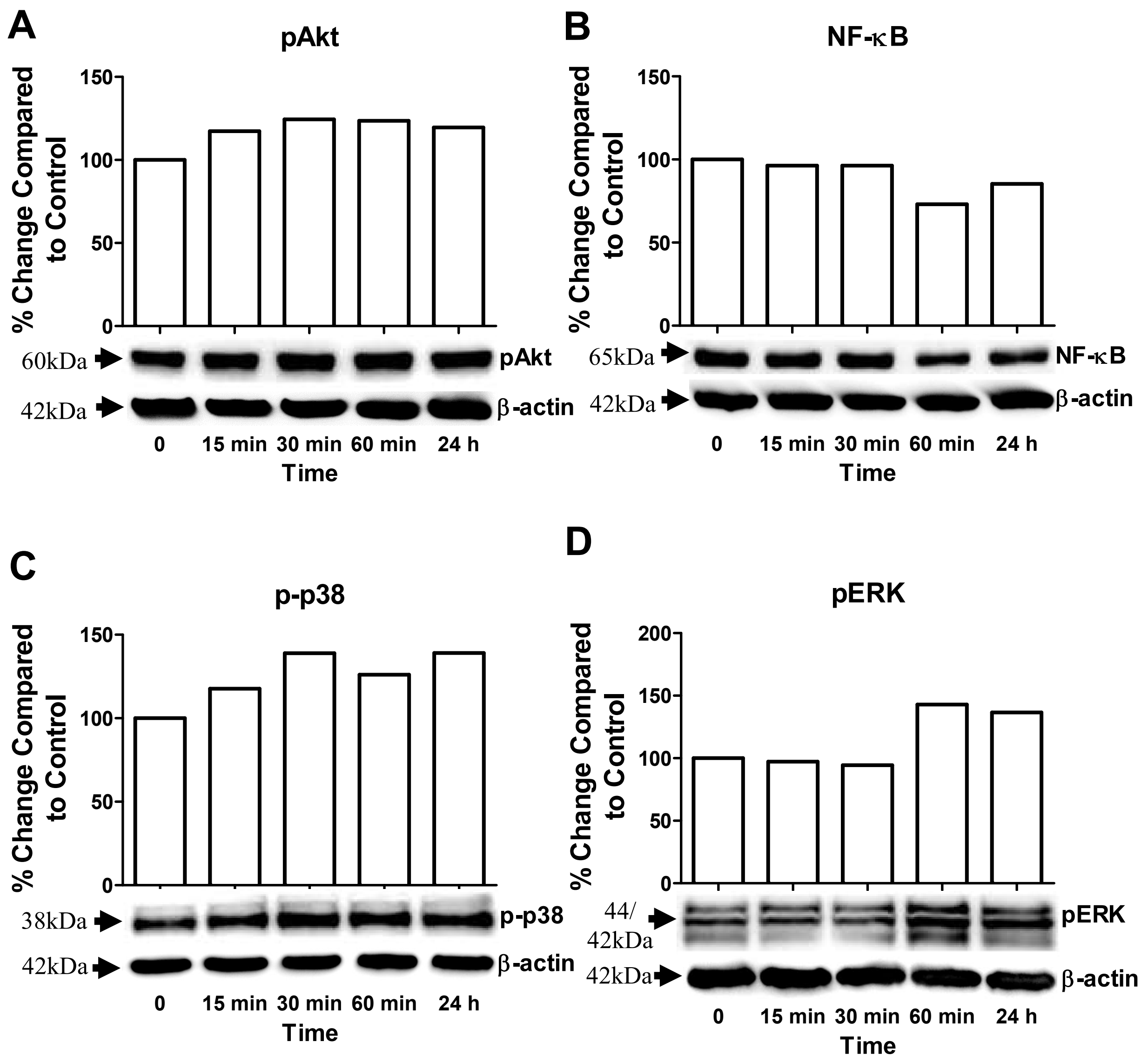

Figure 3-7 *Timeline of APC on p38, NF-κB, ERK1/2, and Akt phosphorylation in MG-63 cells. Confluent MG-63 monolayers were treated with APC and whole cell lysates collected at 0 min, 15 min, 30 min, 60 min, and 24 h post treatment. Proteins were separated by electrophoresis, transferred onto PVDF membrane, and incubated with primary antibodies against p-p38, pERK1/2, NF-κB, and pAkt and relevant secondary antibodies before visualisation and semi-quantification. Results shown are from a single experiment. β-actin was used as a loading agent.*

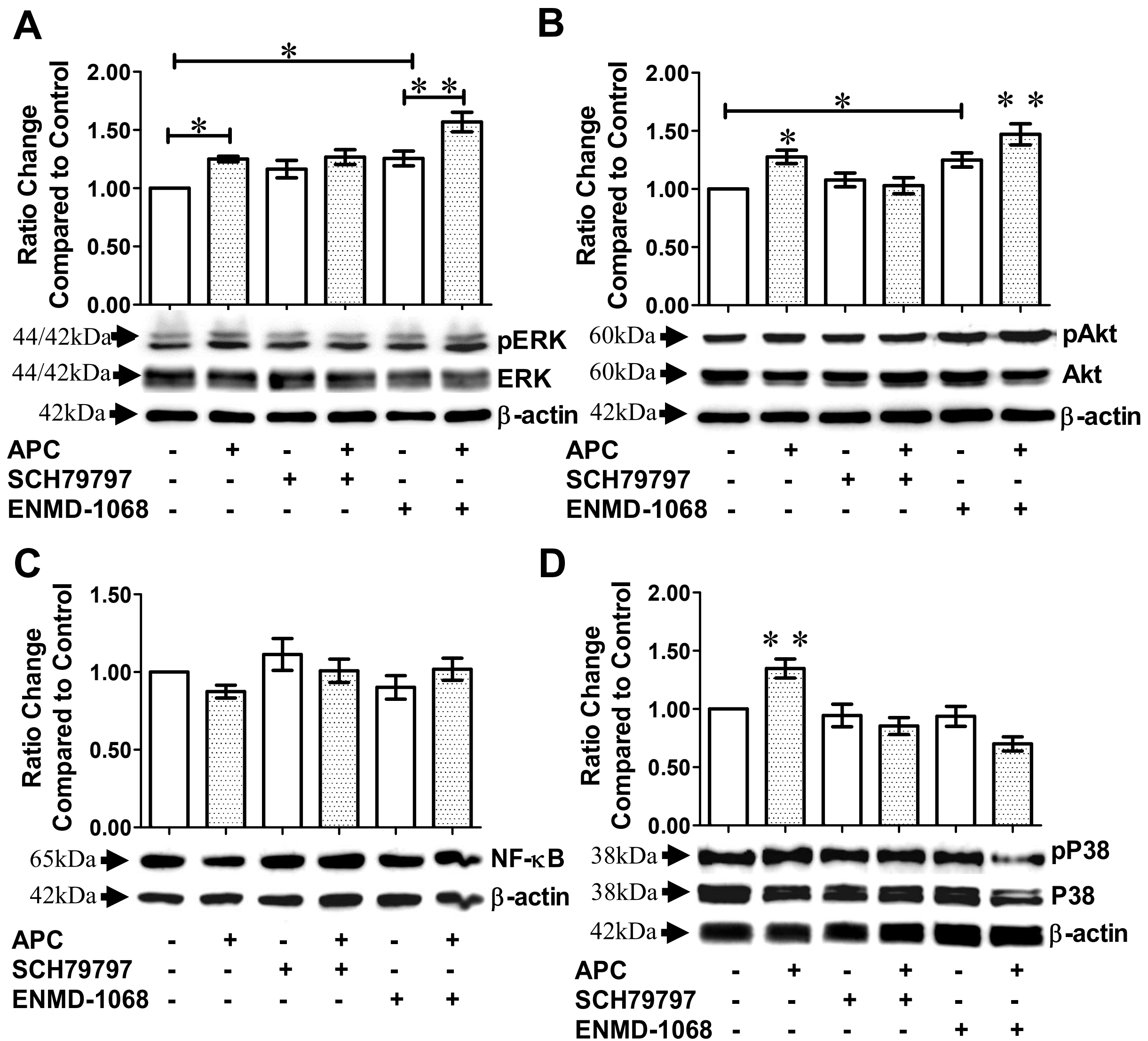

Figure 3-8 *The effect of APC and PAR antagonists on p38, NF-κB, ERK1/2, and Akt in MG-63 cells. Protein analysis followed APC treatment on pERK1/2 (A), pAkt (B), NF-κB (C), and p38 (D). SCH79797 and ENMD-1068 were used to block PAR1 and PAR2 activity respectively. β-actin was used as a loading agent. Statistical analysis was carried out by one-way ANOVA and Newman-Keuls post-test on APC vs control and agonist + APC vs agonist alone. Data represented as mean ±S.E (n=4). * Denotes P<0.05 between treatment and control, ** denotes P<0.01 between treatment and control.*

3.5 Discussion

Osteoblasts are the primary cellular drivers of new bone formation, and are therefore critical for bone repair and remodelling [8, 9, 11]. In this study, we sought to determine how the osteoblast-like MG-63 and MC3T3-E1 cell lines respond to APC treatment. In this regard, we have shown that there is a significant increase in the number of viable osteoblast-like cells, following APC addition to both the human and murine cell cultures. We have demonstrated that EPCR, PAR1, and PAR2 expressed by MG-63 cells are required for APC to exert its effect upon these cells. Furthermore, we also found that APC treatment activates the downstream signalling of the ERK1/2, Akt, and p38 pathways. Overall, these findings demonstrate the potential for APC to enhance osteoblast cell viability, as discussed below.

APC increases the rate of proliferation of many different cell types, which includes normal human osteoblasts [268, 269]. We have confirmed the protective effect of APC on MG-63 and MC3T3-E1 viability, although the response was greater in MG-63 cells. While both cells are osteoblastic in nature, they demonstrate phenotypic differences. MG-63 cells are human osteosarcoma cells with higher rates of proliferation than normal human osteoblasts and express heterogeneous levels of both immature and mature osteoblastic markers [357, 359]. MC3T3-E1 are murine pre-osteoblast cells that express greater levels of alkaline phosphatase and a greater response to vitamin D stimulation than MG-63 cells [355]. These differences can partially account for the slight difference in response to APC. Alternatively or additionally, it may be due to a species related effect of APC. Such effect is apparent in a murine stroke model, where mouse APC is more potent than human APC in neuroprotection against ischemic stroke [389]. Nonetheless, both cell lines are commonly used to model osteoblast responses and our results showed that APC stimulates MG-63 and MC3T3-E1 viability.

Our findings that APC stimulated osteoblastic-like cell viability are consistent with the work of Kurata and colleagues, who showed a similar effect of APC upon MG-63 cells [269]. We were able to demonstrate these effects using the MTT, and trypan blue exclusion assays, and obtained further confirmation with the MUSE® cell viability assay. The study by Kurata *et al* demonstrated APC enhancement of normal human osteoblast DNA incorporation and total cell count over 24 h [268]. In our study, we found that this effect was still detectable at 72 h post-treatment, indicating that a single dose of APC can exert long-lasting effects upon osteoblastic cells.

The Kurata *et al* study [268] reported that 500 nM APC was able to induce a 50% increase in osteoblast number following treatment. In this study, we observe a much more modest effect. One explanation for the difference could lie in the different experimental techniques employed by each study. For instance, in our study, the MTT and cell count assays demonstrated a smaller APC-induced effect, as compared to that measured by the commercial MUSE® assay. The MTT assay measures metabolic status, but it is often used to approximate cell viability and proliferation, as rapidly dividing cells are more metabolically active [390-392]. The MUSE® assay uses the principle of bromodeoxyuridine (BrdU), which utilises DNA incorporation and reflects a different aspect of cell proliferation than MTT. Despite differences in the degree of stimulation reported, all studies consistently demonstrate a robust enhancement of proliferation/viability in different osteoblastic cell types from APC treatment [268, 269].

The MTT and trypan blue exclusion assays used in this study determine cellular viability, which does not strictly correlate with proliferation [393, 394]. Despite this, MTT and trypan blue assays can directly substitute for proliferation in experiments, provided that parameters such as the MTT concentration, the length of incubation, and the confluence of cells are

standardised across the experiments [390-393, 395]. We have applied these parameters in the same manner to experiments in cell lines and cultured them under the same conditions to best enable interpretation of the MTT readings. These assays have been used to describe proliferation in many osteoblast studies [396-400] and are likely a suitable measure of cell proliferation in our study.

We further hypothesised that APC-augmented MG-63 viability would act through its canonical pathway involving EPCR and PARs. Previous papers have demonstrated the presence of these receptors on osteoblasts. Kurata *et al* and Lee *et al* have demonstrated the presence of EPCR on normal human osteoblasts and MG-63 cells respectively, and we confirmed the latter observation in this study [268, 269]. Although other papers have shown the presence of PAR1 and PAR2 in murine osteoblasts [24, 268, 401], we have demonstrated for the first time that PAR1 and PAR2 are present in MG-63 cells, at both a gene and protein level. We further characterised the involvement of PARs in APC-treated osteoblastic cell lines and found APC-mediated viability and signalling was dependent upon PAR1 and PAR2 rather than EPCR in MG-63 cells. This observation was further confirmed in both $Par1^{-/-}$ and $Par2^{-/-}$ osteoblasts.

These results contrast with those from the Kurata *et al* study, which demonstrates EPCR but not PAR1 is required for APC action in normal human osteoblasts [268]. There are several possible explanations for this discrepancy. Firstly, we did not utilise positive controls for the EPCR inhibitors and cannot differentiate between a true lack of inhibition or if the dose of inhibitors was simply too low to inhibit the EPCR pathway. However, we have used the same concentration as previous studies of EPCR inhibition, including the Lee *et al* study of APC and bisphosphonate treatments in MG-63 cells [260, 269]. The Lee *et al* study offers a separate possible explanation for this discrepancy. Lee *et al* demonstrated that EPCR mediates the effect

of APC on alendronate but not zoledronate-treated MG-63 cells [269]. This suggests that APC may act through other signalling mechanisms independent of EPCR. Two previous publications demonstrate EPCR independent effect from APC; the first shows APC stimulation of pERK1/2 through PAR1/sphingosine-1-phosphate receptor (S1P1); and the second shows APC protection against podocyte apoptosis through PAR3 [402, 403]. In the latter, the anti-apoptotic effect of APC also required PAR3 heterodimerisation with PAR2 or PAR1 [402]. As we have demonstrated a PAR1/2 dependent but EPCR independent mechanism, we hypothesise that the effect of APC on MG-63 cells in our study may work through PAR3 or S1P1. Differential responses to EPCR, even within the same cell type, demonstrates that there exists a degree of plasticity in APC-induced cell signalling. Despite this, there is no current experimental data to indicate why APC works via EPCR under some circumstances and not others.

We found that the treatment of MG-63 cells with PAR1 antagonists suppressed cell viability, after accounting for effects due to the vehicle control. However, SCH79797 has been reported to induce off-target effects that can influence the viability of fibroblast, embryonic kidney, and melanoma cell lines [404]. It is was not clear whether the reduction in APC-induced viability was due to these off-target effects. Thus, we determined the involvement of another PAR1 agonist, thrombin, on the effects of MG-63 cells. Activation of PAR1 by thrombin has been reported to stimulate osteoblast proliferation [381, 382, 386, 405]. However, we were unable to reproduce this thrombin-mediated effect on MG-63 cells. Only one paper demonstrated this effect in MG-63 cells, however, they used different culture and assay ([^{3}H] thymidine incorporation) conditions to our work [406]. It is possible that these different experimental conditions, particularly the higher sensitivity of the thymidine incorporation compared to the

MTT assay, may at least partially explain the conflicting results. Despite this, our study has shown that APC, acting through the PAR1 receptor, promotes osteoblastic cell viability.

In contrast to the suppression of osteoblast viability by PAR1 antagonist, PAR2 antagonism alone demonstrated a mild increase in cellular viability, ERK and Akt activity, however, *Par2*$^{-/-}$ osteoblasts did not demonstrate a similar increase in viability as compared to WT osteoblasts. We postulate that this is due to the species difference between murine knockout cell outcomes and antagonist approaches in human cells.

Although both PAR1 and PAR2 signalling were involved in APC-mediated MG-63 viability, we found that PAR2 was only involved in APC-mediated p38 signalling, and PAR1 involvement to be specific in ERK1/2 and Akt signalling. APC acts through different permutations and combinations of receptors to achieve its effects, depending on the cell type. There is precedent for APC signalling through either PAR1 or PAR2 to induce p38, Akt in some studies [266, 292, 407], and both PAR1 and PAR2 for APC-induced pAkt activity in other studies [408]. In the current literature, however, there has been no evidence for the involvement of APC-PAR2 signalling in activating ERK1/2 [258]. Our results here suggest that the individual effects of APC on intracellular proteins, ERK1/2, Akt, and p38, in MG-63 cells rely on particular receptors. However, the use of single time point in our study limits the interpretation of these results. Another possible outcome from SCH79797 and ENMD-1068 inhibition of PAR1 and PAR2 is a shift in the peak response of the intracellular signalling proteins. Further studies using these PAR1 and PAR2 inhibitors over several time points will determine whether this response is due to inhibition of PAR receptors or shifting in signalling protein response.

ERK1/2 is a primary signalling pathway for many cellular activities including proliferation [409, 410], and APC signals through ERK1/2 to induce proliferation in keratinocytes and endothelial cells [248, 371]. ERK1/2 is required for osteoblast proliferation and mediates the proliferative effect of endogenous growth factors, hormones, amino acids, phenols, and mechanical strain on osteoblasts [411-419]. APC promotion of osteoblastic viability was concurrent with its activation of ERK1/2 in our study, which is consistent with recent papers showing APC stimulation of ERK1/2 in MG-63 cells and normal human osteoblasts [268, 269]. ERK1/2 not only regulates osteoblast proliferation, but it also plays a complex role in osteogenic differentiation, possessing both agonistic and antagonistic effects [420]. Lee *et al* demonstrated that APC enhances ERK1/2 and stimulates MG-63 differentiation through augmentation of collagen production and alkaline phosphatase activity [45]. However, the study did not extend to determine whether or not differentiation was dependent upon ERK1/2, thus, it is unclear whether APC signals via the ERK1/2 pathway to induce osteoblast differentiation.

Activation of p38 promotes early and late osteoblast differentiation through different substrates including *Runx2* and *Osterix* [421, 422]. It also mediates BMP-induced osteoblast differentiation [421, 422]. APC can differentially regulate the p38 pathway; stimulating p38 activity in keratinocytes, blood monocytes, and contrastingly, reducing p38 activity in tenocytes, arthritic synovial fibroblasts, and monocytes [184, 276, 277, 292, 343, 377]. It has been shown that APC's inhibition of p38 in keratinocytes occurs specifically via the PAR2 receptor [292], however, PAR2 involvement in other studies was not determined. Here, we demonstrate that APC activates the p38 pathway in MG-63 cells, and this effect is dependent upon the PAR2 receptor. This provides further evidence for the involvement of PAR2 in APC modulation of p38 signalling. Although the activation of p38 promotes osteoblastic

differentiation, and APC has been demonstrated to induce MG-63 differentiation, it is not yet known whether the latter is influenced by the former. It is possible that APC stimulation of osteoblast differentiation may also act through PAR2 and the p38 pathway.

The signalling protein Akt is required for bone formation, skeletal mass regulation and endochondral ossification [32, 423]. Activated Akt exerts a two-fold effect upon osteoblast growth, by stimulating proliferation and protecting against apoptosis [424]. In a similar manner to p38 signalling, Akt mediates BMP-induced osteoblast differentiation, but Akt activation itself also augments osteoblast differentiation and bone mineral density [425, 426]. APC has been shown to induce pAkt in neuronal production, myocardial injury, cutaneous wound healing, and counteraction of inflammation [258, 292, 370, 377, 408]. In this study, APC stimulation of pAkt was consistent with its promotion of MG-63 cell viability, however, we have not investigated whether pAkt mediates other effects of APC. The complex role of Akt in osteoblasts and bone suggests that APC may have additional effects, such as protection against bisphosphonate-induced cell death as demonstrated by Lee *et al* [269].

In contrast to the actions of Akt and p38, stimulation of NF-κB leads to suppression of osteoblast differentiation and mineralisation [427, 428]. NF-κB is primarily up-regulated in cells during states of inflammation, and from their exposure to the cytokines IL-1 and TNF-α; the latter being an inhibitor of bone formation [428, 429]. Several studies have reported the negative regulation of NF-κB by APC treatment: (1) APC inhibits NF-κB in the mononuclear cells of septic patients, but not those of normal patients [430]; (2) APC suppresses lipopolysaccharide stimulated production of TNF-α through suppression of NF-κB in monocytes [277]; (3) APC reduced LPS-induced NF-κB in endotoxemic mice [431]; (4) APC further suppresses tissue plasminogen activator-induced NF-κB and MMP-9 [285]; and (5)

APC inhibits NF-κB activation by alendronate on MG-63 cells [269]. In this study, we did not find NF-κB suppression by APC in MG-63s, and we suggest that it is likely that exogenous inflammatory stimuli are necessary for APC to down-regulate NF-κB in osteoblasts.

3.6 Summary

The viability of osteoblasts is integral to the processes of bone formation and remodelling. The protection of osteoblasts function may improve outcomes in orthopaedic applications. A large number of studies have demonstrated that APC is a potent stimulator of cellular proliferation with the ability to protect different cell types against cellular injury through its receptors EPCR and PARs. In this chapter, we present data to confirm that APC can act on osteoblasts.

We demonstrate that APC promotes the viability of both the MG-63 and MC3T3-E1 osteoblastic cell lines. This was an action dependent upon PAR1 and PAR2 but not EPCR. We also show, for the first time, that APC not only enhances ERK1/2 signalling in MG-63 cells but similarly activates Akt and p38, both of which are proteins essential for osteoblastic differentiation and cell survival. Notably though, the effect from APC on these proteins required different PARs. Overall, these results demonstrate that APC treatment is protective in osteoblast cells and suggest that APC may have therapeutic potential on bone formation.

CHAPTER 4:

APC ENHANCES BMP-2-INDUCED ECTOPIC BONE FORMATION

4 APC Enhances BMP-2-Induced Ectopic Bone Formation

4.1 Introduction

In bone injury and disease, one cause for an inadequate bone healing response is a deficiency in requisite growth factors. This can be the result of reduced or damaged blood supply, external factors including infections, or a large bone defect size [95, 127, 432]. Current treatments to restore osteogenic factors, such as bone grafts, bone transport, and supplementation with recombinant growth factor have their limitations [122, 126, 140]. Recombinant BMP-2, an FDA approved molecular therapy for bone healing, has proven efficacy in animal models and clinical applications [94]. However, the off-label use of recombinant BMP-2 to promote osteogenesis has been increasingly shown to induce complications including inflammation and bone resorption [141]. For example, the use of recombinant BMP-2 to promote osteogenesis has been increasingly shown to induce complications including inflammation and bone resorption [141]. This highlights the need to investigate possible alternative means to increase biological factors.

APC promotes the healing of cutaneous wounds in animal models and humans including rat full-thickness punch biopsy wounds, ischemic flap survival, and long-standing diabetic wounds [290-292, 433]. These actions result because of APC's capability to promote granulation tissue and re-epithelisation through its induction of various growth factors [265, 305].

In light of the results described in Section 3.4.2, where APC was found to induce MG-63 and MC3T3-E1 viability, we sought to further investigate the effect of APC on bone healing, specifically osteogenesis. Traditionally, the assessment of the osteogenic potential of agents is examined *in vitro* by molecular markers, cell biology-based assays, and *in vitro* mineralisation assays [434]. Despite the utility of *in vitro* assays, they are limited in their ability to capture the

complexity of the *in vivo* environment and the interaction between different cell types within the bone microenvironment. Furthermore, the expression of *in vitro* markers does not always correlate with the osteogenic capacity of an agent *in vivo* [435, 436]. For these reasons, we further investigated the effect of APC on bone formation in an *in vivo* setting using a murine model.

Hydroxyapatite/tricalcium phosphate (HA/TCP) is frequently utilised *in vivo* to determine the osteogenic potential of agents. This assay requires the mixing of potential agents with HA/TCP and implantation into mice. This method is relatively easy to perform and also provides reproducible results. However, micro-computed tomography (μCT) is unable to quantitate the newly formed bone and differentiate it from implanted HA/TCP granules [434]. Schindeler *et al* highlighted that an alternative method that can be used as a rapid *in vivo* screening assay [437]. Using a modified version of this approach, we implanted collagen scaffolds containing recombinant human BMP-2 into the hind limb musculature of a mouse. The addition of BMP-2 induces ectopic bone formation and leads to rapid induction of a bone nodule over 3 weeks that can be readily quantified using μCT [437].

We hypothesised that APC would (1) adjunctively augment BMP-2-induced ectopic bone formation, (2) act through a PAR1/PAR2-dependent pathway, and (3) suppress osteoclast numbers.

4.2 Aims

The overall objective of this chapter was to investigate the effect of APC on BMP-2-induced ectopic bone formation. Specifically, we aimed to:

1. determine the effect of APC on bone formation in an *in vivo* model;
2. examine the underlying mechanism of APC on ectopic bone formation including the effect on osteoclasts; and
3. examine whether a PAR1/PAR2-dependent pathway was implicated in APC's actions.

4.3 Methods

4.3.1 APC and BMP-2-Induced Bone Formation in WT and *Par*$^{-/-}$ Mice

The effect of APC on BMP-2-induced bone formation was assessed using an ectopic bone formation model. Treatments of 10 or 25 µg recombinant human APC (Eli Lilly) was combined with 10 µg recombinant human BMP-2 (Medtronic) in 10 µL of water, which was then pipetted onto collagen sponges. These treated collagen sponges were then implanted bilaterally into intramuscular pockets of *Par1*$^{-/-}$, *Par2*$^{-/-}$, or WT control mice (all mice possessed a C57BL/6J genetic background), according to the groups in Table 2-7 (Section 2.3.2). *Par*$^{-/-}$ mice were genotyped as per Section 2.3.1. All animal experiments were approved by the Animal Care and Ethics Committee for the Children's Medical Research Institute and The Children's Hospital Westmead (Protocol K294).

Ectopic bone formation was monitored radiographically using X-ray (Faxitron X-ray Corp) at 2 and 3 weeks. Fixed bone nodules were further analysed using µCT and CTAn Software for bone volume, tissue volume, and trabecular structure. Nodule midsections were reconstructed using 20 slices of scanned images to demonstrate trabecular complexity.

4.3.2 Osteoclast Numbers in Ectopic Bone Nodules

To evaluate osteoclast numbers in bone nodules, TRAP staining was performed on histologic sections of bone nodules. Bone nodules were fixed, cryoembedded and sectioned as per Section 2.3.4. TRAP staining was conducted to determine osteoclast numbers. Cryosections were rehydrated in PBS, and then further incubated in Tris-HCl buffer before transfer into Na-Acetate buffer. The tissues were then incubated in filtered TRAP staining solution, then washed, counterstained with haematoxylin, and coverslipped (Section 2.3.4). Osteoclast numbers were assessed using microscopy and Bioquant Software (BioQuant).

4.3.3 EPCR, PAR1, and PAR2 Staining in Ectopic Bone Nodules

To assess PAR1 and PAR2 staining in ectopic bone nodules, sections were cut onto cryofilm, adhered to slides using chitosan. Staining for EPCR, PAR1 and PAR2 was conducted using antibodies from Table 2-1 as per Section 2.3.5. Staining was counterstained with haematoxylin and coverslipped in Euckitt solution. Staining was visualised and scanned on ScanScope.

4.3.4 Statistics

Statistical analyses and data graphing were performed as per Section 2.4. *P*-values less than 0.05 were considered statistically significant. All duplicated values were presented as mean ± standard error (SE). N indicates the number of bone nodules.

4.4 Results

4.4.1 APC Increases BMP-2-Induced Bone Formation

Our previous *in vitro* results (Section 3.4.2) suggested the potential for APC to act on bone formation through its stimulatory actions on osteoblasts. In this chapter, the capacity of APC to augment BMP-2-induced bone formation was examined in a mouse ectopic bone formation model. Scaffolds containing BMP-2 and APC were compared to scaffolds infused with BMP-2 alone. After 3 weeks, bone nodules were formed and quantified by X-ray and µCT (Figure 4-1 & 4-2).

Representative bone nodules in each treatment group were compiled to examine the relationship between XR images and µCT results. Qualitative X-rays demonstrated nodule formation after 3 weeks with varying levels of structural complexity and size. Quantitation of nodules was then carried out after µCT to determine total volume and bone volume. Whole nodule modelling was constructed to provide 3D confirmation of nodule sizes and shapes seen from X-ray (Figure 4-1). Three-dimensional µCT reconstructions showed enhanced bone formation in APC-treated specimens consistent with the XR images (Figure 4-1). Cross-sectional reconstruction of 20 slices within nodules also demonstrated an increased trabecular complexity in both APC treatment groups (Figure 4-1).

The examination of X-ray images taken at the 3-week timepoint, revealed an increase in the volumetric size of the nodules, following both BMP-2 + 10 µg APC or BMP-2 + 25 µg APC treatment, as compared with BMP-2 treatment alone (Figure 4-1). This suggested that APC has a positive effect on ectopic bone formation. Indeed, quantification of µCT data using CTAn software revealed that inclusion of only 25 µg APC led to a significant increase in bone volume (BV) by 74% ($P<0.01$; Figure 4-2A). The 10 µg of APC inclusion had no significant effect on

BV (32%, P=0.07; Figure 4-2A). Similarly, 25 µg of APC inclusion led to a significant increase in total tissue volume (TV) by 104% (P<0.01) but not the 10 µg of APC (40%, P=0.09; Figure 4-2B). These results indicate that APC increased the area of bone remodelling. However, there was no apparent change in the ratio of bone volume to total volume (BV/TV) with APC treatment, demonstrating that APC did not affect the density of bone nodules (Figure 4-2C). This is the first study to demonstrate that APC can increase bone volume *in vivo*.

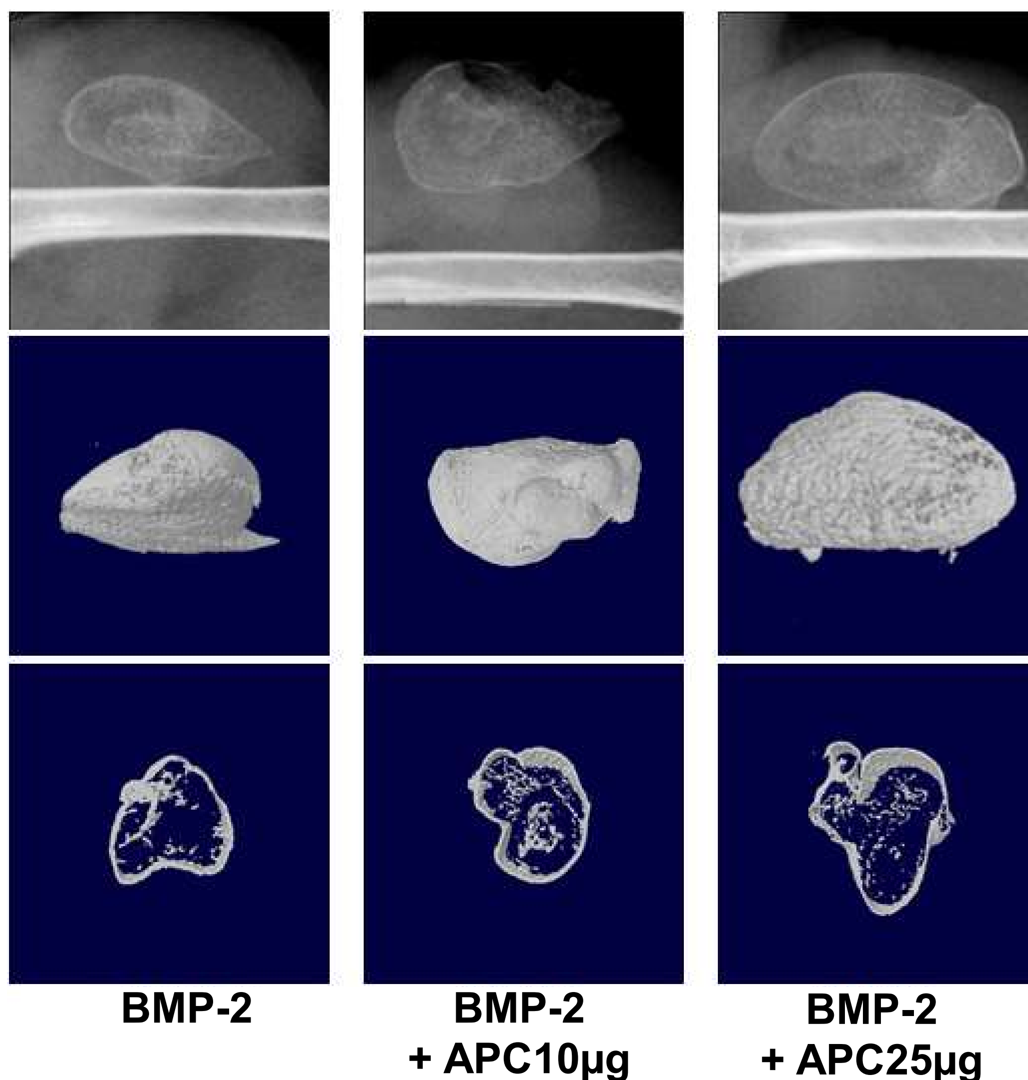

Figure 4-1 *Representative bone nodules selected based on median bone volume, from X-ray imaging and µCT reconstructions. Figure shows representative X-rays and 3D µCT models of whole nodules and cross-sectional compilation of 20 slices after 3 weeks of BMP ± APC incorporation.*

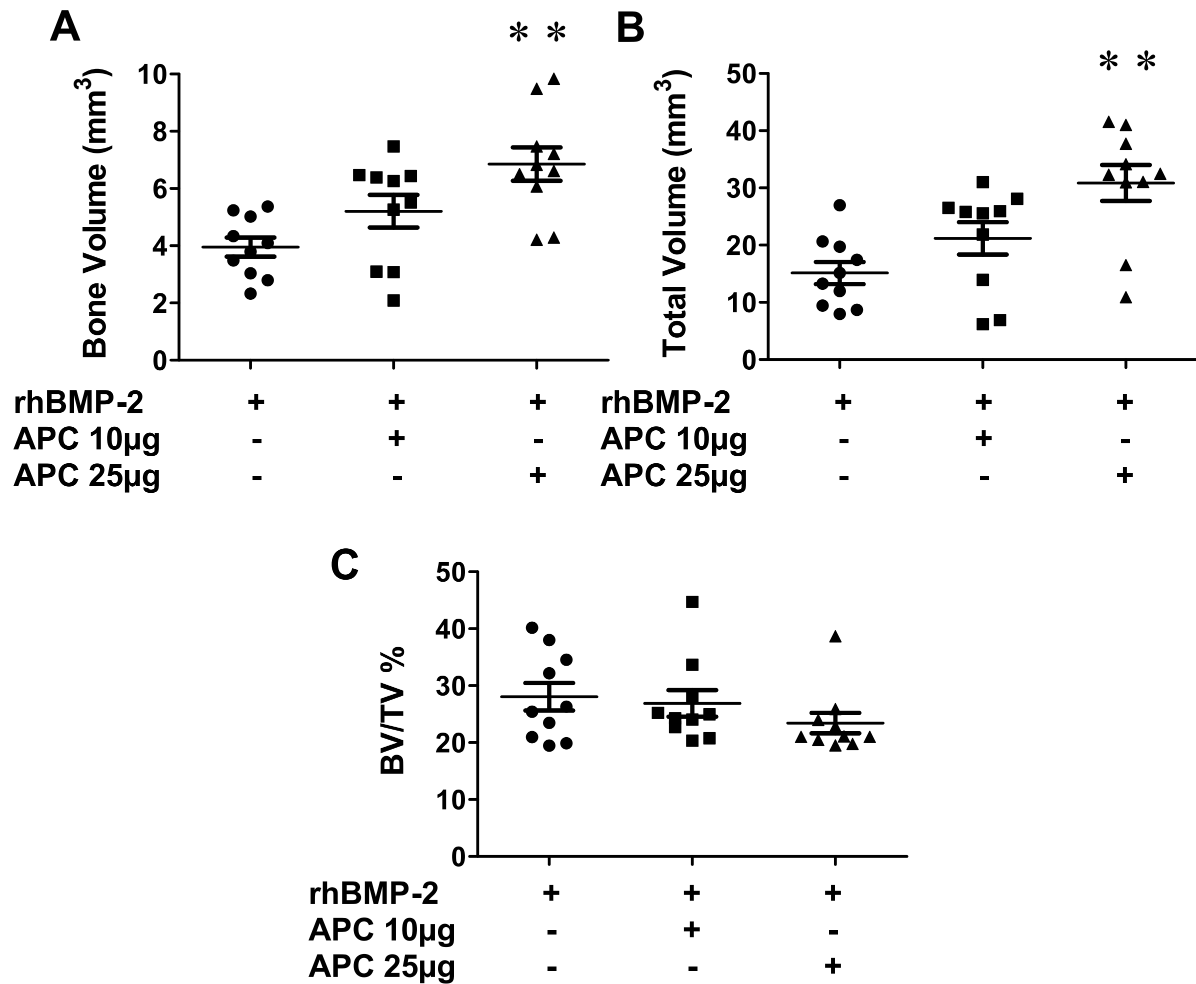

Figure 4-2 *The effect of APC on BMP-2-induced bone formation model. CT analysis was used to calculate bone volume (A), total volume (B), and bone volume to total volume (BV/TV, C) ratio in nodules treated with BMP-2 ± 10 or 25 µg APC. Statistical analysis was carried out by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=10). ** Denotes P<0.01 between treatment and control.*

4.4.2 APC Increases Osteoclasts in Bone Nodules

Increases in net bone in the BMP-2 ectopic bone formation assay could be indicative of either increased bone anabolism or of suppressed bone resorption. To examine this further, TRAP staining was performed to examine osteoclast number (OcN) (Figure 4-3A).

No decrease was seen in osteoclasts numbers, suggesting that increases in bone volume were not influenced by an anti-catabolic effect of APC. Indeed, a 20% increase in TRAP+ cell number was observed with 25 µg APC ($P<0.05$; Figure 4-3B), as compared to BMP-2 alone. Similarly for bone volume and total tissue volume, no differences were seen with 10 µg of APC (10%, $P=0.2$; Figure 4-3B).

Osteoclasts were noted to be adherent to the bone surface, unlike with bisphosphonate treatment where they are detached from the bone surface [438-440]. When osteoclast numbers were normalised to respective bone surface areas, there was no significant effect of APC (Figure 4-3C). These results suggest that the stimulation of bone formation by APC is an anabolic effect and the concurrent increase in osteoclast number resulted from an increase in bone surface area.

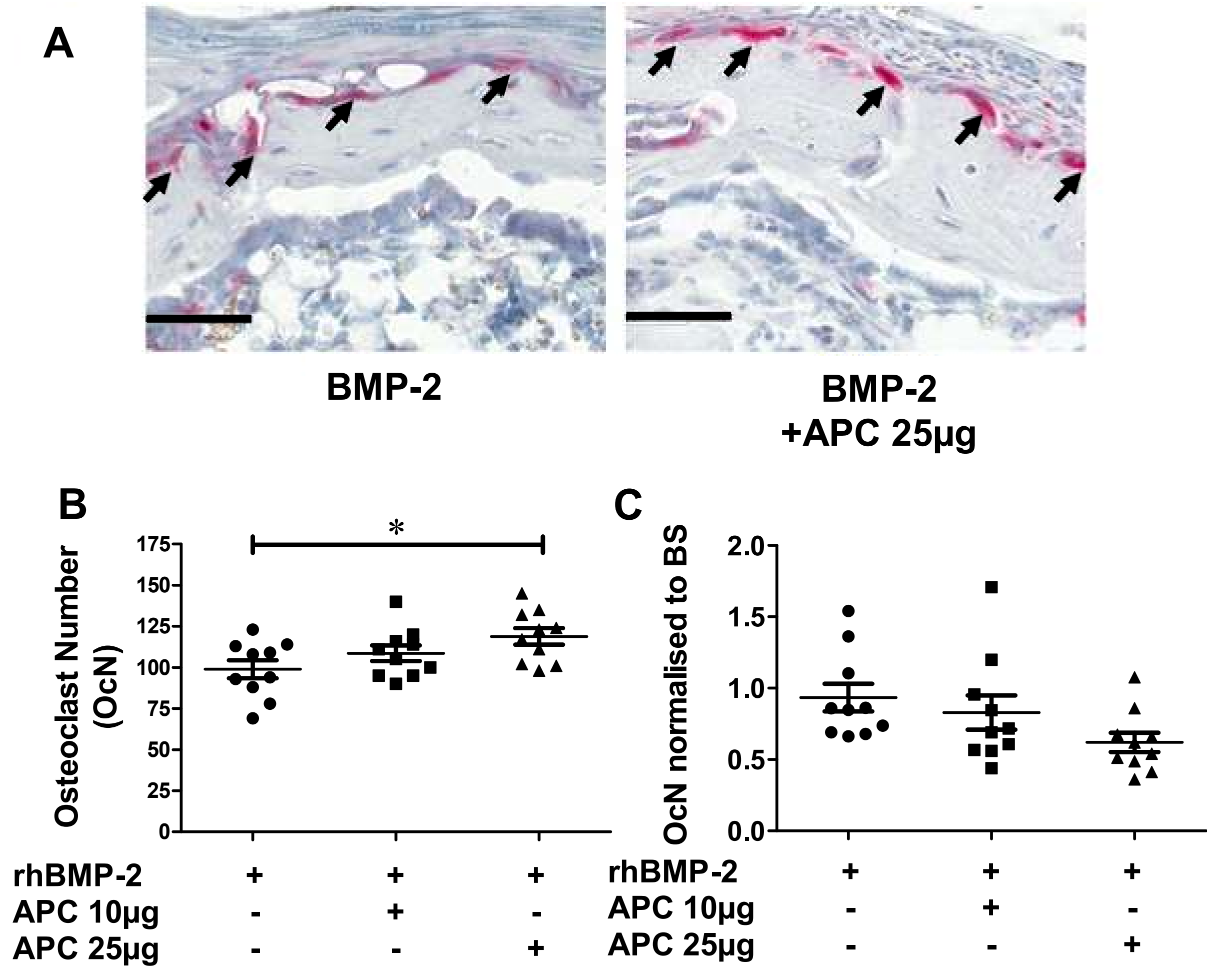

Figure 4-3 *The effect of APC on osteoclast activity in bone nodules. Osteoclasts were stained for TRAP (red) enzymatic staining (A). Positive cells were counted in five random fields of view at 20X and graphed as osteoclast numbers (OcN, B), and normalised to bone surface area (BS, C). Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=10). * Denotes P<0.05 between treatment and control. Scale bar 50 μm.*

4.4.3 APC Does Not Affect EPCR and PAR Expression in Bone Nodules

As our previous *in vitro* data (Section 3.4.4) demonstrated a role for PAR1 and PAR2 in APC-induced MG-63 cell viability, we sought to investigate whether or not EPCR and PAR receptors were up-regulated in APC-infused nodules following 3 weeks of treatment.

Our immunohistochemical staining revealed EPCR-positive cells to be sporadically distributed throughout the bone nodule (Figure 4-4). EPCR staining, however, did not appear to be expressed on bone lining cells or cuboidal cells on the bone surface. Overall, there was no significant difference in EPCR expression between APC-treated bone nodules, and those treated with BMP-2 alone (10 µg APC, 11%, P=0.48; 25 µg APC, 2%, P=0.88).

Similarly to EPCR, PAR1 and PAR2-positive cells were dispersed throughout bone nodules, and this was not significantly altered in APC-treated nodules (Figure 4-5). The level of PAR1 expression in the 10 µg or 25 µg APC treatment groups was not significantly different to that of control nodules (4%, P=0.83 and 15%, P=0.41 respectively). Likewise, PAR2 expression resulted in no change (5%, P=0.74) after 10 or 25 µg APC (15%, P=0.18) as compared to control.

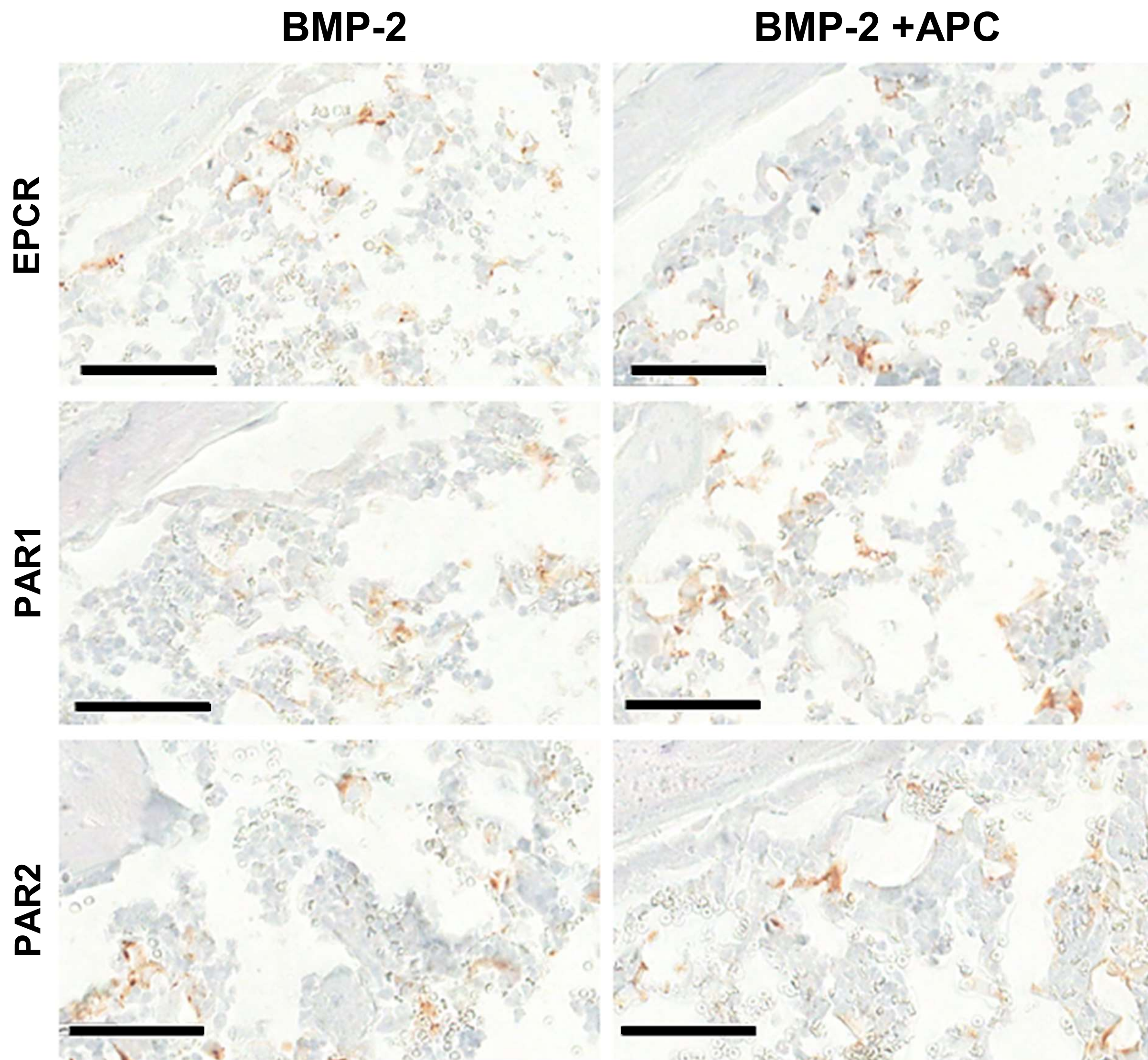

Figure 4-4 *The expression of EPCR, PAR1, and PAR2 in bone nodules. IHC was performed using EPCR, PAR1, and PAR2 antibodies on bone nodules treated with BMP-2 ± 25 µg APC. Slides were scanned using a ScanScope scanner and processed in ImageScope. Scale bar indicates 60 µm.*

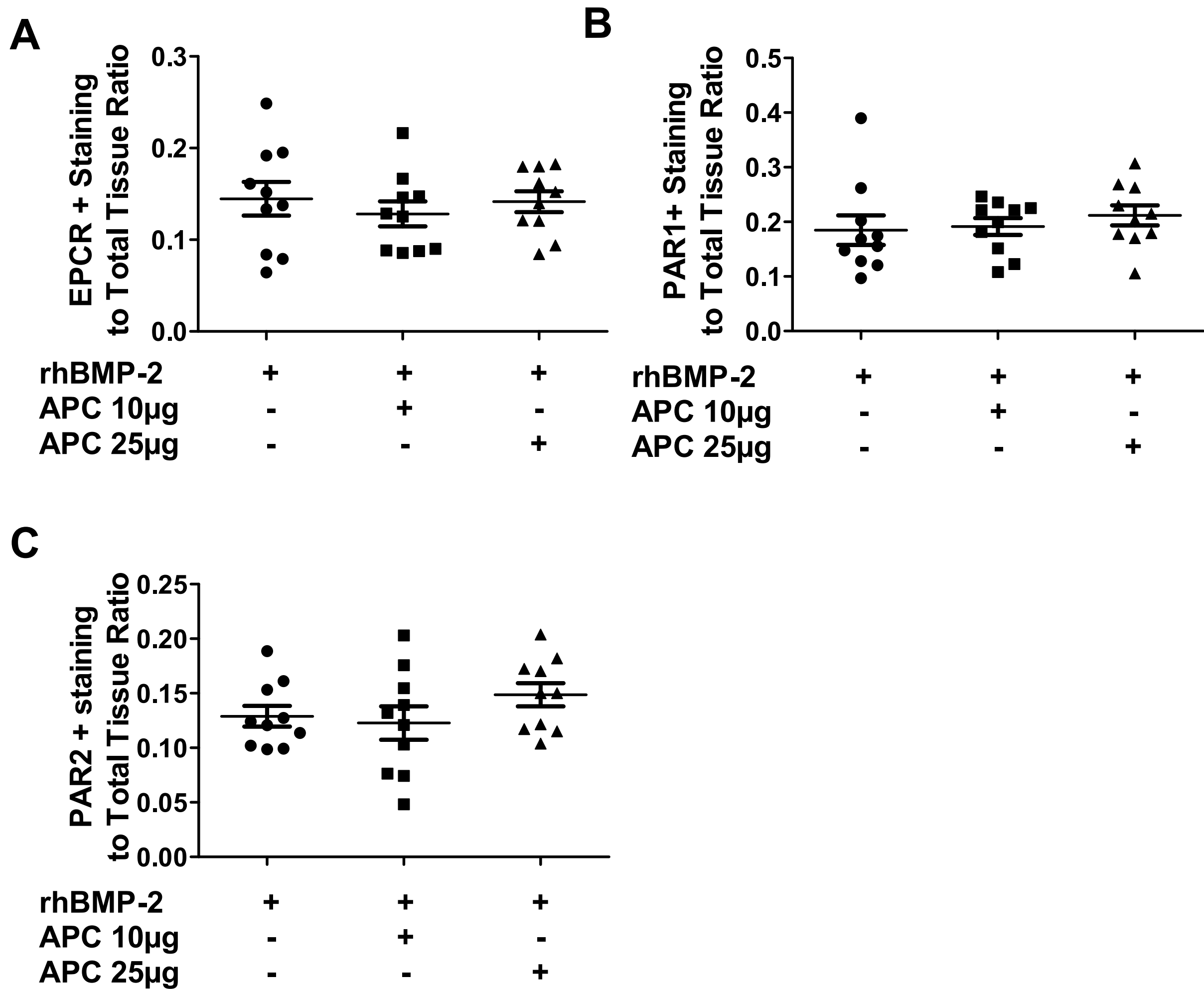

Figure 4-5 *Quantification of EPCR (A), PAR1 (B), and PAR2 (C) in bone nodules. Stained nodules were analysed using ImageScope for positive staining to total tissue ratio. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=10).*

4.4.4 APC-Induced Bone Formation Requires PAR1 but Not PAR2

In Section 3.4.4, we observed that the cell viability of an *in vitro* osteoblast model was dependent upon PAR signalling. Following this observation, we sought to investigate the role of PARs in APC-induced ectopic bone formation. We compared the effects of APC treatment on *Par1*$^{-/-}$, *Par2*$^{-/-}$, and wild-type (WT) mice. Initially, mice were treated with 25 μg APC, which we had experimentally determined to be the optimal APC dose to administer (Section 4.4.1). Then, we investigated whether BMP-2-induced ectopic bone formation was further impacted by an increased APC dose. To this end an additional treatment group of the study was created, whereby a higher dose of 50 μg APC was administered to WT mice.

Experiments with WT mice were conducted using the conditions described in Section 4.4.1. XR images, representative 3D μCT reconstructions, and quantitative μCT results after 3 weeks were compiled in Figure 4-6. X-rays and μCT indicated that inclusion of 25 μg or 50 μg APC led to increases of 43% or 74% in total volume, respectively ($P<0.05$, $P<0.01$; Figure 4-7) and 32% or 46% increase in bone volume ($P<0.05$ for both; Figure 4-7). There was no significant change in BV/TV following APC treatment.

Interestingly, in *Par1*$^{-/-}$ mice, there was no significant difference in bone volume (45%, $P=0.19$; Figure 4-8 & 4-9), total volume (46%, $P=0.27$), or BV/TV (7%, $P=0.58$) in response to APC treatment. However, in *Par2*$^{-/-}$ mice, APC significantly increased total volume by 49% ($P<0.05$) but did not increase bone volume (42%, $P=0.06$; Figure 4-8 & 4-9), nor BV/TV as compared to control (8%, $P=0.41$). These results suggest that PAR1 rather than PAR2 was involved in mediating APC's anabolic effect on ectopic bone formation.

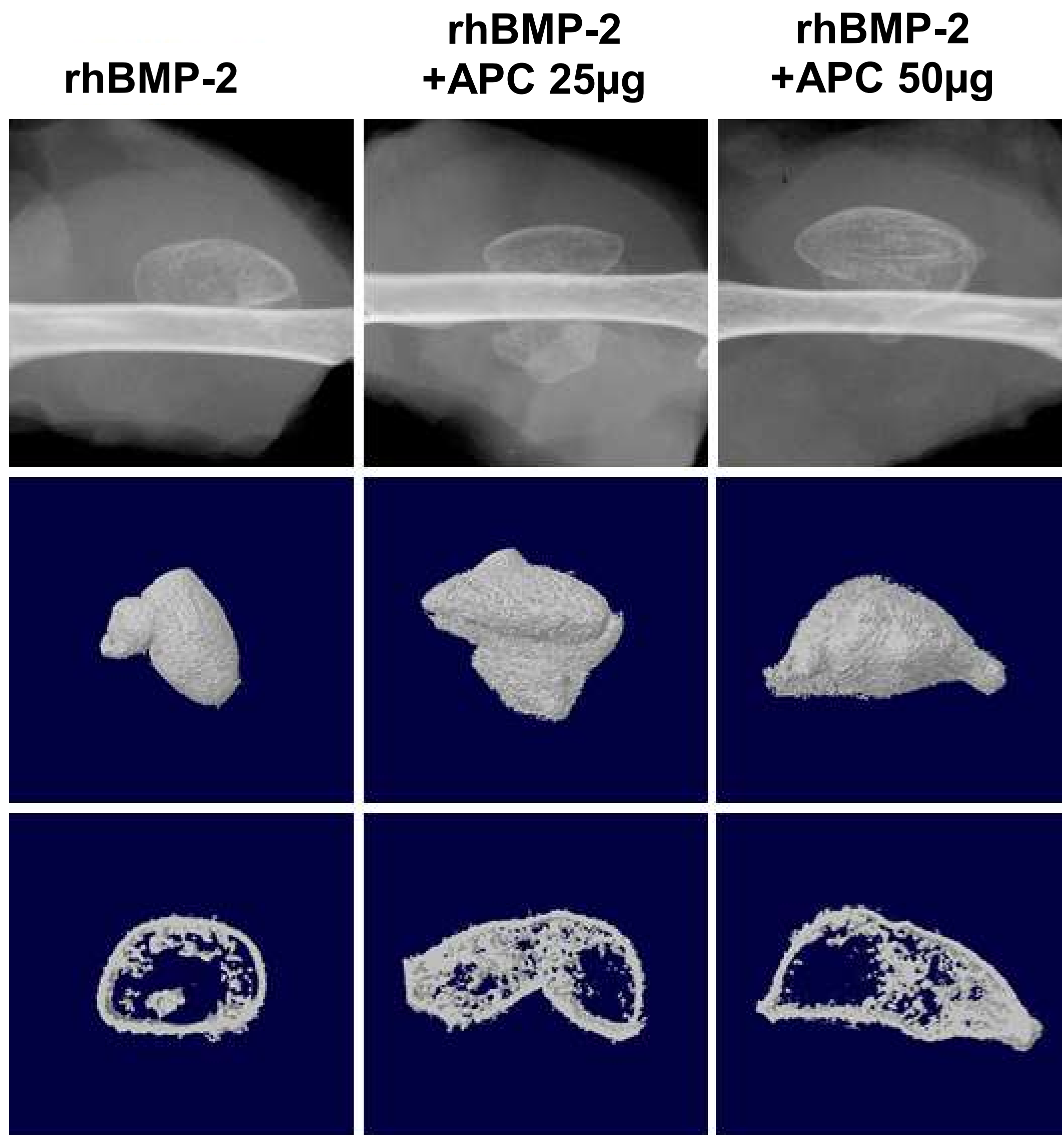

Figure 4-6 *The effect of APC on bone nodules via X-ray and μCT reconstruction in WT mice. The figure shows representative nodules X-rays, 3D μCT reconstructed models, and cross-sectional compilation of 20 slices after 3 weeks of BMP ± APC incorporation.*

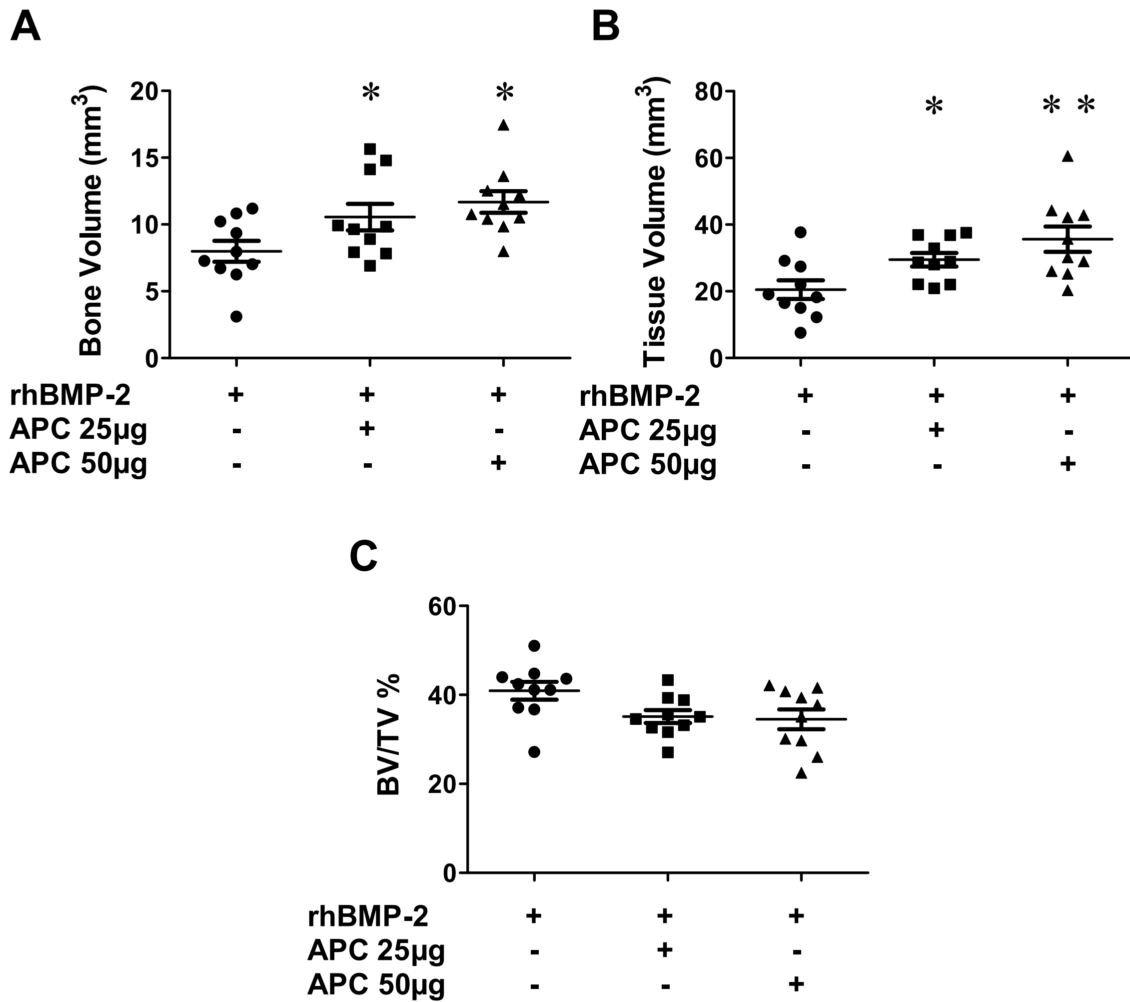

Figure 4-7 *The effect of higher doses of APC on bone nodules via μCT analysis in WT mice. CT analysis was performed on bone volume (A), total volume (B), and the ratio of bone volume to total volume (BV/TV) (C). Statistical analysis was carried out by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=10). * Denotes P<0.05, ** denotes P<0.01 between treatment and control.*

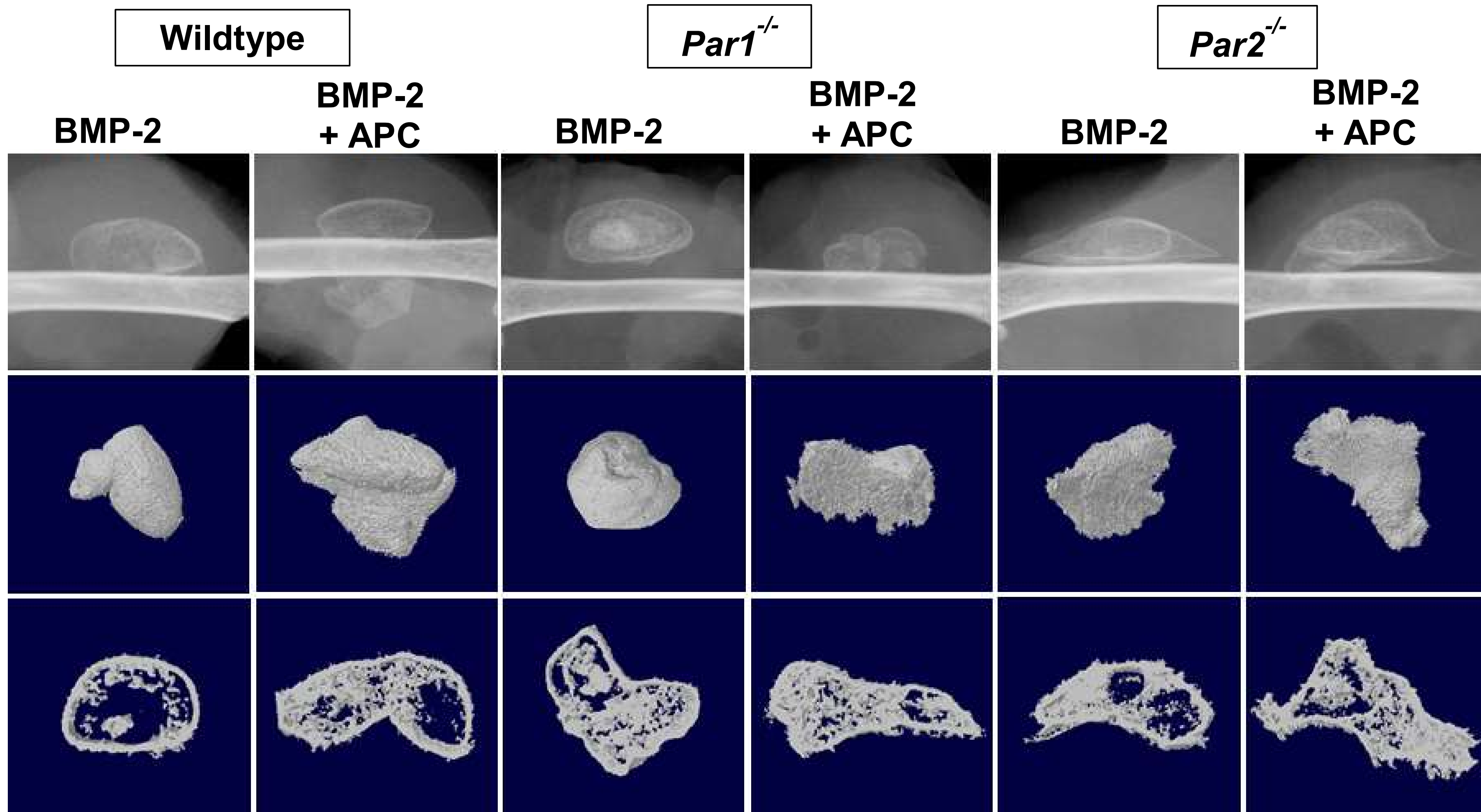

Figure 4-8 *The effect of APC 25 µg on nodules via X-ray and µCT reconstruction in WT, Par1$^{-/-}$, and Par2$^{-/-}$ mice. The figure shows representative nodule X-rays, 3D µCT reconstructed models and cross-sectional compilation of 20 slices.*

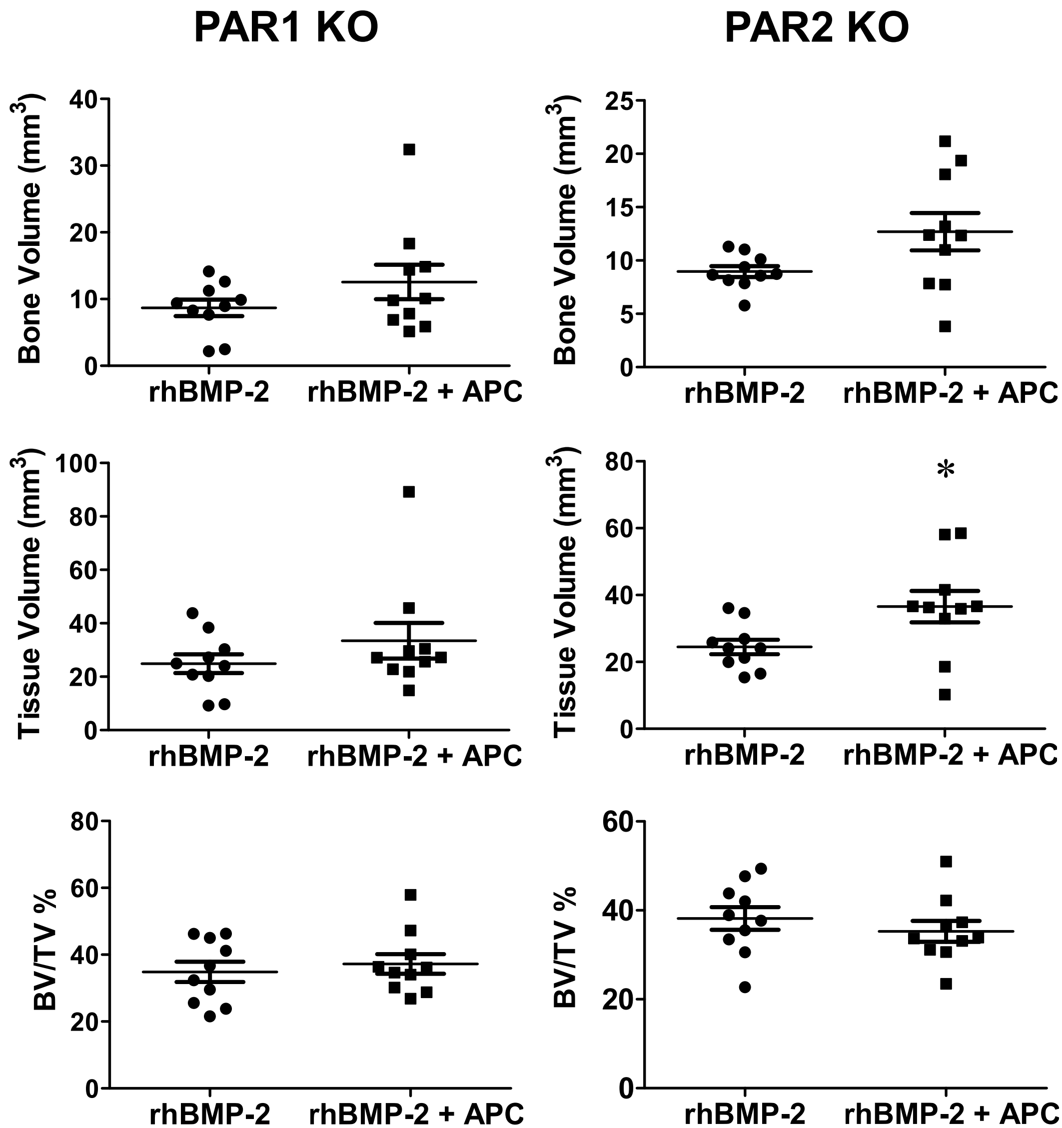

Figure 4-9 *The effect of APC on bone nodules via µCT analysis in Par1$^{-/-}$ or Par2$^{-/-}$ mice. CT analysis was performed on bone volume, total volume, as well as the ratio of bone volume to total volume (BV/TV). Statistical analysis was carried out by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=10). * Denotes P<0.05 between treatment and control.*

4.5 Discussion

Previous studies have demonstrated that the clinical administration of APC has therapeutic benefits on the healing of recalcitrant orthopaedic wounds in patients [309]. We have shown that APC can protect the viability of cultured MG-63 osteoblastic cells via a PAR1- and PAR2-dependent mechanism (Section 3.4.4). In this chapter we sought to further investigate whether APC could also stimulate bone formation. Indeed, we found that local co-delivery of APC augmented BMP-2-induced bone formation *in vivo* and increased osteoclast numbers within bone nodules. Experiments with knockout mouse models revealed that this action of APC was PAR1-dependent, however, PAR2 expression was found not to be required.

In this chapter, we utilised an ectopic bone formation model which requires BMP-2 for bone induction [437]. This model provides an ideal *in vivo* screening tool for determining the anabolic effects of agents, such as APC, on bone remodelling. We observed, by X-ray imaging, a substantial enhancement of bone formation in response to APC, and this increase was confirmed with μCT quantification. These *in vivo* findings, consistent with those of our previous *in vitro* studies, show that APC has an anabolic effect on bone formation [268, 269, 309]. This is a novel finding that highlights the physiological importance of the biological interactions between local coagulation and bone factors. One limitation of the study design is that blind evaluation of μCT outcomes could have reduced the bias of the results. However, we were able to replicate these results in further studies of APC in WT and *Par*$^{-/-}$ mice.

The nature of the *in vivo* model we utilised meant that we were unable to ascertain which effects were due to the specific action of APC, and those which arose from the interaction between APC and BMP-2. APC has not been previously shown to be osteogenic or capable of stimulating ectopic bone formation. Therefore, we assayed the quantitative effect of APC on

ectopic bone formation by utilising APC in combination with BMP-2. It will be necessary for future studies to implement *in vivo* models that are not dependent upon exogenous BMPs, to address the osteogenic potential of APC when used alone.

Aside from the osteogenic potential of APC, it may also functionally interact with BMP-2, in a manner similar to the interactions that have been reported to occur between BMP-2 and other coagulation factors. A prime example is heparin, which binds to BMP-2, leading to increased stabilisation and prolonged half-life, and thus an increase in BMP-2-induced osteoblast differentiation in both *in vitro* and *in vivo* models [441-443]. In contrast, heparin treatment alone actually decreases bone mineral density, suggesting the above effects on bone are mediated via a specific interaction with BMP-2 [444, 445]. Similarly to the effects of APC described in this chapter, heparin addition to BMP-2 induces a dose-dependent increase of mineralised bone tissue in ectopic bone formation as compared to BMP-2 alone [446]. Notably, heparin can also bind to the serine protease domain of APC [447, 448], so it is possible that APC interacts with heparin to potentiate BMP-2-generated ectopic bone formation.

The effect of APC on enhanced bone formation may not only be due to increased osteoblastic viability but also other aspects of osteoblastogenesis. For instance, Lee *et al* have demonstrated that APC enhances osteoblast differentiation through the induction of type I collagen and alkaline phosphatase, both markers of osteoblast maturation [269]. Currently, we have no data to support the role of APC in the induction of osteoblastogenesis within bone nodules as we have not studied the effect of APC on osteoblast histomorphometric parameters in these bone nodules. However, we propose that APC augmentation of bone formation is likely to occur secondary to the multiple effects it has on the osteoblastic lineage; including the stimulation of osteoblastic differentiation and enhancement of cell viability. We suggest that further studies

with APC in ectopic bone nodules can utilise alkaline phosphatase markers to determine the number and effect of APC on osteoblasts within these nodules.

As APC was capable of enhancing bone formation, we sought to further determine whether this effect was reliant on an anabolic action or an anti-catabolic action. Histology of nodules revealed a modest increase in osteoclast number, despite a large increase in bone formation. However, this catabolic effect was diminished when normalised to bone surface area, suggesting that the effect was proportional to the increase in bone surface area. Taken together with APC's stimulation of osteoblasts and its increase of trabecular complexity, it appears that APC can induce dual increases in anabolism and catabolism, with the net effect being anabolic [70]. It is feasible that suppression of osteoclast activity using anti-catabolic drugs such as bisphosphonates may lead to further increases in total bone in response to APC.

The potential benefit of APC in combination with bisphosphonates is not only limited to the inhibition of osteoclasts. Lee *et al* demonstrated the differential effects of APC on osteoblast viability and differentiation in combination with bisphosphonates [269]. The addition of APC protects against pamidronate and zoledronate-induced cell death but enhances alendronate-induced MG-63 apoptosis [269]. The addition of APC to alendronate enhances calcium deposition whereas addition to pamidronate and zoledronate abolishes this effect [269]. APC also enhances type I collagen in all the presence of all 3 bisphosphonates [269]. These disparate effects of APC on bisphosphonate-treated MG-63 cells makes it difficult to predict how combined treatment will translate *in vivo* and whether these combination treatments can enhance bone formation. However, we suggest that APC enhancement of osteoblast viability in the presence of pamidronate and zoledronate, and its promotion of soft tissue healing may

be beneficial in some cases of bisphosphonate-related osteonecrosis of the jaw, a condition which requires both bone and soft tissue healing.

Existing studies have also utilised bisphosphonates in combination with BMP-2 to provide both anabolic and anti-catabolic effects in bone repair models, with mostly positive results [169]. The combined delivery of BMP-2 with the bisphosphonate minodronate in a rat intramuscular ectopic bone formation model demonstrates increased bone area, bone strength, and reduced osteoclast numbers over BMP-2 alone [449]. The combination of BMP-2 with another bisphosphonate, ibandronate, has been trialled in ischemic osteonecrosis of the femoral head where it increased trabecular number, bone volume, and thickness at the same time as it suppressed osteoclast numbers [450]. Additionally, combined bisphosphonate-BMP-2 treatment is effective in the genetic orthopaedic model of neurofibromatosis type 1 (NF1), a condition manifested by increased bone catabolism and decreased anabolism [451]. The delivery of local BMP-2 with systemic zoledronate injection maximised bone production and reduced osteoclast numbers in wild-type and NF1 deficient mice [452]. Despite the promising results from these studies, higher doses of bisphosphonates can result in impaired orthopaedic function, even in the presence of BMP-2 [169, 453]. This suggests that bisphosphonate augmentation of the effects of BMP-2 requires a low dose of bisphosphonates. It is yet unknown whether the triple combination of BMP-2, bisphosphonates, and APC can further improve the existing outcomes of dual BMP-2-bisphosphonate treatment. This would depend on whether APC addition can lower the required dosage of bisphosphonate without compromising orthopaedic outcomes. Alternatively, as APC is protective against bisphosphonates in MG-63 cells, APC addition to high doses of bisphosphonates may defend against the adverse effects of bisphosphonates.

As we found EPCR and PAR involvement in APC-induced effects on MG-63 cells, we further determined their role in APC-augmented bone formation. APC has been shown to up-regulate EPCR expression in keratinocytes [260], however, it is unknown whether APC or BMP-2 regulates EPCR, PAR1 or PAR2 expression in osteoblasts. We found no difference in EPCR or PAR expression in the presence or absence of APC when bone nodules were excised after their formation at 3 weeks. We also did not notice EPCR or PAR expression on bone lining cells or cuboidal cells on bone surfaces. One explanation for this is that APC may have exerted its effects early in the ectopic bone formation process and thus EPCR and PAR receptors on bone lining cells were not upregulated at 3 weeks. We did not conduct pharmacokinetic or pharmacodynamic experiments to determine the distribution or half-life of APC, so we cannot be certain whether APC affected the expression of these receptors at earlier time points, and whether this effect has subsided over time.

PAR1 and PAR2 play important roles in bone healing with PAR1 primarily mediating the proliferative effects of thrombin on osteoblasts [385, 405]. *Par1*$^{-/-}$ mice have reduced mineralisation and greater osteoclast presence in the early stages of healing but no morphological differences in the later stages of healing [385]. PAR1 has been involved in many actions of APC including its protection of endothelial cells, neurons, podocytes against apoptosis and injury, its maintenance of the endothelial barrier, its induction of a wound healing phenotype in keratinocytes, and importantly its mediation of APC's angiogenic effects [261, 262, 342, 353, 371, 372, 454]. Kurata *et al* have shown that PAR1 is not required for the proliferative effects of APC on normal human osteoblasts. In contrast, we have shown in Section 3.3.3 that PAR1 was involved in APC-mediated MG-63 and MC3T3-E1 viability. To further investigate the involvement of PAR1 in bone formation, we applied APC to the ectopic bone formation model in *Par1*$^{-/-}$ mice and found that APC-induced bone volume and total

volume was abolished, confirming that PAR1 is required for APC-augmented ectopic bone formation. There was considerable variability largely due to a single outlier in the APC-treated group. However, statistical analysis after the removal of this outlier also showed no significant difference, confirming the important role of PAR1 in APC and BMP-2-induced ectopic bone formation.

Similarly to *Par1*$^{-/-}$, *Par2*$^{-/-}$ mice exhibit decreased mineralisation of callus formation in femur fractures compared to WT animals, as assessed by bone volume fraction and total mineral density [455]. However, the genetic deletion of PAR2 does not result in delayed fracture healing [455]. Currently, the role of PAR2 in osteoblasts is unclear, although, it has been shown to stimulate intracellular calcium levels, and type I collagen expression, and to have no apparent effect on alkaline phosphatase activity [24]. PAR2 can also regulate RANKL/OPG ratio in osteoblasts to decrease osteoclast differentiation [456]. In the current study using *Par2*$^{-/-}$ mice, APC elevated total volume but not bone volume or BV/TV. The lack of significance may be attributed to the unexpected larger biological variation seen in the *Par2*$^{-/-}$ mice as compared to both WT and *Par1*$^{-/-}$ mice. A greater sample size of *Par2*$^{-/-}$ mice may show that APC does induce bone volume.

4.6 Summary

The healing of bone injuries is both frequently and dramatically impeded by a lack of biological factors. To date, a large body of experimentally derived evidence has demonstrated the ability of APC to significantly enhance the healing of soft tissue wounds. In this thesis, we present novel data that for the first time points to the additional clinical utility that APC may possess in the treatment of bone fractures.

Firstly, in Section 3.4.2, we demonstrated that APC enhances the viability of osteoblastic cell lines, and we hypothesised that APC would enhance ectopic bone formation in a rapid screening *in vivo* model. In this chapter, we tested this hypothesis, and we did indeed observe that APC augmented BMP-2-induced ectopic bone formation, an effect that was found to be PAR1-dependent.

APC treatment led to an increase in osteoclast numbers. This suggests that APC works via multi-faceted actions, on different cells within the bone environment including osteoblasts and osteoclasts. The discovery of these novel findings show that the healing abilities of APC may also be extended to the treatment of bone injuries. We further propose that it will now be important to investigate APC in other orthopaedic models, particularly those of fracture healing.

CHAPTER 5:

THE EFFECT OF APC ON

CLOSED FRACTURE HEALING

5 The Effect of APC on Closed Fracture Healing

5.1 Introduction

The union of a fracture depends on an adequate cellular environment, sufficient growth factors, a bone matrix, absence of infection, and mechanical stability [70, 90, 97, 457, 458]. In addition, factors such as age, gender, smoking, systemic corticosteroid therapy, diabetes, fracture severity, the metabolic and nutritional state of the patient, and whether fracture was open or closed, can all contribute to delayed healing [146, 459-466]. In the previous chapters, we have found that APC augmented osteoblast viability *in vitro* and it worked adjunctively to enhance BMP-2-induced bone formation *in vivo*. This potential for APC to enhance bone formation suggested that it may have therapeutic effects in fracture healing.

Both ectopic bone formation and fracture healing share similarities, including the recruitment of local factors into the area and the induction of new bone formation [94, 467]. BMPs stimulate the migration of mesenchymal stem cells to the site of implantation [52]. These progenitor cells first differentiate into chondrocytes, forming islands of cartilage that are similar to the cartilaginous soft callous formation observed in fracture healing [468, 469]. This is followed by vascular invasion, hypertrophy and mineralisation of chondrocytes, which is also comparable to the hypertrophy of soft callus in fracture healing [470]. Finally, the subsequent migration of osteoblasts into the site, bone formation, and remodelling parallels the hard callus and remodelling stages of fracture healing [469, 470].

Different models of fracture healing are available to assess the repair of long bones after a fracture. A common model of bone repair in rodents is a closed fracture, generated by blunt trauma [471]. As closed fractures involve less damage to soft tissue than open fracture models, they can achieve greater experimental reproducibility by minimising the variability introduced

by local wound healing [471]. Also, the study of osteoblastic and osteoclastic lineages in open fracture models of bone healing is complicated by myogenic progenitors that contribute to the repair process [472]. Furthermore, it has been observed that moderate soft tissue trauma delays bone healing in the early phases, but advances regeneration at the later stages [473]. Of the available models, the mouse closed tibial fracture models have been described as the most faithful replication of common clinical fractures in terms of anatomical site, aetiology, and fixation in this species [474]. For these reasons we chose to study the effect of APC in this model system.

BMP-2 promotes bone formation and repair of critical size defects in animal models [469, 475]. In rodent models of fracture healing, the expression of BMP-2 protein is present in intramembranous and endochondral ossification at early stages but also present in trabecular bone at later stages [476, 477]. A deficiency of BMP-2 in mice results in delayed formation of secondary ossification centres at an early age and a complete absence of fracture healing occurs later in life once weight-bearing commences [51]. Clinical use of rhBMP-2 with a collagen sponge carrier has been approved by FDA for use as a bone graft in conjunction with internal stabilisation to heal a fresh open fracture of the tibia [169, 478]. Therapeutic use of BMP in non-union demonstrates a comparable time to union as bone graft substitutes and BMP application avoids the need for a donor site [479, 480]. However, off-label BMP application can induce complications including immunogenic responses, heterotopic bone formation, premature bone catabolism, local inflammation and oedema, graft failure, pseudarthrosis, and surgical site infection or complications [141, 142, 169, 475]. This suggests that many factors must be considered before the clinical use of BMPs.

The application of APC with topical negative pressure was shown to promote soft tissue healing in patients with chronic wounds post-orthopaedic surgery that had failed to respond to conventional therapy [309]. Based upon this observation, along with our previous findings that APC enhanced both osteoblast cell line viability and ectopic bone formation; we hypothesised that in a fracture healing model, exogenous APC treatment will promote bone repair. This effect may be mediated by enhancement of premature callus remodelling by osteoclasts.

5.2 Aims

Following results from the previous chapter, we further examined the potential effect of APC on fracture healing *in vivo*. Specifically, we aimed to:

1. determine the effect of APC on healing in closed murine fractures over 3, 7, and 21 days, and whether APC can enhance bridging of fractures or callus formation; and
2. determine the effect of APC on osteoclast numbers in fracture calluses.

5.3 Methods

5.3.1 Murine Closed Fracture Model

To investigate the effect of APC on fracture healing, a closed murine fracture model was employed. Closed fractures were induced in 8 week old female C57BL6 mice under anaesthesia and allowed to heal over 21 days. APC treatment was administered by anterolateral and posteromedial axis injections into the site of injury. Mice were subsequently treated either with biweekly treatments of 25 μg APC over 3 weeks (i.e. 25 μg ×5) or a 50 μg bolus dose, to compare between single dose and multiple dosage treatments of APC (Table 2-8, Section 2.3.3) Fracture healing endpoints were at 3, 10, and 21 days.

Three samples of fracture calluses were collected from treated mice on days 3 and 10. Ten samples were collected per treatment on day 21. Fractures were radiographed by X-ray (XR) at 3, 7, 10, 14, and 21 days, and visually assessed for union. Calluses at day 21 were analysed using μCT and CTAn as per Section 2.3.2. All animal experiments were approved by the Animal Care and Ethics Committee for the Children's Medical Research Institute and The Children's Hospital Westmead (Protocol K248).

5.3.2 Histology of Fractures

After fractures were scanned in μCT, they were processed for histology, similarly to ectopic bone nodules. Briefly, the samples were fixed overnight in 4% PFA, stored in 70% alcohol for 24 h, and then incubated in 30% sucrose overnight. Fractures were then cryoembedded in Tissue-Tek OCT Compound (Sakura Finetek). Cryosections were cut until a parasagittal section of the fracture was reached and further sectioned to 5 μm by cryostat (Leica, CM1900) onto cryofilm (Section Lab-Co Ltd). Sections were then adhered as per Section 2.3.4.

5.3.3 Staining of Fractures

After histology was completed, sections were stained for TRAP similarly as ectopic bone nodules to assess osteoclast numbers in calluses (Section 4.3.2). In brief, TRAP staining was conducted using naphthol ASBI phosphate substrate, tartaric acid, new fuchsine dye, and sodium nitrite after washing in buffers. Once stained, cryosections were then counterstained with haematoxylin, coverslipped, and osteoclast numbers were counted using Bioquant Software (BioQuant).

5.3.4 Statistics

Statistical analyses and data graphing were performed as per Section 2.4. *P* values less than 0.05 were considered statistically significant. Results are presented as mean ± standard error (SE) performed in duplicate. Fisher's exact test was employed to assess union where appropriate.

5.4 Results

5.4.1 APC Treatment Does Not Enhance Closed Fracture Healing

X-rays of fractures from 3, 10, and 21 days illustrated that tibia were broken in all animals at the time of surgery, with close alignment of the broken ends, small degrees of axial rotation, as well as minimal damage to the surrounding soft tissues (Figures 5-1, 5-2, 5-3). Fibulas were broken in 36 out of all 48 mice. On day 3, there was no new bone formation seen on radiographs (Figure 5-1). After 7 days, there was no radiographic evidence of calluses (Figure 5-3). By day 10, X-rays indicated the early formation of calluses at ends of the fracture site although none of the fractures were bridged by this callus, regardless of treatments. At 14 days, 50% of all fractures were bridged by callus in the control group, 40% in the 25 μg×5 APC group, and 40% in the 50 μg APC bolus group. At day 21 all fractures formed fully enclosed hard calluses. Illustrative X-ray images of mid-tibial fractures are presented in Figure 5-3.

By day 14, injection into the fracture site was made difficult by the hard callus formation and APC (25 μg×5) treatments beyond this time were injected locally into the soft tissue and allowed to passively diffuse into the hard tissue. Prior to the completion of the experiment, at day 16, one of the mice died due to unknown causes.

X-ray and 3D modelling of representative fracture at 21 days did not demonstrate any obvious increase in volume that could be associated with APC treatment (Figure 5-2). All of the fractures achieved union regardless of treatment, and Fisher's exact test yielded P=1 between treatment groups and control. 3D μCT reconstructions of coronal fracture cross-sections showed no obvious change in callus architecture in APC-treated specimens (Figure 5-4).

Quantification of total tissue volume (TV) indicated that there was no statistical significance between APC treatment and saline control. Biweekly treatments of 25 µg or 50 g bolus of APC induced no difference (75%, *P*=0.28; average TV=0.14 mm^3, SE=0.04 mm^3 and 61%, *P*=0.2; average TV=0.23 mm^3, SE=0.05 mm^3 respectively; Figure 5-5A) as compared to control (average TV=0.14 mm^3, SE= 0.04 mm^3; Figure 5-5A). There was a large variation in tissue volume due to an outlier in APC 25 µg (×5) treatment. The removal of this outlier resulted in no significant difference between APC treatment and control (22%, *P*=0.63; Figure 5-5A).

Quantification of bone volume (BV) indicated that there was no statistically significant change between biweekly APC 25 µg or 50 µg of APC bolus treatment (20%, *P*=0.64, average BV=0.08 mm^3, SE=0.02 mm^3 and 31%, *P*=0.44, average BV=0.09 mm^3, SE=0.02 mm^3 respectively; Figure 5-5) as compared to saline control (average BV=0.06 mm^3, SE=0.02 mm^3, Figure 5-5). Bone volume over tissue volume (BV/TV) assessment revealed no significant changes between biweekly APC 25 µg treatment or 50 µg of APC bolus treatment as compared to control (20%, *P*=0.11, average BV/TV = 43%, SE = 5.5% and 22%, *P*=0.06, average BV/TV = 42%, SE = 3.3%; Figure 5-5C).

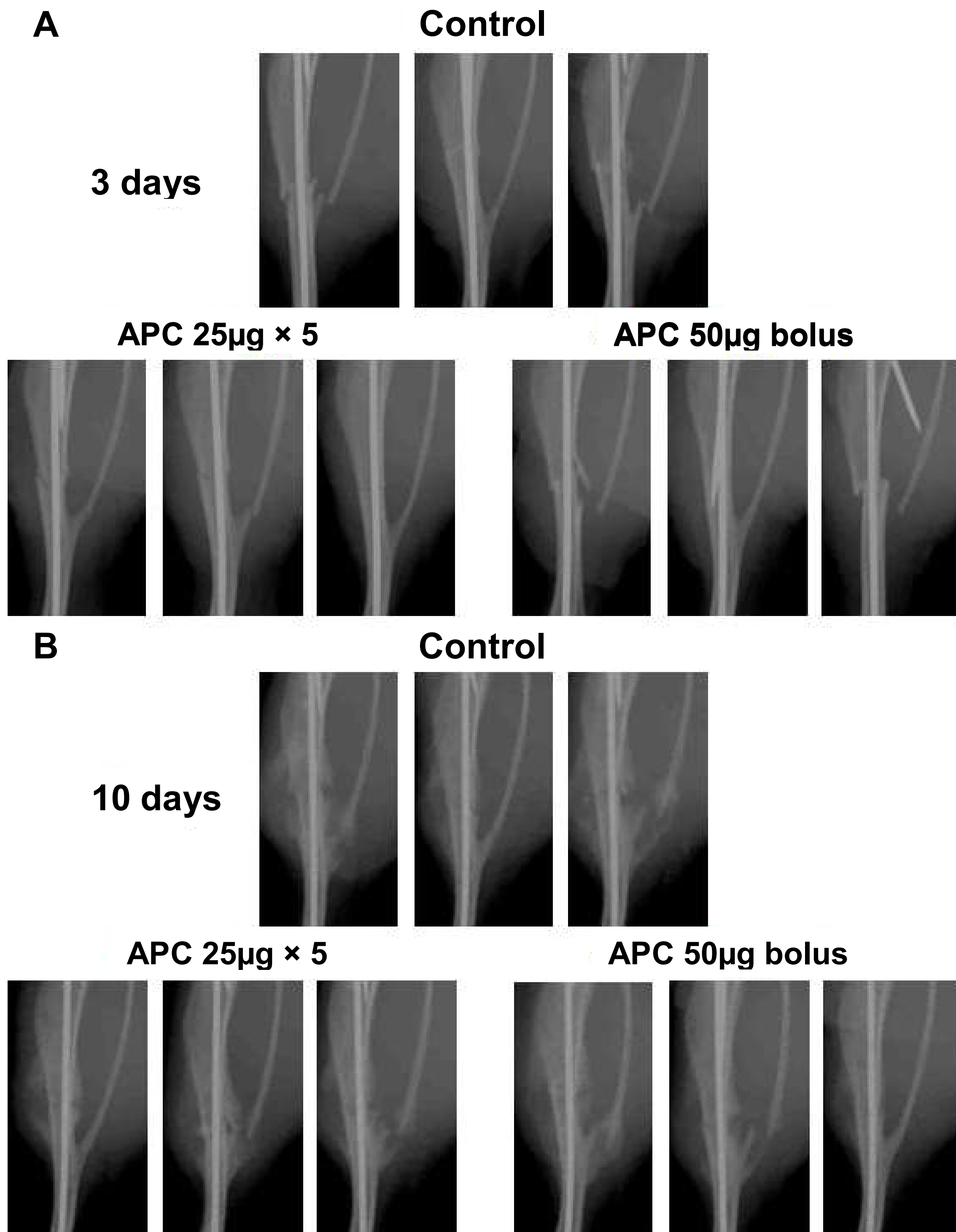

Figure 5-1 *X-rays of murine closed fracture healing on days 3 and 10. Biweekly treatments of APC (25 µg, x5), APC (50 µg, bolus), or saline control were injected into fracture calluses. X-rays were taken with intact intramedullary pins at 3 days (n=3 per group, A) and 10 days (n=3, B) per group.*

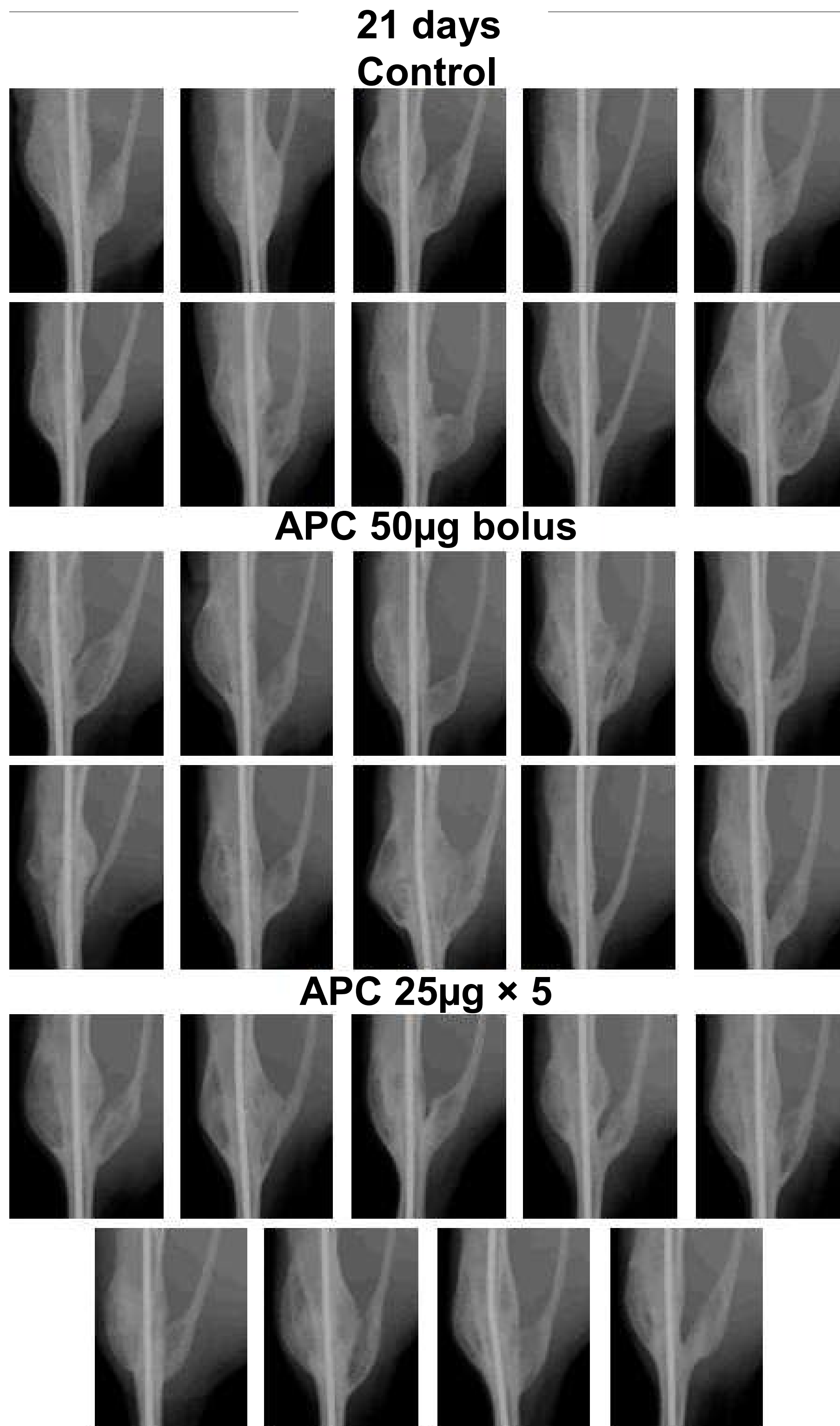

Figure 5-2 *X-rays of murine closed fracture healing on day 21. Biweekly treatments of APC (25 µg, x5), APC (50 µg, bolus), or saline control were injected into fracture calluses. X-rays were taken after 21 days. N=10 per group except APC 50 µg (n=9).*

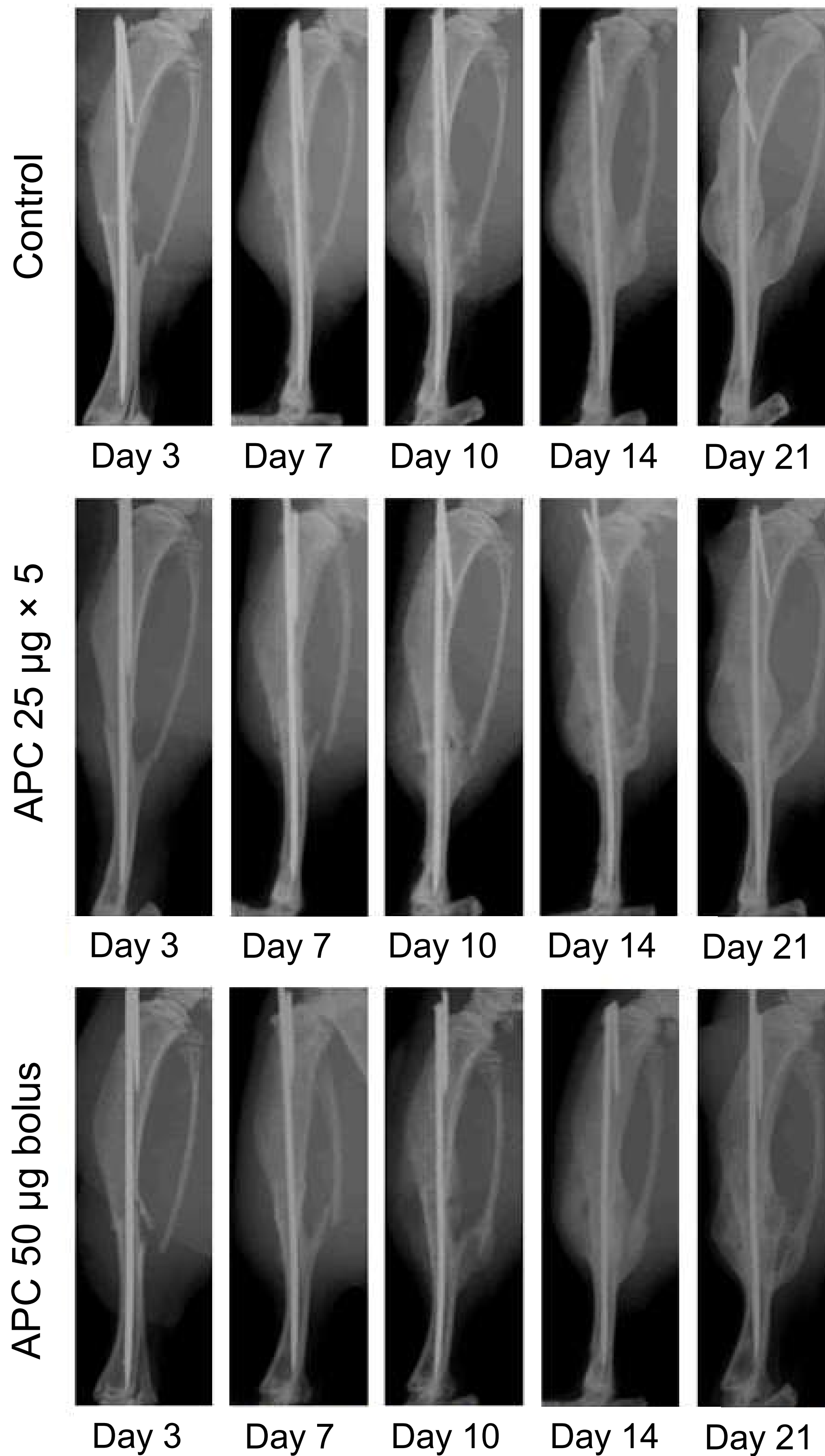

Figure 5-3 *Representative X-rays of closed mid-tibial fractures. Control and APC-treated closed fractures were X-rayed on days 3, 7, 10, 14, and 21.*

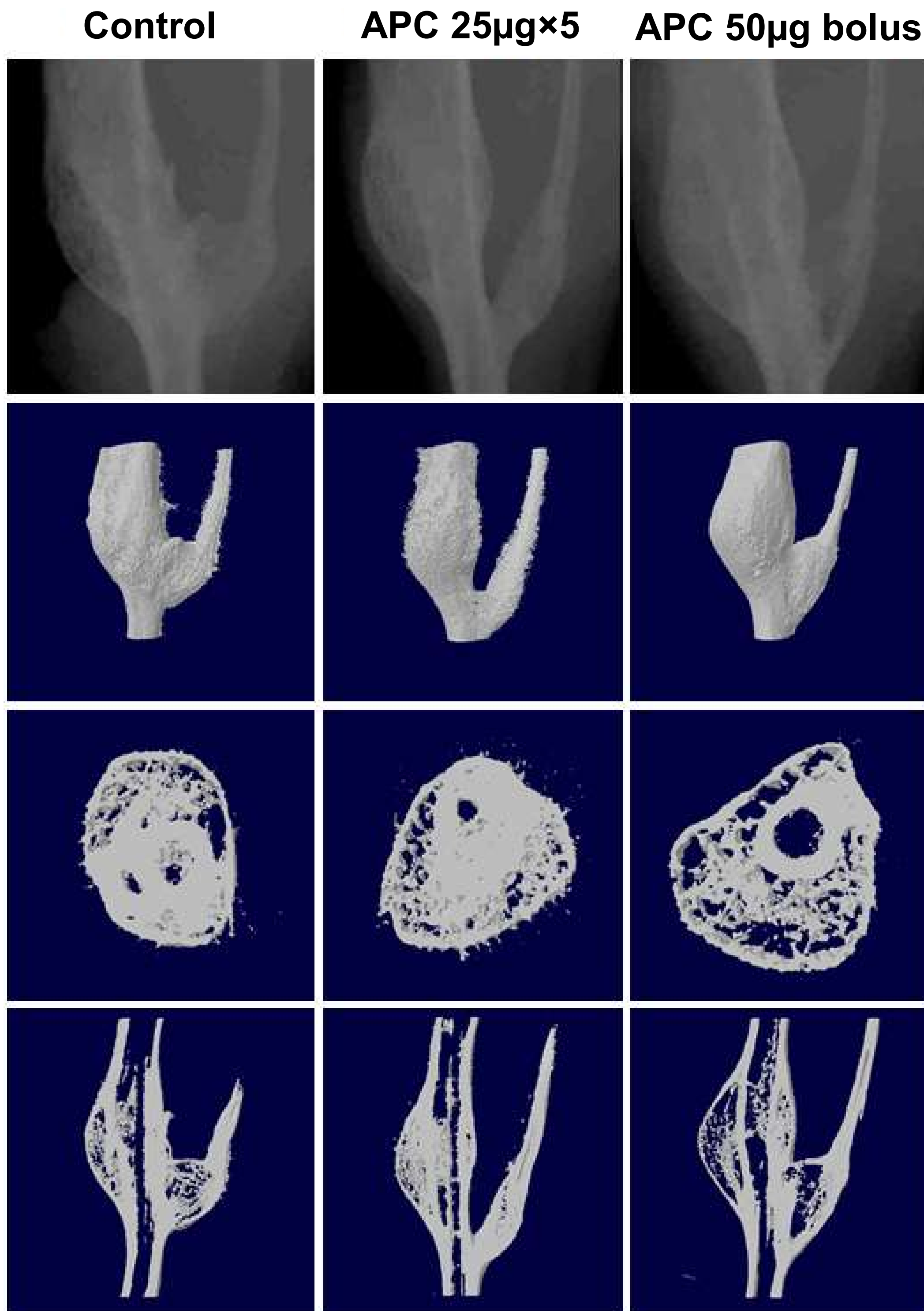

Figure 5-4 *The effect of APC treatment on murine closed fracture healing X-ray, 3D reconstruction, and transverse/coronal cross sections. Representative closed fractures from Figure 5-2 were X-rayed at 21 days, reconstructed as 3D models, reconstructed from 20 slices of transverse, and coronal cross sections.*

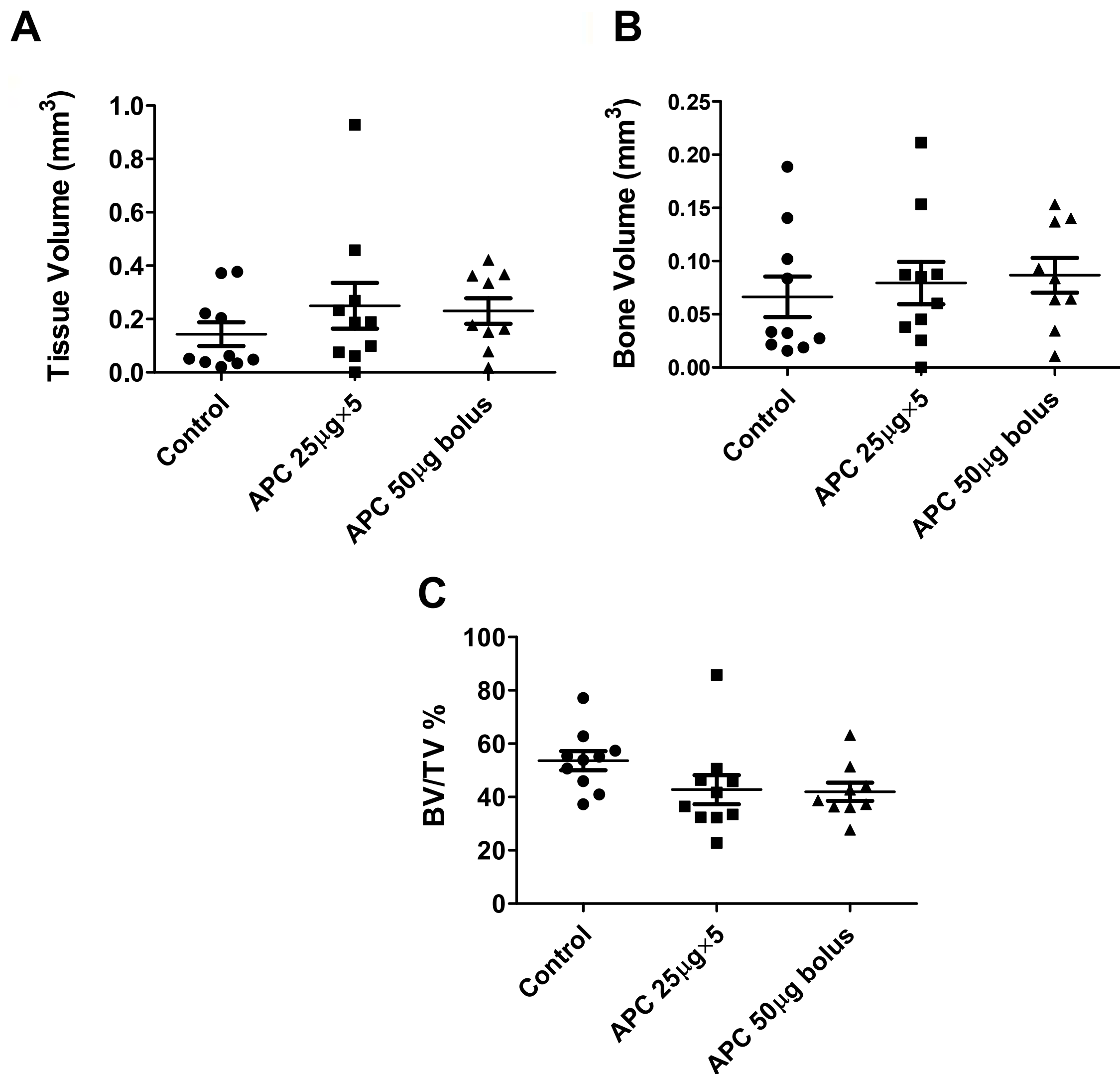

Figure 5-5 *The effect of APC treatment on murine closed fracture healing via µCT analysis. Closed fractures treated with biweekly APC 25 µg ×5 or APC 50 µg bolus were assessed for total volume (A), bone volume (B), or bone volume/total volume ratio (BV/TV) (C), after 3 weeks. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E (n=10, except APC 50 µg at 21 days where n=9).*

5.4.2 APC Does Not Affect Osteoclast Numbers in Fractures

TRAP staining was performed to determine the effect of APC on osteoclast number (Figure 5-6). APC treatment of 25 µg×5 or 50 µg bolus had no effect on TRAP+ cells in the callus at 21 days (17%, *P*=0.1, 22%, *P*=0.1 respectively; Figure 5-6). This was consistent with the lack of change in callus size (i.e. total volume).

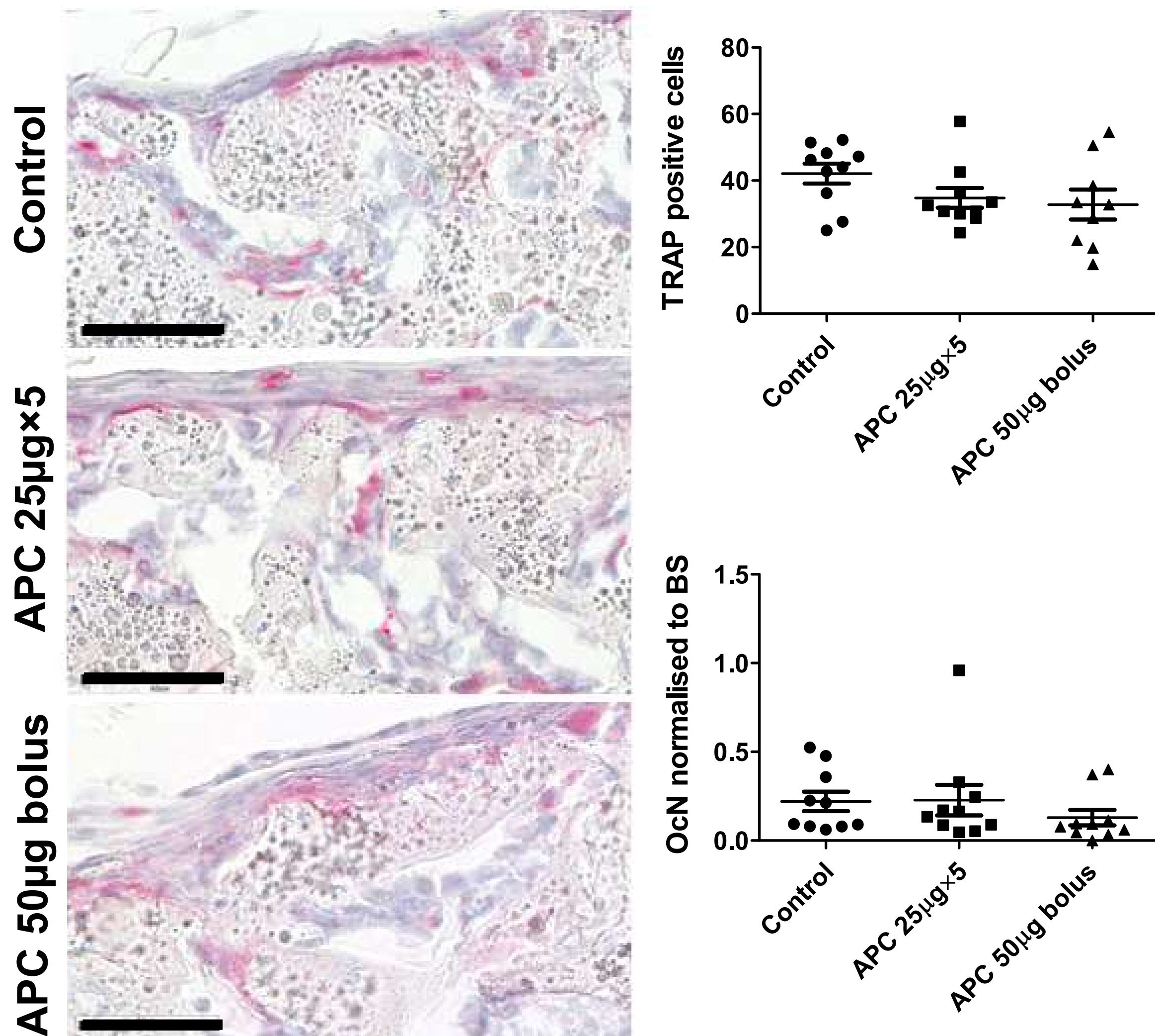

Figure 5-6 *The effect of APC treatment on osteoclast number in closed fractures. After 3 weeks of treatment, fracture calluses were sectioned and osteoclasts were stained for TRAP (red). TRAP-positive cells were counted in five random fields of view at 20X. Results were graphed as osteoclast numbers (OcN) and normalised to bone surface area (BS). Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data represented as mean ± S.E. (n=10, except APC 50 µg bolus where n=9). Scale bar indicates 60µm.*

5.5 Discussion

APC is well-documented to promote soft tissue healing, and in Section 4.4.1 we showed that APC can augment BMP-2-induced ectopic bone formation [290, 291, 309]. Here, we sought to additionally investigate the potential of APC in the treatment of healing bone fractures. However, assessment of the primary outcome found no radiographic evidence for enhanced callus formation or shortened bridging time following APC treatment. Nor did we observe an effect from APC on the number of TRAP-stained osteoclasts in this model. This outcome differs from the positive effects of APC we had previously observed on BMP-2-induced ectopic bone nodules. These findings are at first indicative of APC not possessing the ability to accelerate bone fracture healing; however, several limitations of the experimental model need to be addressed, before we can confidently draw such a conclusion.

One major limitation of this study was that we chose to utilise a murine closed midshaft fracture model. Such fractures models are low energy trauma, with little disruption of periosteum and sufficient vascular supply for regenerative factors provision at the site of injury. Therefore, closed fracture models demonstrate reduced time to union and requires little intervention [369, 472, 481]. Indeed, all fractures observed in this study rapidly healed within 3 weeks and possessed prominent callus formation. In hindsight, any therapeutic effect of APC might not be observable in such a system that already heals efficiently without intervention. For this reason, investigation of APC should be carried out in complex fracture models that are more challenging to heal. For example, open fracture models exhibit extensive damage to the periosteum, which compromises the healing process by preventing vascular ingrowth and migration of osteoprogenitor cells [90, 369, 472, 482]. Clinically, open fractures also require aggressive debridement to prevent infection, which can further deprive the access of osteoprogenitors to the healing site, increasing the risk of non-union [95, 127, 309]. The ability

of APC to heal soft tissue could also further benefit open fracture healing [290, 291, 309]. The use of a distal tibial model, where limited accessibility to osteoprogenitor cells from the bone marrow or soft tissue, could present another method of investigating whether APC can recruit essential factors to the fracture site [369, 483]. APC heals by induction of angiogenesis and restoration of biological factors [248, 265, 292, 484], suggesting that it may have more potential in a high trauma situation such as open fractures.

Another animal model in which APC may be utilised is one of osteomyelitis. Models species that are commonly utilised to study osteomyelitis are rabbits, rats, and mice, all of which can readily undergo surgical interventions that include the use of metal implants and screws [485]. Rats have become the animal of choice in osteomyelitis models due to the detailed knowledge of their immune system, their size, ease of handling, and lower costs. They also enable bioluminescent imaging of bacteria's metabolic activity without the need to sacrifice the animal, thereby markedly reducing cost and the number of animals required for such studies [485]. Our lab has recently developed an osteomyelitis rat model induced by the inoculation of open fractures with *Staphylococcus aureus* bacteria [486]. The potential for APC to heal orthopaedic wounds with underlying osteomyelitis suggests that it may also benefit osteomyelitis in animal models [309]. We hypothesise that this effect, at least partly, will rely on the angiogenic properties of APC; the increased local blood supply enabling a more efficient removal of pathogens, that is typically only achieved surgically.

A key difference between the ectopic bone formation study described in Chapter 4 and the fracture healing study in this chapter was the use of BMP-2 in the ectopic model. As previously discussed in Section 4.5, there may be an additive effect resulting from combined APC and BMP-2 treatment. The application of BMP-2 with APC together in an open fracture or critical

defect rat model would be an additional method of investigating the combined effects of these agents. BMPs have been long investigated in critical defect, delayed, and non-union models, however, they are limited by some of their adverse side-effects [15-17, 28, 29]. Combination therapy warrants future investigation, as it may allow a reduction of BMP's therapeutic dose and therefore minimise its associated side-effects.

APC efficacy is dependent on a sufficient concentration reaching the target tissue. This can be limited by several factors. Firstly, APC has a short half-life of ~15 min, and may be quickly depleted [487]. If so, a single bolus treatment may not be sufficient to treat fractures. However, there is a precedent for a single dose of APC exerting cytoprotective and healing effects over more than 12 days in a rat skin wound healing model [265]. Despite this, we did not find an increase in callus BV or TV with the bolus APC application. Furthermore, osteoclast numbers were not affected by APC treatments at 3 weeks. It is possible that osteoclast numbers at earlier time points could have been altered by APC.

Timing of APC delivery was an issue that we attempted to address in this study by using two separate dosing regimens. It is well known that the beginning of fracture healing requires an inflammatory stage comprising of IL-1, IL-6, and TNF−α secretion which is essential for chemotaxis of osteoprogenitors and initiating the healing process [70, 89]. APC down-regulates inflammatory cytokines, including TNF-α, IL-6, and IL-1, through the suppression of NF-κB [265, 270, 274, 294, 301, 324]. This suggests that early treatment of fractures with high APC doses may suppress the inflammatory response thereby delaying the healing process. Although APC treatment of 25 μg was carried out biweekly so that the effect of APC on the later phases of fracture healing was also incorporated, we still saw no evidence of fracture healing. To overcome the potential anti-inflammatory effects of APC on early fracture healing,

APC treatment can be applied in fracture responses which exhibit abnormal levels of inflammation including osteomyelitis/high impact traumatic fractures.

Lastly, it may have been the case that APC did not reach therapeutically effective concentrations, due to the use of soft-tissue injection as our method of drug delivery. Administration of APC into the injury site became very difficult after callus formation, and therefore most injections had to be alternatively delivered into soft tissue surrounding the calluses. Although we were unable to ascertain as such, it may be the case that little or no APC was able to diffuse from these injection sites into callus [488]. This is similar to the application of BMPs where a controlled local delivery was required due to their short half-life. This issue was resolved for rhBMP-2 using collagen scaffolds, which provided greater sustained release when compared to buffer alone [489]. Based on this, we propose that future studies utilising sustained release such as collagen scaffolds, may provide a linear release of APC and demonstrate efficacy [490, 491].

5.6 Summary

Fracture repair is an important process that is dependent on vasculature and mechanical stability. Current treatments for fracture repair, although providing some clinical benefit, are associated with several limitations including inflammation, immunogenic responses, induction of bone resorption, and surgical site infection. This suggests the need for alternative therapies in fracture repair.

In Chapter 3 and 4, we demonstrated that APC can both enhance osteoblastic viability and ectopic bone formation, which suggested that it may be an effective therapy in bone repair. Nevertheless, in a murine tibial fracture model, two dosing regimens of APC resulted in no

changes in fracture healing, including union rates, callus BV and TV, and osteoclast numbers. Several limitations of this model were identified, particularly in terms of the robustness of healing seen in untreated controls and the delivery of the APC. Further studies using alternative models that show more deficient healing responses and/or feature infection may be appropriate for investigating the orthopaedic effects of APC.

CHAPTER 6:

APC SUPPRESSES VIABILITY AND INFLAMMATION IN OA AND RA HUMAN BONE-DERIVED CELLS (HBDCs)

6 APC Suppresses Viability and Inflammation in OA and RA HBDCs

6.1 Introduction

Bone homeostasis is balanced by the processes of osteoblastic bone formation and osteoclastic bone resorption [8, 9, 11, 83]. This homeostasis is altered in diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA). In RA, chronic inflammation with intense cytokine production, inflammatory cell infiltration, and synovial fibroblast proliferation leads to aggressive pannus formation [492]. Cytokines, including TNF-α, IL-1β, IL-6, and IL-17 inhibit osteoblast maturation through the down-regulation of alkaline phosphatase and type I collagen expression, and stimulates osteoclast formation and activation [196, 201]. RA osteoblasts adjacent to inflammatory synovium express a paucity of mature markers and an inability to mineralise bone, which together with an increased rate of bone resorption, leads to an overall decrease in bone formation [194, 195, 201, 493]. Synovial pannus also mediates the degradation of cartilage and bone matrix by proteinases such as MMP-2 and MMP-9 [183]. These pathological changes result in irreversible joint damage.

In contrast to RA, OA is not traditionally considered an inflammatory arthritis. However, acute synovial inflammation does occur in the early stages of OA, and this is characterised by synovial hypertrophy, hyperplasia, and increased lymphocyte infiltration [494]. Pro-inflammatory cytokines within the OA joint, including TNF-α and IL-1β, are responsible for enhanced catabolism by inhibiting ECM synthesis by chondrocytes, and stimulating secretion of proteolytic enzymes such as MMP-2 and MMP-9 [225, 283, 495]. TNF-α and IL-1β also stimulate IL-6 production by osteoblasts, which deregulates osteoblast-osteoclast coupling through the RANKL/OPG signalling axis, resulting in aberrant bone resorption [233, 496]. This leads to hypomineralisation, subchondral sclerosis and osteophyte formation in joints [209, 224, 435].

APC is a natural anticoagulant with potent anti-inflammatory and cytoprotective properties [245, 247]. Previously, our laboratory identified elevated PC/APC antigen and APC activity in RA synovial fluid as compared with OA synovial fluid [281]. The same study also found that APC co-localises with the anti-inflammatory marker MMP-2 in both RA and OA synovial tissue. Furthermore, APC stimulates MMP-2 activation in both RA and OA synovial fibroblasts and suppresses pro-inflammatory MMP-9 expression in RA synovial fibroblasts. Combined, this data is indicative of the anti-inflammatory effects of APC on arthritic synovial fibroblasts [184]. Furthermore, APC reduces RA synovial fibroblast proliferation and down-regulates TNF-α-induced phosphorylation of p38, JNK, and Akt [377]. Similarly in RA monocytes, APC inhibits monocyte activation and decreases TNF-α-induced MMP-9 secretion through suppression of NF-kB, and p38 phosphorylation [184, 293]. In cartilage, PC/APC antigen is present on chondrocytes and in areas of fibrillation [284]. Exogenous APC treatment activates MMP-2 and MMP-9 to augment collagen breakdown, and the release of aggrecan, glycosaminoglycans (GAGs), and hydroxyproline in OA cartilage [283, 284]. It is evident from these studies that APC plays an important role in arthritis, however to date, there have been no studies investigating the action of APC upon OA and RA osteoblasts.

We have shown that APC enhances MG-63 cell viability via EPCR and PAR1 (Section 3.4.2 & 3.4.4) [268]. On the basis of these results and the scientific literature we hypothesised that APC would: (1) enhance OA and RA osteoblast viability through activation of EPCR and PARs; (2) modulate downstream ERK1/2, p38, and Akt signalling activity; (3) attenuate pro-inflammatory cytokines TNF-α, IL-1, IL-6, and IL-17 in OA and RA HBDCs; and (4) inhibit TNF-α stimulated cytokine and MMP production.

6.2 Aims

The overall aim of this chapter was to examine the role of APC on OA and RA osteoblast viability and inflammation. Specifically, we aimed to:

1. examine the morphology of OA and RA subchondral tissue;
2. determine the phenotype of OA and RA human bone-derived cells (HBDCs);
3. investigate the effect of APC on OA and RA HBDC viability;
4. examine the expression of PC, EPCR, PAR1, and PAR2 on OA and RA bone sections and human bone-derived cells (HBDCs) and the implication of these receptors in APC's effects on HBDCs;
5. measure the effect of thrombin on HBDCs;
6. elucidate the role of APC on signalling mediators, ERK1/2, Akt, p38, and NF-κB activity in HBDCs;
7. measure the effects of APC in the presence or absence of TNF-α on IL-1β, IL-6, and IL-17 cytokine secretion in HBDCs; and
8. determine the effect of APC in the presence or absence of TNF-α on MMP-2 and -9 secretion in HBDCs.

6.3 Methods

6.3.1 Histology of OA and RA Tissue

A total of 4 OA and 4 RA condyle samples were collected, decalcified, and fixed as per Section 2.2.1. Condyles were embedded in paraffin and then sectioned before staining with H&E and Toluidine Blue to assess joint morphology and proteoglycan loss (Section 2.2.2-2.2.3). Sections were also assessed by IHC for PC/APC (0.2 ng/mL), PAR1, PAR2, and EPCR expression (as previously described in Section 2.2.4) in arthritic bone. Slides were then scanned using ScanScope and visualised and assessed for qualitative changes using ImageScope software.

6.3.2 HBDC Osteoblast Culture

Four sets of OA HBDCs was derived from 4 patients and 3 sets of RA HBDCs were derived from 3 patients. These cells were cultured by sequential collagenase digestion, as per Section 2.1.3, and grown in 75 cm^2 tissue culture flasks containing α-MEM media (Gibco, 11900-024) supplemented with 10% FBS and penicillin/streptomycin.

6.3.3 APC and TNF-α Treatment on IL-1β, IL-6 and IL-17 Production in HBDCs

To determine the effect of APC and/or TNF-α treatment on the cytokine profile of OA and RA HBDCs, cultured monolayers grown as per Section 6.3.2 were first assessed for basal secretion of TNF-α, IL-1β, IL-6, and IL-17. Then, the cells were treated with either 1, 10, or 100 ng/mL of TNF-α; or 10 μg/mL of APC; or both, in which TNF-α was added to the culture 30 min prior to APC addition. Then, to quantify the amount of secreted cytokines including IL-1β, IL-6, IL-17, culture supernatants were collected at 24 h post-treatment and analysed by ELISA (Section 2.1.10) using a plate reader (BioRad), and then the data was analysed using GraphPad Prism 5 software.

6.3.4 HBDC Phenotype and Receptor Expression

HBDCs were assessed for colony forming capacity by CFU-f (as per Section 2.1.4), differentiated in osteogenic media and stained for alkaline phosphatase (Section 2.1.5). Then HBDCs were allowed to mineralise in osteogenic media over 28 days and stained for calcium deposits by Alizarin Red S staining (Section 2.1.7). Primary HBDCs were cultured in chamber wells and assessed for PAR and EPCR expression using immunocytochemistry (Section 2.1.6). HBDC cells grown into monolayers were harvested for subsequent extraction and purification of total RNA. Then, RT-PCR was performed in quadruplicates to determine gene expression of EPCR, PAR1, and PAR2, each normalised against β-actin expression as the internal control

(Section 2.1.9). Cell lysates from monolayers were also analysed by ELISA to assess EPCR protein expression levels (Section 2.1.10).

6.3.5 APC and Receptor Involvement in HBDC Viability

The effect of APC upon HBDC viability was measured using the MTT and trypan blue dye exclusion assays. The treatment of HBDCs with APC was first assessed in 10% FCS-containing media, which after optimisation, was reduced to 2% FCS. HBDCs were then incubated with 0.1, 1, 10 µg/mL of APC or 0.1, 1, 10 U of thrombin over 24, 48, or 72 h. EPCR blocking and non-blocking antibodies, as well as PAR antagonists, were employed 30 min prior to APC addition to determine EPCR and PAR1/2 involvement in APC-mediated viability by MTT. This was then confirmed with trypan blue exclusion dye assay as per Section 2.1.8. PAR1 antagonist SCH79797 was originally reconstituted in DMSO but was further diluted in PBS for experiments. SCH79797 was diluted to 0.1, 1, or 10 µM in and assessed against respective concentrations of vehicle DMSO. Three and four sets of isolated HBDCs were used to compile RA and OA graphs respectively.

6.3.6 APC Treatment on Activation of Signalling Proteins ERK, Akt, NF-κB, and P38

Whole cell lysates extracted from cultured HBDCs were analysed by western blotting (Section 2.1.11) to determine the effect of APC treatment upon the expression of the intracellular signalling proteins ERK, Akt, NF-κB, and p38. In brief, cell cultures were treated with APC for 60 minutes prior to extraction of whole cell lysates. Proteins were separated by electrophoresis and then electro-transferred onto a PVDF membrane, incubated with primary antibodies (as per Table 2.5), and then relevant HRP-conjugated secondary antibodies. Membrane were visualised using an ECL system and ImageQuant LAS 4000 (GE). Densitometric analysis of protein bands was performed using MultiGauge software (FujiFilm).

6.3.7 APC and TNF-α Treatment on MMPs in HBDCs

To determine the effects of APC and TNF-α treatment upon MMP activity in HBDCs, gelatin zymography was used as per Section 2.1.12. Baseline levels of pro- and active- MMP-2 and -9 were determined prior to and after the addition of APC, TNF-α, or combined treatments. Media from treated cells was isolated and then their protein concentrations determined and standardised. Samples were then resolved by electrophoresis through zymography gels, which were then developed, stained, and destained. Visualisation and semi-quantification were carried out on ImageQuant LAS 4000 (GE) and analysed similarly to western blotting.

6.3.8 Statistics

One-way ANOVA, Student-Newman-Keuls post-hoc test, and data graphing were performed using GraphPad Prism 5 (GraphPad) as per Section 2.4. N refers to the number of HBDC samples unless otherwise stated.

6.4 Results

6.4.1 Morphology of OA and RA Bone

To determine the morphology of bone tissue in OA and RA, a total of 4 OA and 4 RA subchondral bone samples were collected, fixed, and stained. From available demographic information, 3 RA samples and all OA samples came from female patients (Table 6-1). The number of RA samples available for this project was limited due to the overall reduction in joint replacement surgery performed on RA patients treated with biological drugs. Recent literature demonstrates reductions in the rate of joint replacement surgery in the biologic era, suggesting that improved biologics reduce the need for joint replacement [497-499]. Thus, to increase the sample size, two RA knuckle samples were obtained from hand surgery, with the remainder being femoral condyles (Table 6-1).

Table 6-1 *Patient demographics for histology samples.*

Disease	Sample #	Gender	Age	Joint
OA	1	F	58	Femoral condyle
	2	F	68	Femoral condyle
	3	F	83	Femoral condyle
	4	F	76	Femoral condyle
RA	1	F	43	Femoral condyle
	2	F	67	Femoral condyle
	3	F	Unknown	Knuckle
	4	Unknown	Unknown	Knuckle

Toluidine blue staining, which determines proteoglycan and GAG levels, was first used to detect cartilage damage in OA and RA tissue. Fast green counterstaining allowed visual assessment of collagen content. Fibrillation in the superficial zone of the cartilage was evident in all RA and OA samples. This was associated with GAGs and proteoglycans loss, as indicated by the decrease in toluidine blue stain on the superficial cartilage layer (Figure 6-1). Although both RA knuckles had less proteoglycan content than RA condyles, there was a considerable difference between the two knuckles (Figure 6-2). There was no clear difference between cartilage GAG loss in OA and RA condyles.

There was evidence of increased bone turnover in both RA and OA samples, with the presence of an osteophyte at the condyle edge in the OA sample, resorption of the cartilage tidemark in both samples, and marked hypertrophy of the synovium also in both samples (indicated by arrows in Figure 6-1).

H&E staining of RA and OA samples also showed synovial tissue hyperplasia, as well as a number of bone lining cells on the bone surfaces, and presence of blood vessels in osteophytes. However, there was no obvious visual difference between OA and RA condyles in the parameters previously mentioned (Figure 6-1).

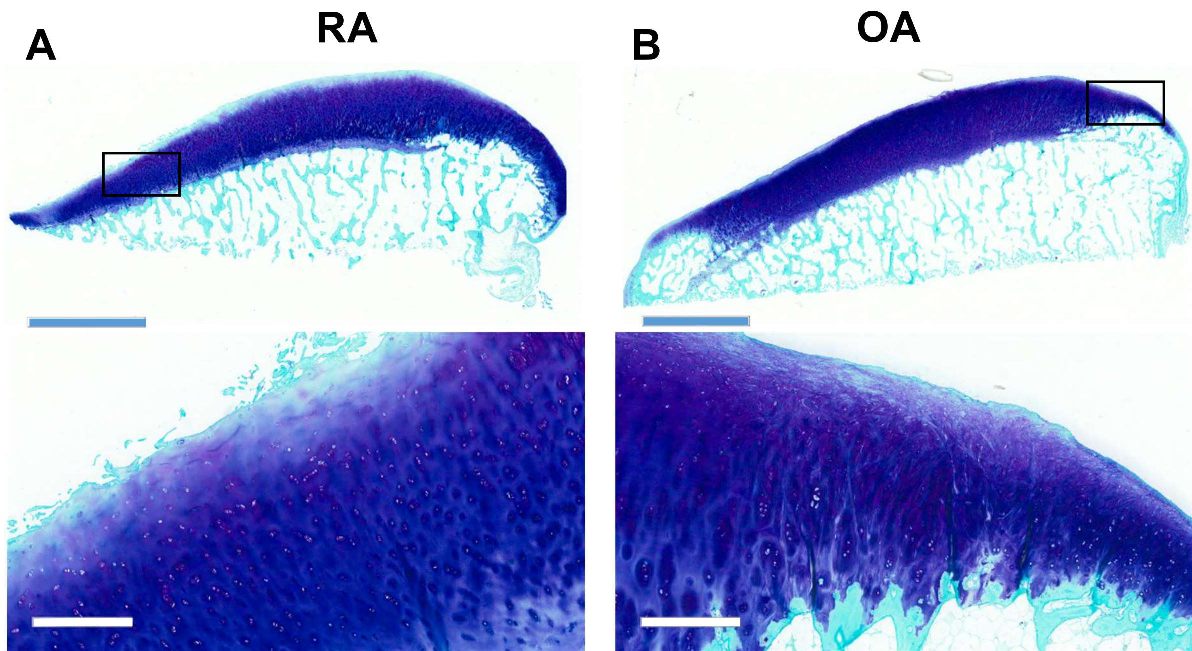

Figure 6-1 *Toluidine levels in representative RA (A) and OA condyles (B). There is evidence of proteoglycan loss, resorbed cartilage tidemark, and synovial hyperplasia in both OA and RA subchondral bone samples. An osteophyte is also present in the OA condyle. Blue scale bars indicate 5 mm, white scale bars indicate 500 µm.*

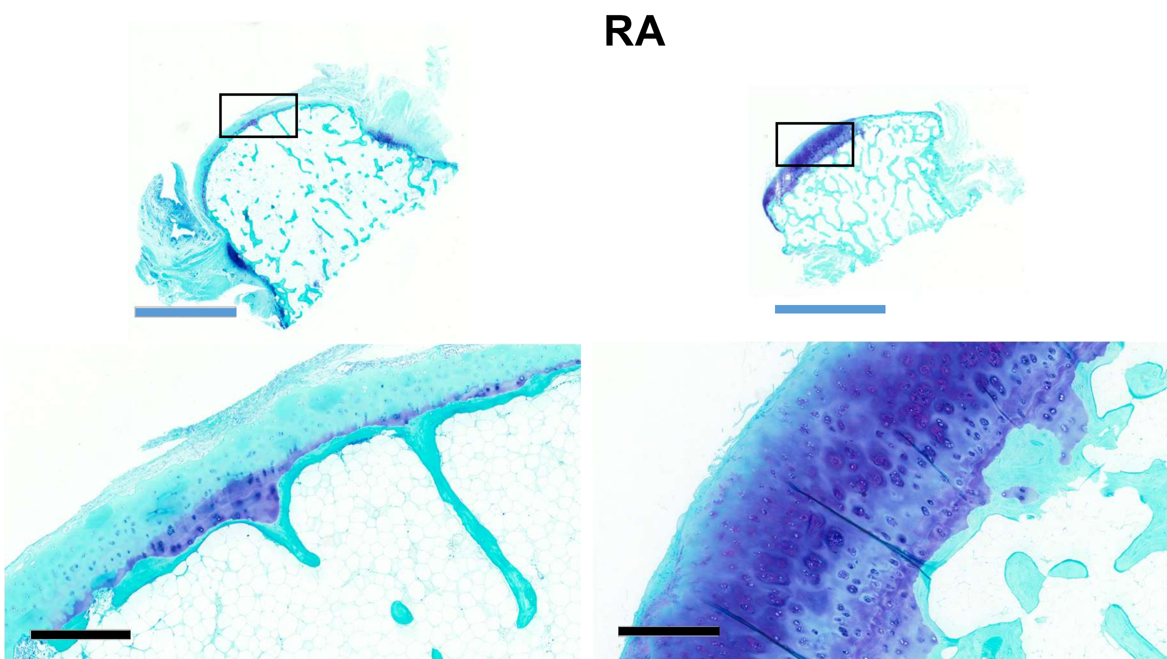

Figure 6-2 *RA knuckles stained with toluidine blue and fast green. Blue scale bars indicate 4 mm, black scale bars indicate 500 µm.*

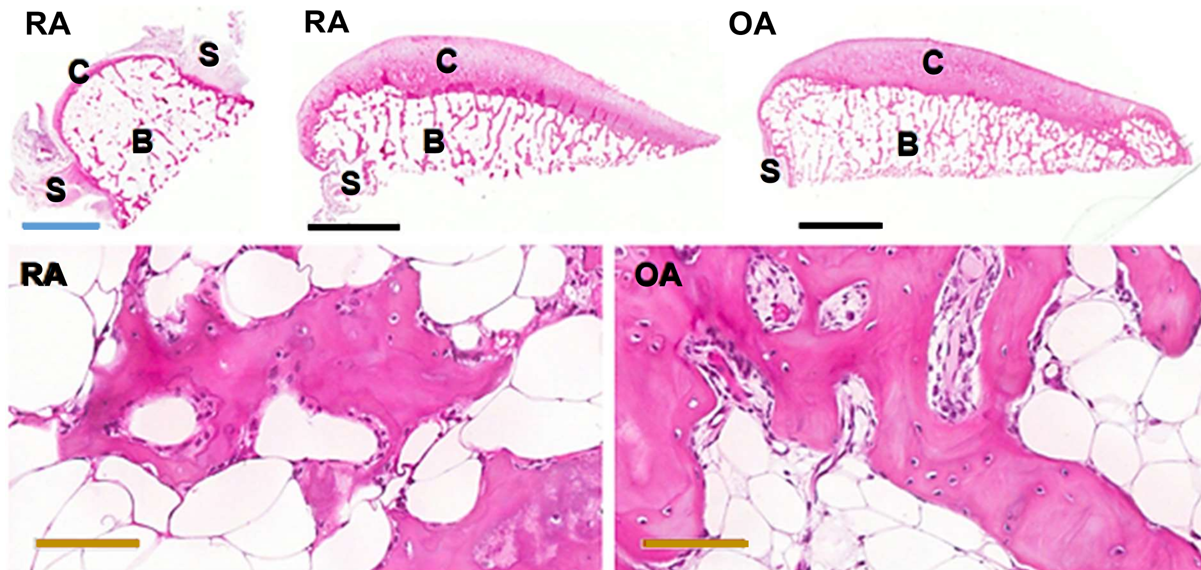

Figure 6-3 *H&E of RA knuckle, RA condyle, and OA condyle. Beneath are magnified views of osteophytes in both OA and RA. C = cartilage, B = bone, S = synovium. Blue scale bar indicates 4 mm, black scale bars indicate 5 mm, and brown scale bars indicate 200 µm.*

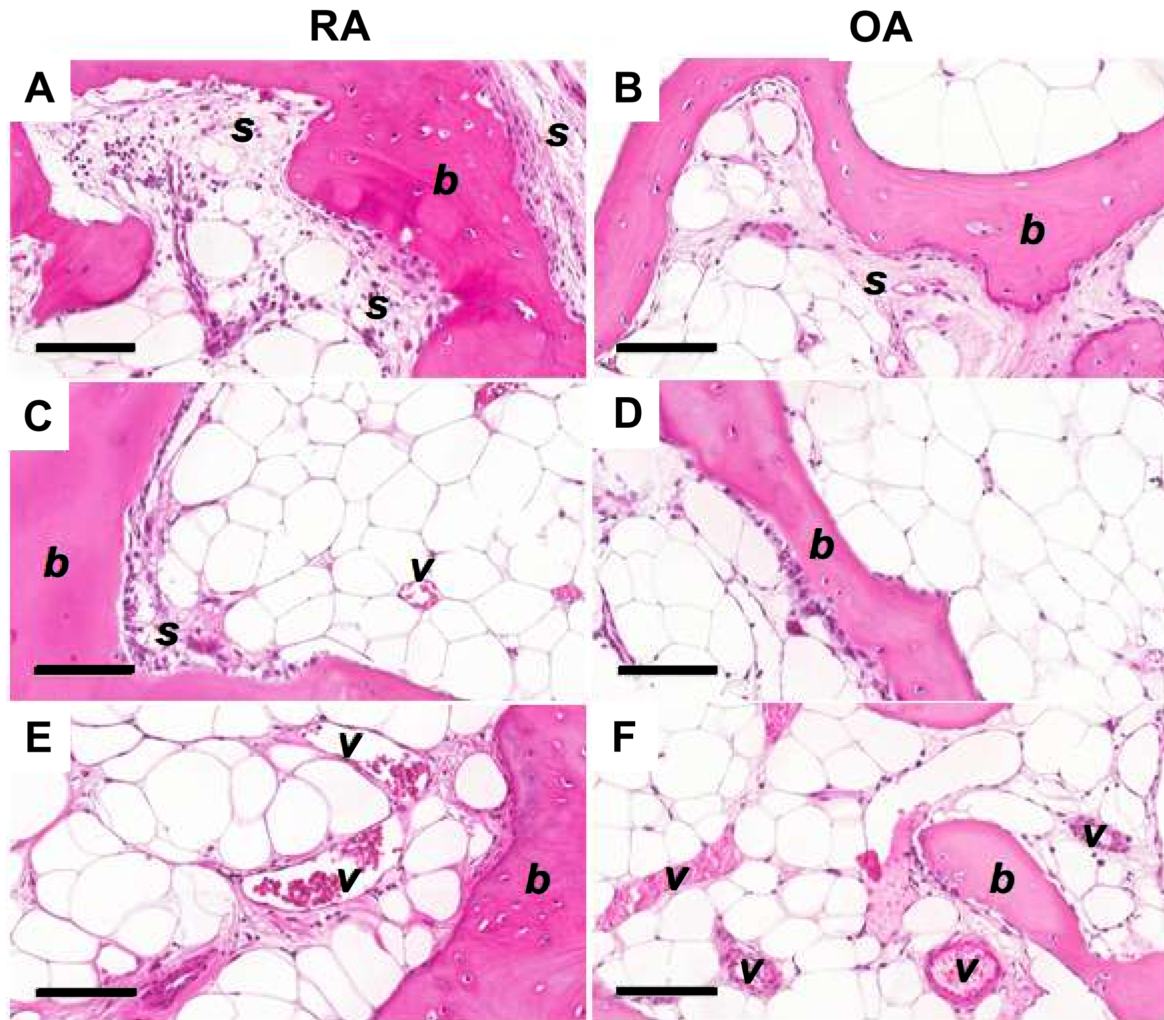

Figure 6-4 *H&E staining of OA and RA tissue surfaces. Femoral condyle sections showed synovial hyperplasia (A&B), bone lining cells (C&D), and the presence of blood vessels (E&F). Tissues labelled with b for bone, s for synovium, and v for vessels. Scale bar 100 µm.*

6.4.2 OA and RA HBDCs are Heterogeneous Populations

The characterisation of HBDCs using CFU-f, alkaline phosphatase staining and osteoblast differentiation was employed to help determine the success of osteoblast isolation.

During the first cell culture passage, the percentage of positive colonies (defined as clusters consisting of more than 100 cells) was 15% for OA HBDCs and 9% for RA HBDCs (Figure 6-5 A&B), which indicates heterogeneity of the isolated cell population [366]. This was consistent with a previous study that has shown that HBDC culture contains cells of the osteogenic lineage at different stages of maturation [364]. Subsequent CFU-f in passage four cultures demonstrated a marked increase in the number size of colonies (Figure 6-5C), where up to 30% were CFU-f positive.

HBDCs were then cultured to confluence in osteogenic media and stained for alkaline phosphatase, a mid-stage marker for the osteoblast lineage, to further confirm the osteoblastic phenotype of HBDCs. They were also terminally differentiated to ensure that they can form bone in osteogenic media and that they were capable of mineralisation as determined by Alizarin Red staining of calcium.

It appeared macroscopically that fewer RA HBDCs expressed alkaline phosphatase than OA HBDCs (Figure 6-6). Differentiation was conducted in the presence of L-ascorbic acid and β-glycerophosphate (Figure 6-7). OA cells readily formed calcium deposit after 28 days of differentiation, whereas RA cells did not form any calcium deposits (Figure 6-7). This may be related to the pathology of RA osteoblasts as previous literature has shown that these cells fail to mineralise bone matrix [194].

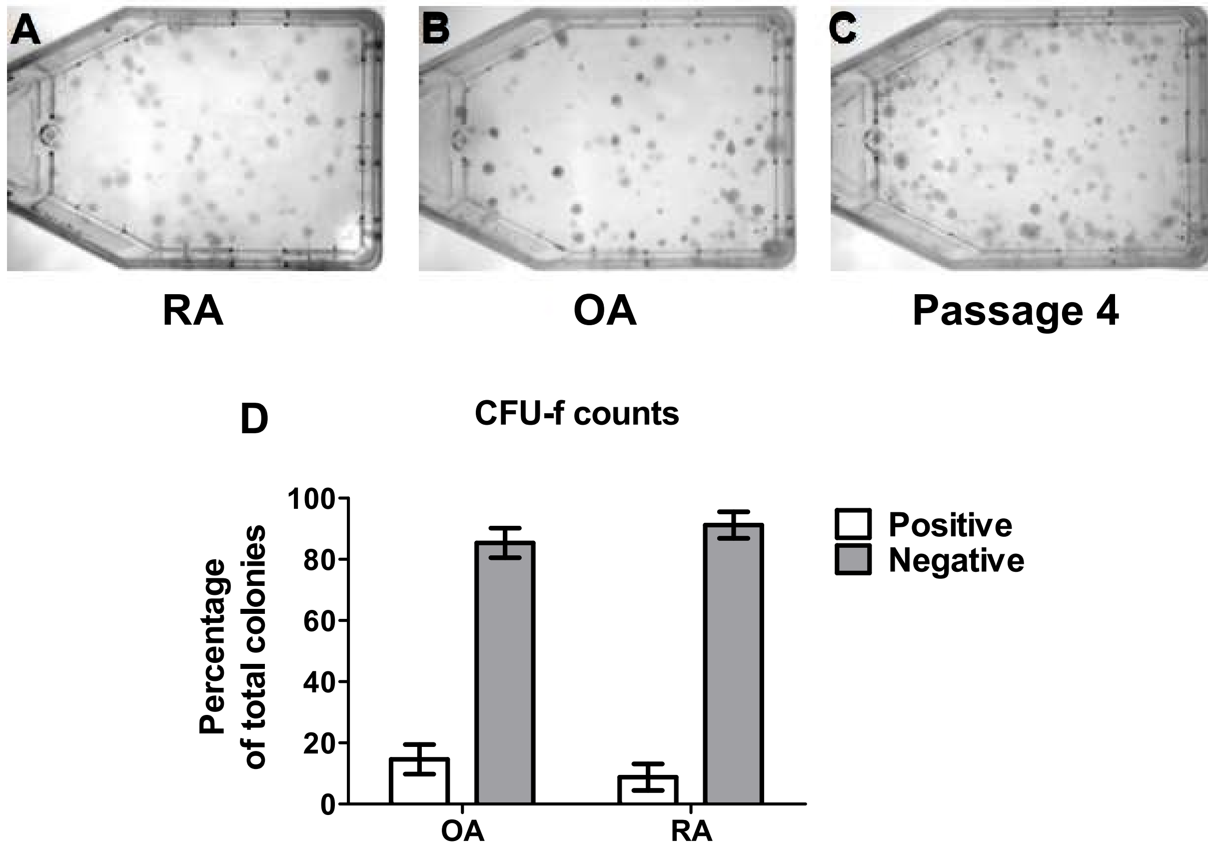

Figure 6-5 *CFU-fs OA and RA HBDCs. Passage 1 RA (A) & OA HBDCs (B) and passage 4 OA HBDCs (C) were seeded at 200 cells/5cm² flask and cultured for 10 days before fixing and staining with Giemsa. Four sets of OA HBDCs and three sets of RA HBDCs at passage 1 were used to plot positive and negative CFU-fs (D). Positive CFU-f was defined as colonies with distinctive borders that counted greater than 100 cells.*

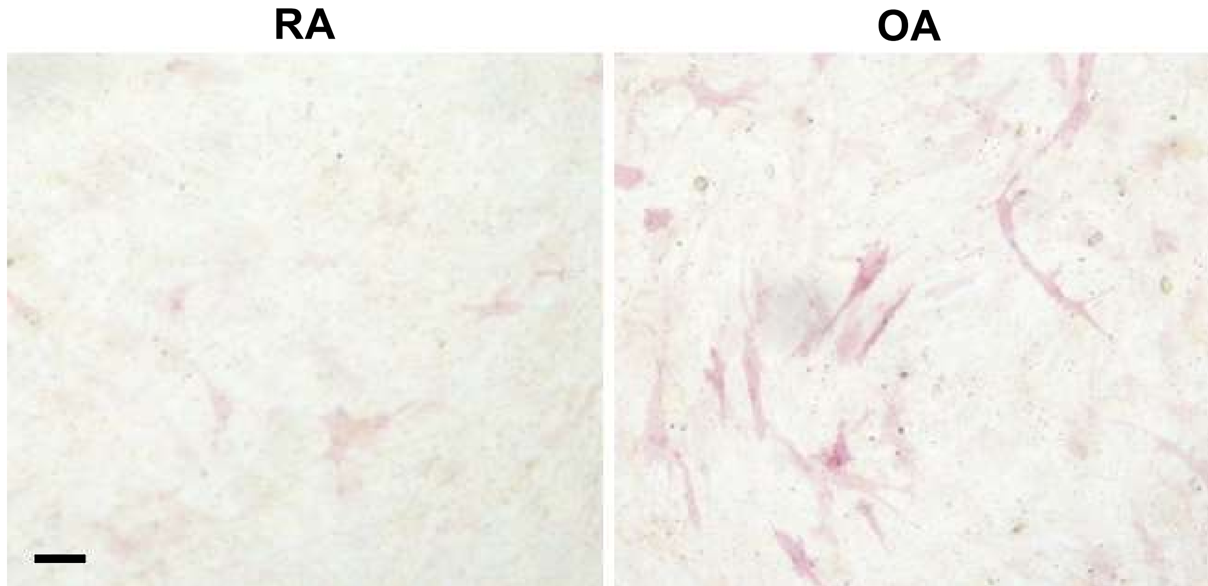

Figure 6-6 *Alkaline phosphatase staining in representative OA and RA HBDCs grown to confluence.*

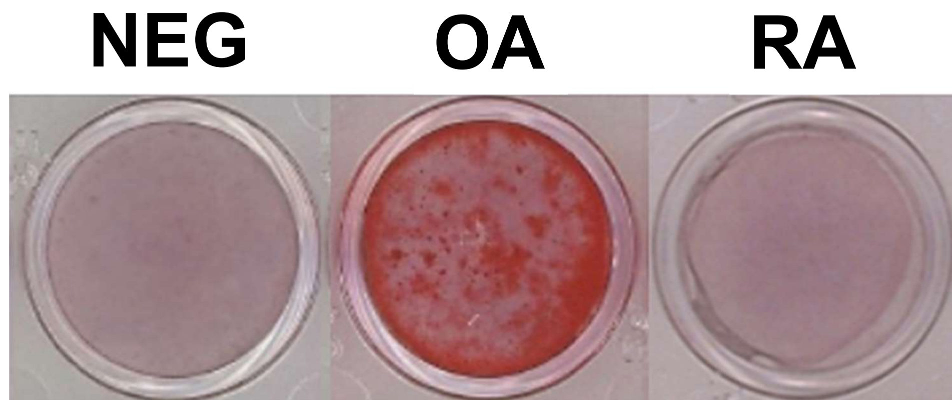

Figure 6-7 *Osteogenic differentiation of OA HBDC and RA HBDC. OA or RA cells were seeded at 2×10^5 and then treated with 50 µg/mL of L-ascorbic acid and 10 mM of β–glycerophosphate for 28 days and then stained for calcium deposits with Alizarin Red. As a negative control, OA HBDCs were seeded at the same density and grown in growth media rather than osteogenic media.*

6.4.3 PC/APC and Receptors are Expressed in OA and RA Bone and HBDCs

The presence of PC has previously been investigated in fracture haematomas of normal bone [268]. Here we investigated its presence in OA and RA subchondral bone [268]. We have also found the expression of APC receptors on MG-63 cells and their involvement in APC-mediated viability in Section 3.4.1 & 3.4.4. We further examined the presence of EPCR, PAR1, and PAR2 in both arthritic subchondral bone and primary HBDCs.

PC, EPCR, PAR1, and PAR2 expression was found in both OA and RA tissue (Figure 6-8). There were no clear differences observed between the intensity of PC or receptor staining of OA and RA tissues. PC, EPCR, and PAR2 staining on OA and RA bone tissue were heterogeneous and localised to some cuboidal shaped osteoblasts and bone lining cells (Figure 6-8 A&B, C&D, G&H). PAR1 stained most bone surfaces and many cuboidal and bone lining cells (Figure 6-8 E&F). Osteocytes in the embedded bone matrix were not positive for PC or the receptors. Neither PC nor its receptors were specifically located to areas of high remodelling.

There was also no obvious visual correlation between PC and receptor staining. PC and PAR1 staining was controlled against rabbit IgG, EPCR against goat IgG, and PAR2 against mouse IgG at the same concentrations (Figure 6-8 I-K).

Immunocytochemistry staining and qualitative PCR confirmed that EPCR, PAR1, and PAR2 were expressed by OA and RA HBDCs at both gene and protein levels (Figure 6-9, 6-10). Gene expression levels were normalised against β-actin mRNA. Positive immunostaining was controlled similarly to subchondral bone staining (Figure 6-9, 6-10).

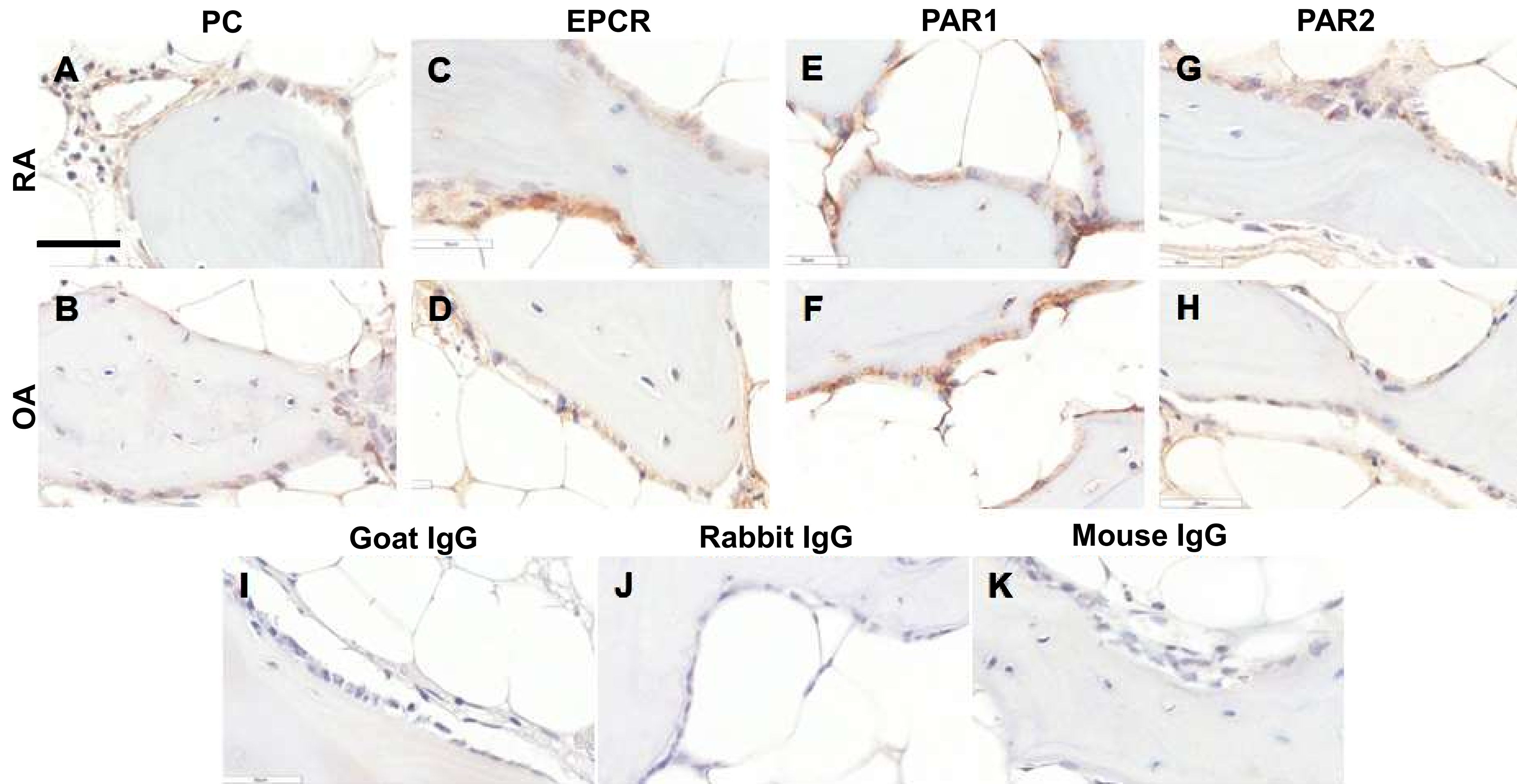

Figure 6-8 *PC, EPCR, PAR1, and PAR2 staining in RA and OA subchondral bone. Sections were cut, dewaxed, incubated overnight with PC (A&B), EPCR (C&D), PAR1 (E&F), and PAR2 (G&H) antibodies or rabbit, mouse, and goat IgG as negative controls (I-K). Then slides were conjugated to secondary antibodies, stained with DAB, and counterstained with haematoxylin. Scale bar indicates 50 µm.*

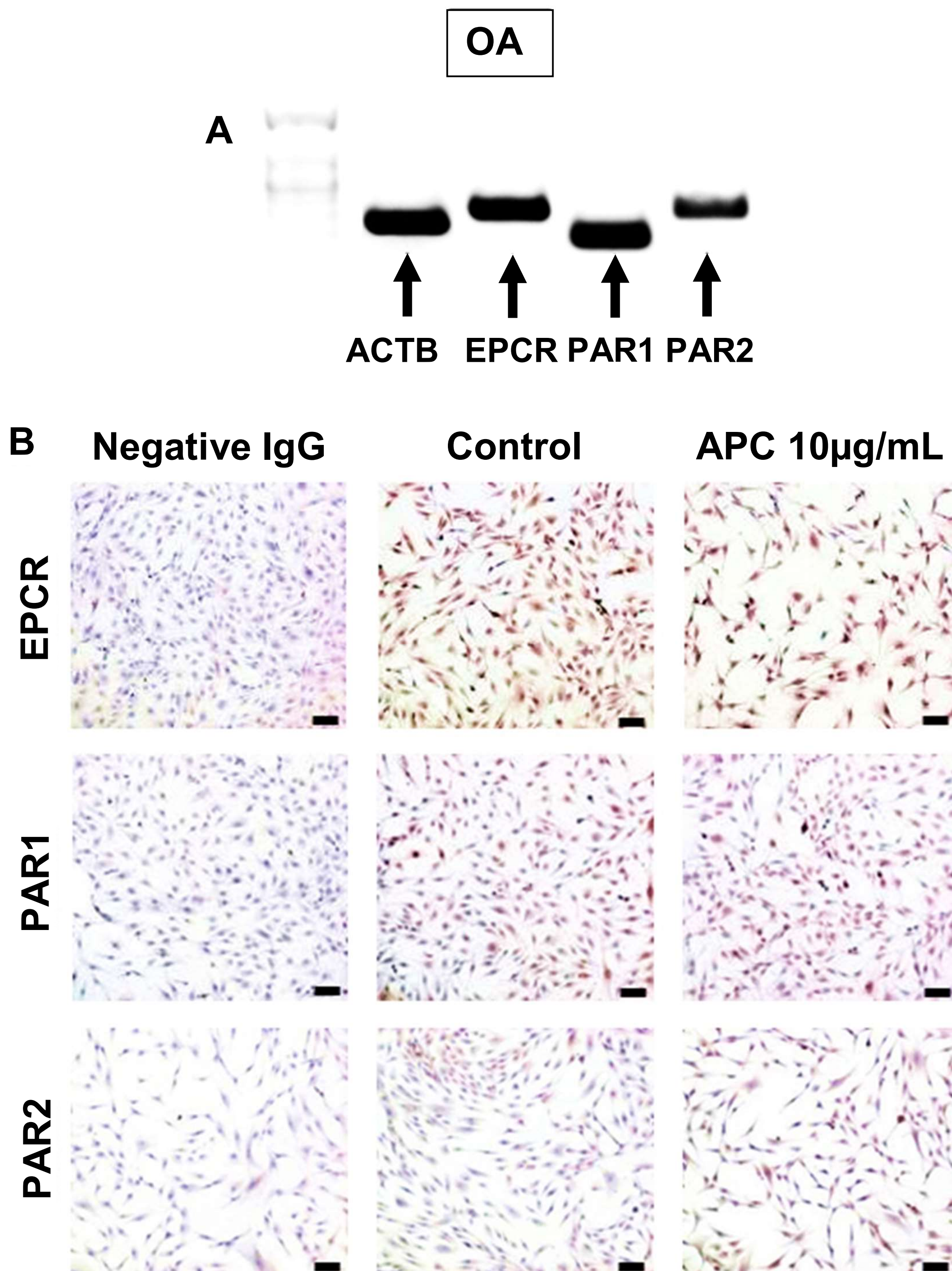

Figure 6-9 *Immunohistochemistry and PCR was conducted to measure EPCR, PAR1, and PAR2 protein and mRNA expression, respectively, in OA HBDCs. RT-PCR was conducted on mRNA extracted from untreated OA HBDC to determine qualitative levels of β-actin/ACTB, EPCR, PAR1, and PAR2 (A). Immunocytochemistry staining was performed using a goat anti-EPCR antibody, a rabbit anti-PAR1 antibody, or a mouse anti-PAR2 antibody and their respective negative control IgGs. Slides were then visualised with DAB and counterstained with haematoxylin (B). Scale Bar equals 50 µm.*

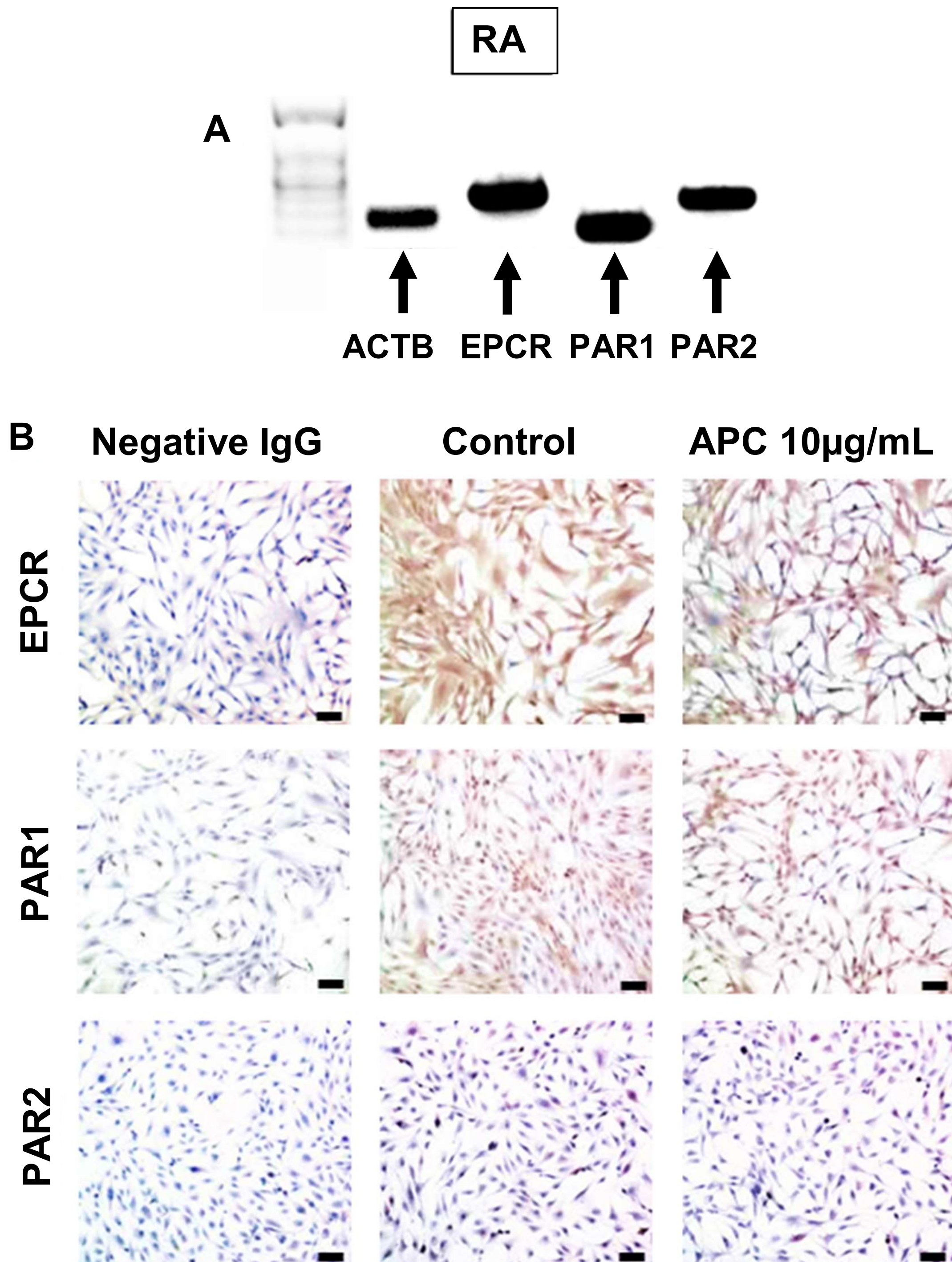

Figure 6-10 *Immunohistochemistry and PCR was conducted to measure EPCR, PAR1, and PAR2 protein and mRNA expression, respectively, in RA HBDCs. RT-PCR was conducted on mRNA extracted from untreated RA HBDC to determine qualitative levels of β-actin/ACTB, EPCR, PAR1, and PAR2 (A). Immunocytochemistry staining was performed using a goat anti-EPCR antibody, a rabbit anti-PAR1 antibody, or a mouse anti-PAR2 antibody and their respective negative control IgGs. Slides were then visualised with DAB and counterstained with haematoxylin (B). Scale Bar equals 50 µm.*

Visual assessment of EPCR expression by immunocytochemistry showed reduced expression in OA as compared to RA HBDCs, however, there was no difference in expression of PAR1 or PAR2. APC treatment of OA or RA HBDCs did not substantially alter EPCR, PAR1, or PAR2 staining (Figure 6-9, 6-10).

To further evaluate whether there was a quantitative difference in EPCR expression between OA and RA HBDCs, the more sensitive and quantitative ELISA technique was used. RA HBDCs expressed a mean of 37.4 ng/mL of EPCR, which was significantly higher than the mean 7.8 ng/mL of EPCR found in OA cells (*P*<0.05; Figure 6-11).

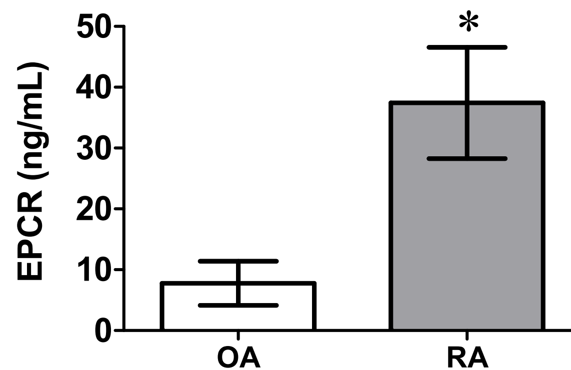

Figure 6-11 *ELISA of EPCR expression in OA and RA HBDCs. Untreated OA and RA HBDCs lysates were assayed for EPCR by ELISA. Protein loading was corrected for by BCA assay. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05 between treatment and control.*

6.4.4 APC Decreases OA HBDC Viability

As we have previously shown that APC enhances MG-63 viability, similar studies were performed in HBDCs. The effect of APC was first examined on 4 sets of OA HBDCs and 3 sets of RA HBDCs, cultured in normal growth medium (αMEM with 10% FCS). No difference in viability was found from APC treatment on RA and OA HBDC at 72 h (Figure 6-12).

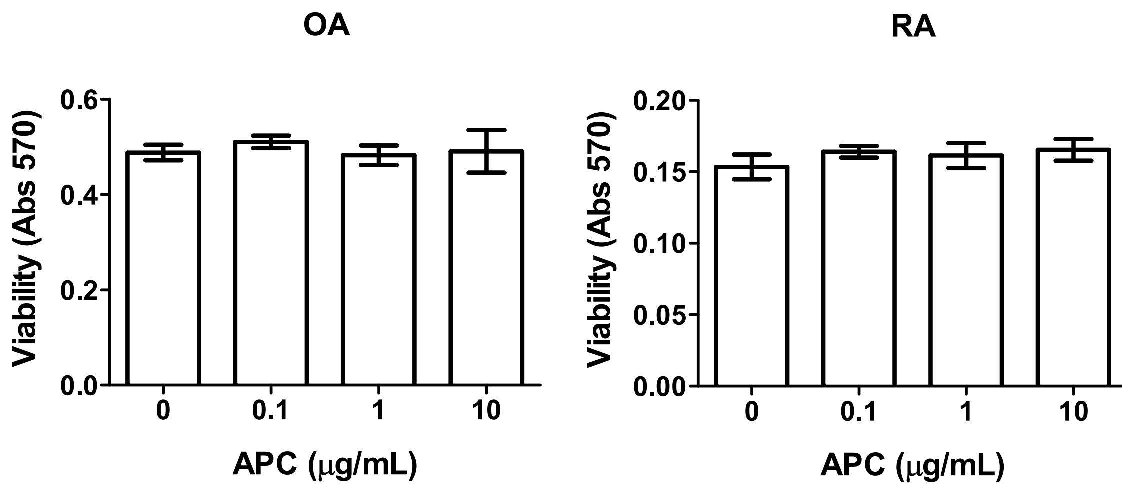

Figure 6-12 *The effect of APC on cells cultured in 10% FCS. RA or OA HBDCs were seeded and treated with 0.1, 1, or 10 μg/mL of APC in α-MEM + 10% FCS and MTT performed 72 h after stimulation. Statistical analysis was performed by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3).*

Since inhibitors present in the serum may mask APC's effect, the serum in media was reduced to 2% FCS. Using the serum-reduced media, we determined the baseline cell proliferation for RA and OA cells over 72 h and found a greater increase in MTT readings of OA cells as compared to RA cells (25%, $P<0.01$). This suggested a slower growth rate for RA cells (Figure 6-13).

We further treated OA and RA HBDCs with APC in serum-reduced media. Treatment of OA HBDCs with APC over 24 h showed no significant changes in MTT absorbance but significant decreases of 20% or greater ($P<0.05$; Figure 6-14) in cell numbers. At 48 h, treatment with 10 μg/mL of APC significantly decreased both cell numbers and MTT readings by 21% ($P<0.05$ for both assays; Figure 6-14). At 72 h, there was a continued suppression of MTT reading by 19% after 10 μg/mL of APC treatment ($P<0.05$; Figure 6-14), and likewise a 26% suppression of cell numbers ($P<0.05$).

Overall, these effects showed that APC was able to suppress OA HBDC viability, contrasting with APC's effect on MG-63 cells. All subsequent OA and RA HBDC cell viability experiments utilised 10 µg/mL of APC as the optimal treatment dose.

Treatment of RA HBDCs with APC in serum-reduced media over 24 h had no significant impact upon on MTT absorbance (Figure 6-15). Over 48 h, APC treatment had no effect on MTT reading in RA cells (23%, *P*=0.06; Figure 6-15). At 72 h, APC had no effect. APC also had no significant effect on RA HBDC counts at 24, 48 or 72 h. The lack of significance for the aforementioned results may be attributed to the small sample size (n=3) in RA HBDCs. Due to the low numbers of replacements surgeries for RA patients, these were the only samples available for this study.

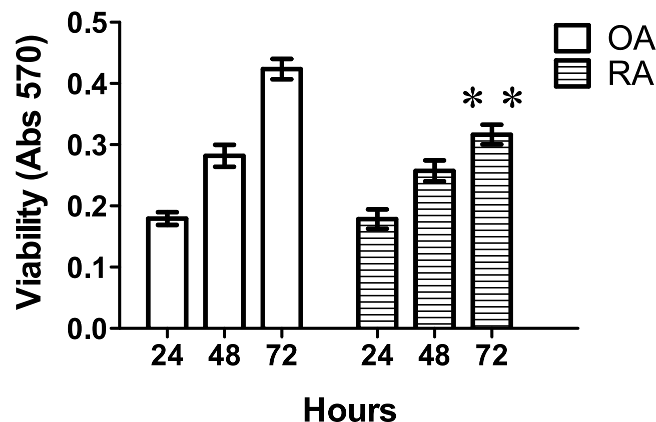

Figure 6-13 *The effect of 2% serum on OA and RA HBDCs. Cells were seeded and maintained in 2% FCS α-MEM and MTT assays were conducted 24, 48, and 72 h. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). ** Denotes P<0.01 between 72 h OA and RA HBDC MTT reading.*

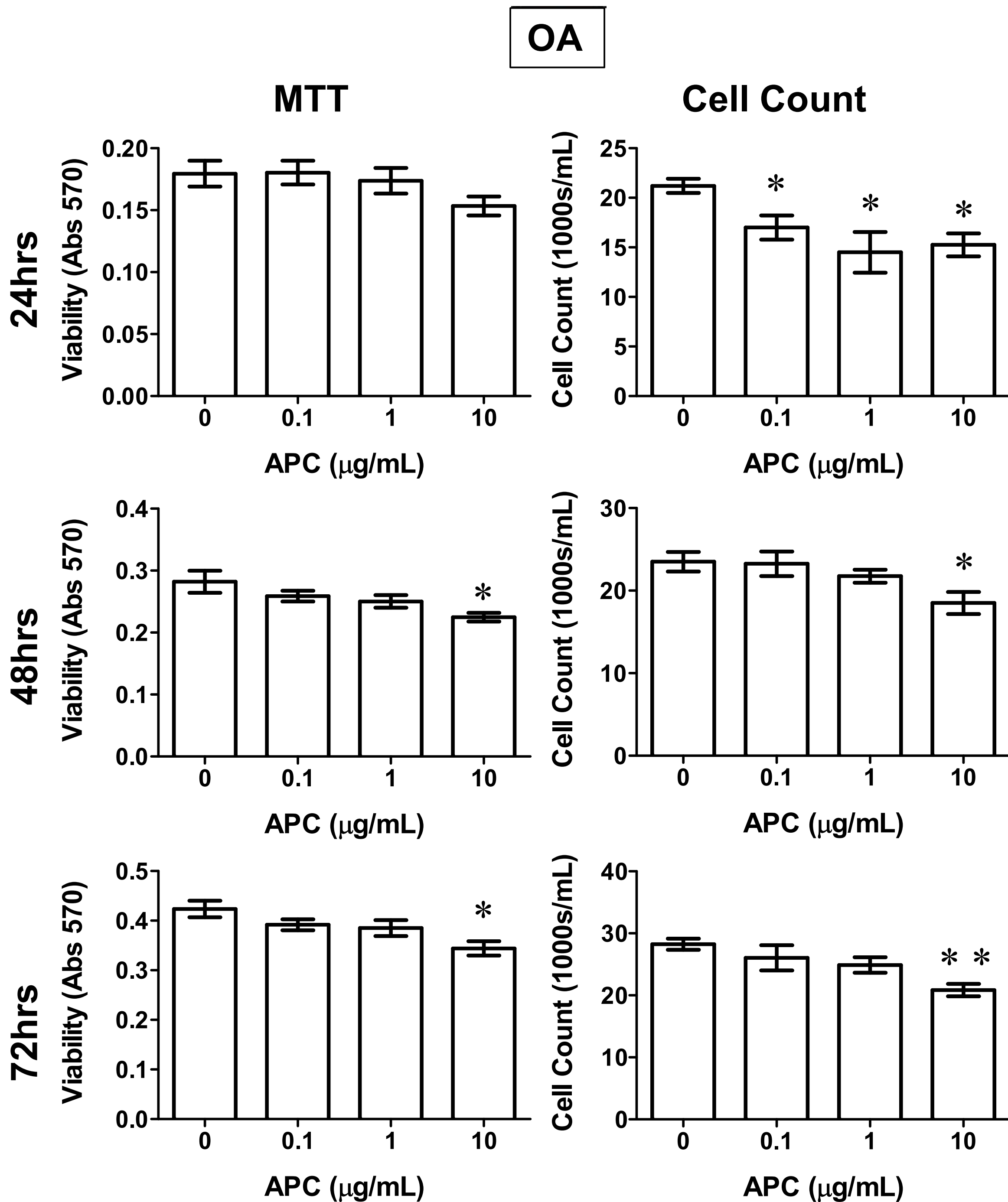

Figure 6-14 *The effect of APC on OA HBDC viability over 72 h. Cells were serum-reduced overnight and then treated with 0.1, 1, or 10 µg/mL of APC. After 24, 48, or 72 h, cell viability was measured by MTT and trypan blue exclusion. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (n=4). * Denotes P<0.05 and ** denotes P<0.01 between treatment and control.*

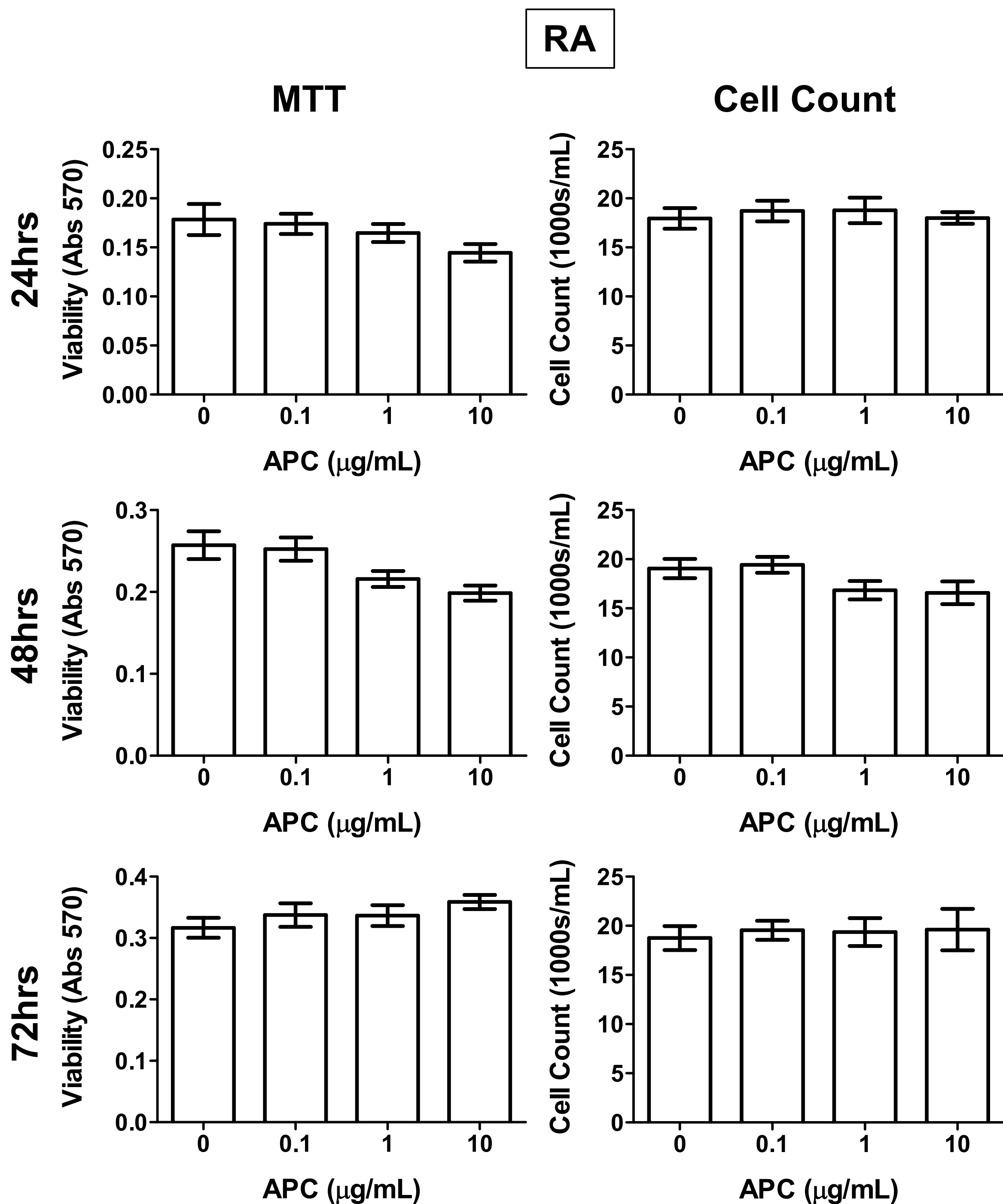

Figure 6-15 *The effect of APC on RA HBDC viability over 72 h. Cells were serum reduced overnight and then treated with 0.1, 1, or 10 μg/mL of APC. After 24, 48, or 72 h, cell viability was measured by MTT and trypan blue exclusion. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (n=3). * Denotes P<0.05 and ** denotes P<0.01 between treatment and control.*

6.4.5 PAR1 and PAR2 are Required for APC's Actions

It was shown in Chapter 3 that PARs are involved in APC's actions on MG-63 viability. We further investigated the involvement of EPCR and PARs on the mediation of APC activity in OA & RA HBDCs.

Consistent with previous experiments, APC treatment was associated with a 14% reduction in OA HBDC viability as compared to the untreated controls ($P<0.05$; Figure 6-16). Blocking EPCR with an RCR-252 antibody prior to a 48 h treatment with APC abolished the APC-stimulated suppression of OA cell viability. The non-blocking control antibody, RCR-92, had no significant effect. These data suggests that APC's mechanism of action in OA HBDCs is mediated at least partly through EPCR, which contrasts with APC's actions in MG-63 cells (Section 3.4.4).

PAR1 antagonist (SCH79797) or PAR2 antagonist (ENMD-1068) were used to block PAR1 or PAR2 signalling, respectively. SCH79797 or ENMD-1068 treatment prior to APC addition led to the abolishment of APC-mediated down-regulation of viability. SCH79797 alone significantly suppressed viability by 50% ($P<0.01$; Figure 6-16) and APC treatment post SCH79797 yielded no difference (2%, $P=0.89$). Similarly, APC treatment post ENMD-1068 addition resulted in no difference (6%, $P=0.45$). These results suggest that APC may work through both PAR1 and PAR2 in OA HBDCs.

In RA cells, neither RCR-92, RCR-252 nor ENMD-1068 resulted in any significant changes in viability. APC treatment did not affect MTT viability in RA HBDCs (11%, P=0.06; Figure 6-16). SCH79797 treatment alone caused a significant decrease (28%, $P<0.05$) in RA HBDC viability (Figure 6-16), which was maintained even after APC treatment.

The marked suppression of OA and RA viability by SCH79797 was confirmed in a study controlled against vehicle DMSO (Figure 6-17). Compared to DMSO control, SCH79797 (10 μM) treatment alone decreased viability over 48 h by 23% (P<0.01) in OA HBDC and 23% (P<0.05) in RA HBDC. These results indicate that DMSO did not cause the inhibition of PAR1 activity by SCH79797.

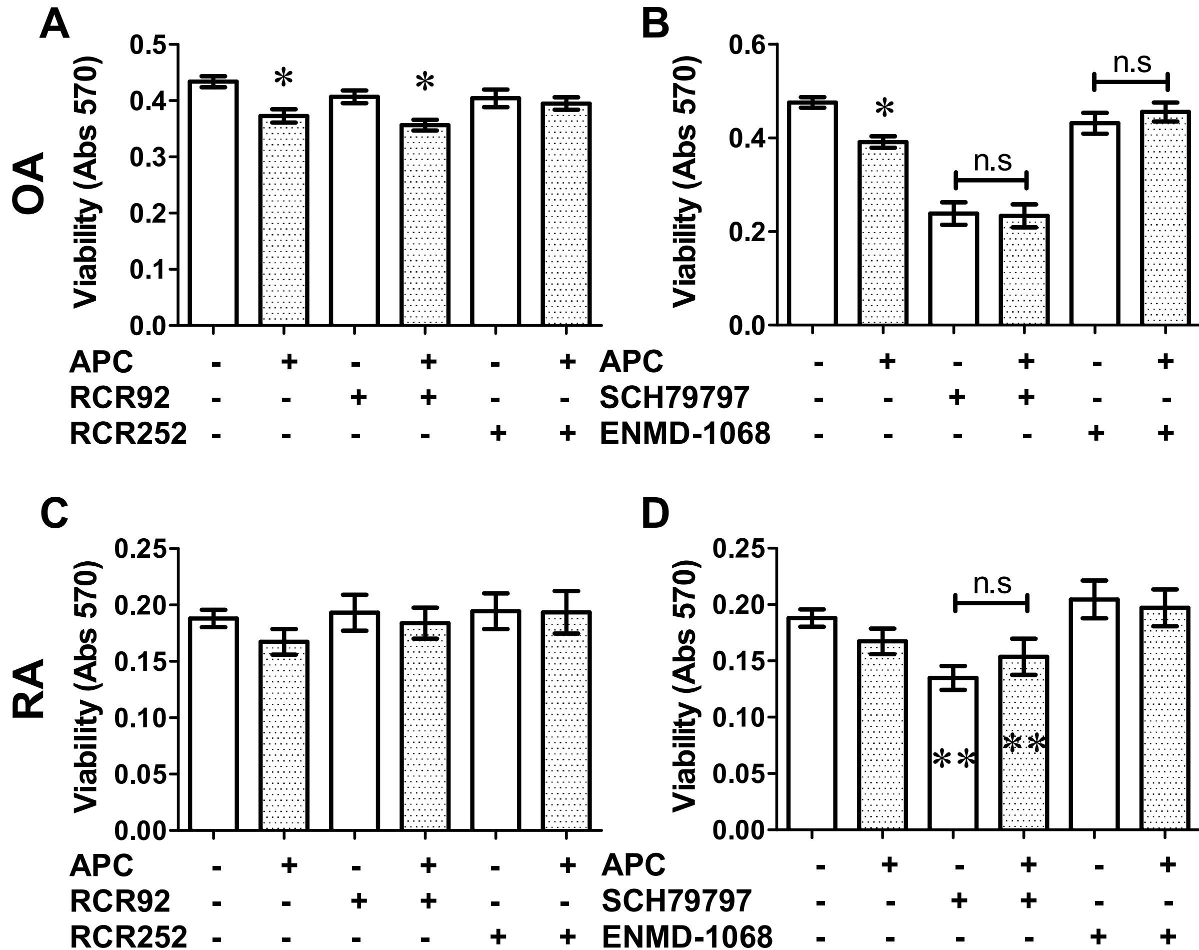

Figure 6-16 *The involvement of EPCR, PAR1 and PAR2 on APC-induced OA and RA cellular viability. OA and RA HBDCs were pre-incubated with either 10 μg/mL of RCR-92 (EPCR non-blocking antibody) or 10 μg/mL of RCR-252 (EPCR blocking antibody) (A&C), 10 μM of PAR1 antagonist SCH79797 or 10 μM of PAR2 antagonist ENMD-1068 (B & D) for 30 min prior to 48 h of 10 μg/mL of APC treatment. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05 between treatment and control.*

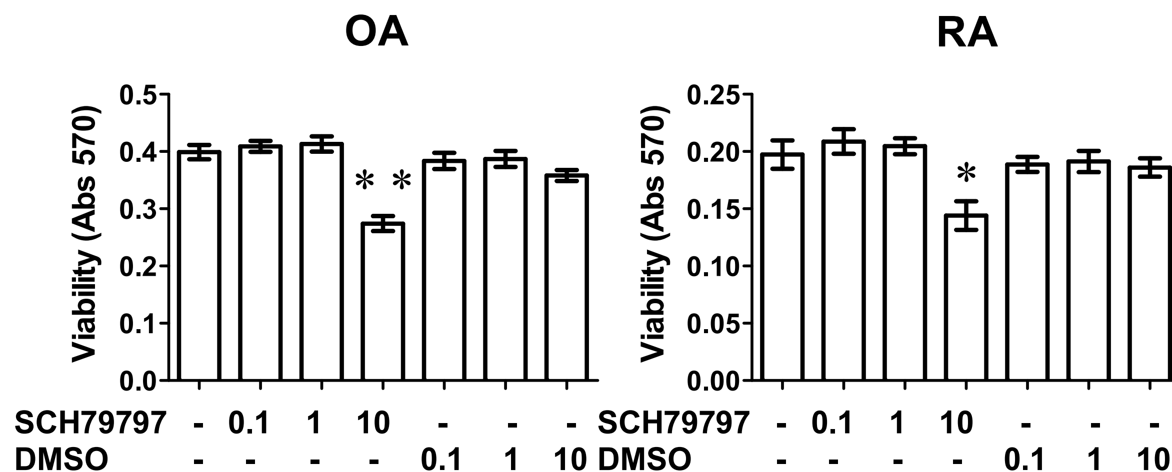

Figure 6-17 *Dose-dependent effect of SCH79797 in OA and RA HBDCs. Cells were pre-incubated with either PAR1 antagonist SCH79797 at 0.1, 1, or 10 µM or respective DMSO controls. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05, ** denotes P<0.01 between SCH79797 and DMSO control.*

6.4.6 Thrombin Does Not Alter HBDC Viability

As PAR1 was found to be involved in APC's actions on HBDCs, we hypothesised that another PAR1 agonist, thrombin, could induce a similar decrease in OA and RA HBDC viability. Assessment by MTT assay showed no significant difference in either OA or RA HBDC viability after 0.1-10 U/mL of thrombin treatment (Figure 6-18).

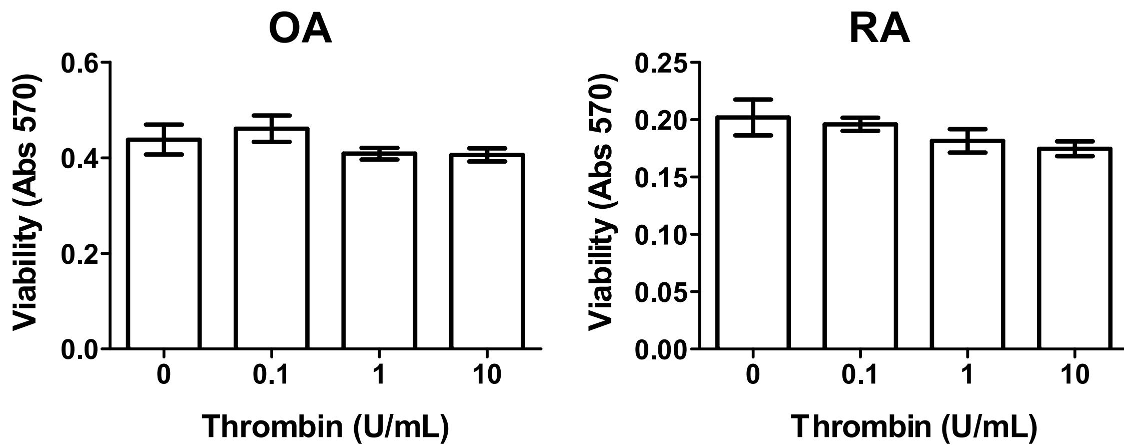

Figure 6-18 *Thrombin treatment of OA and RA HBDCs. OA and RA HBDC were serum-reduced overnight and then treated with 0.1, 1, or 10 U/mL of thrombin and cell viability was measured by MTT. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E. (OA n=4, RA n=3).*

6.4.7 APC Down-regulates ERK1/2, Enhances p27 in OA HBDCs

APC stimulates mitogenesis through signalling of pERK1/2, Akt, p21, p27, and p38 in RA synovial fibroblasts [368, 377]. We previously showed APC can stimulate pERK1/2, pAkt, and p38 in MG-63 cells (Section 3.4.5) and these and NF-κB were examined in cultured OA and RA HBDCs.

APC increased p27 (56%, $P<0.05$; Figure 6-19), decreased pERK1/2 (26%, $P<0.05$) in OA cells but did not significantly affect p21 ($P=0.06$), p-p38 ($P=0.69$), NF-κB ($P=0.64$), or pAkt (10%, $P=0.15$). In RA cells, APC did not significantly influence any of these intracellular signalling proteins (Figure 6-19).

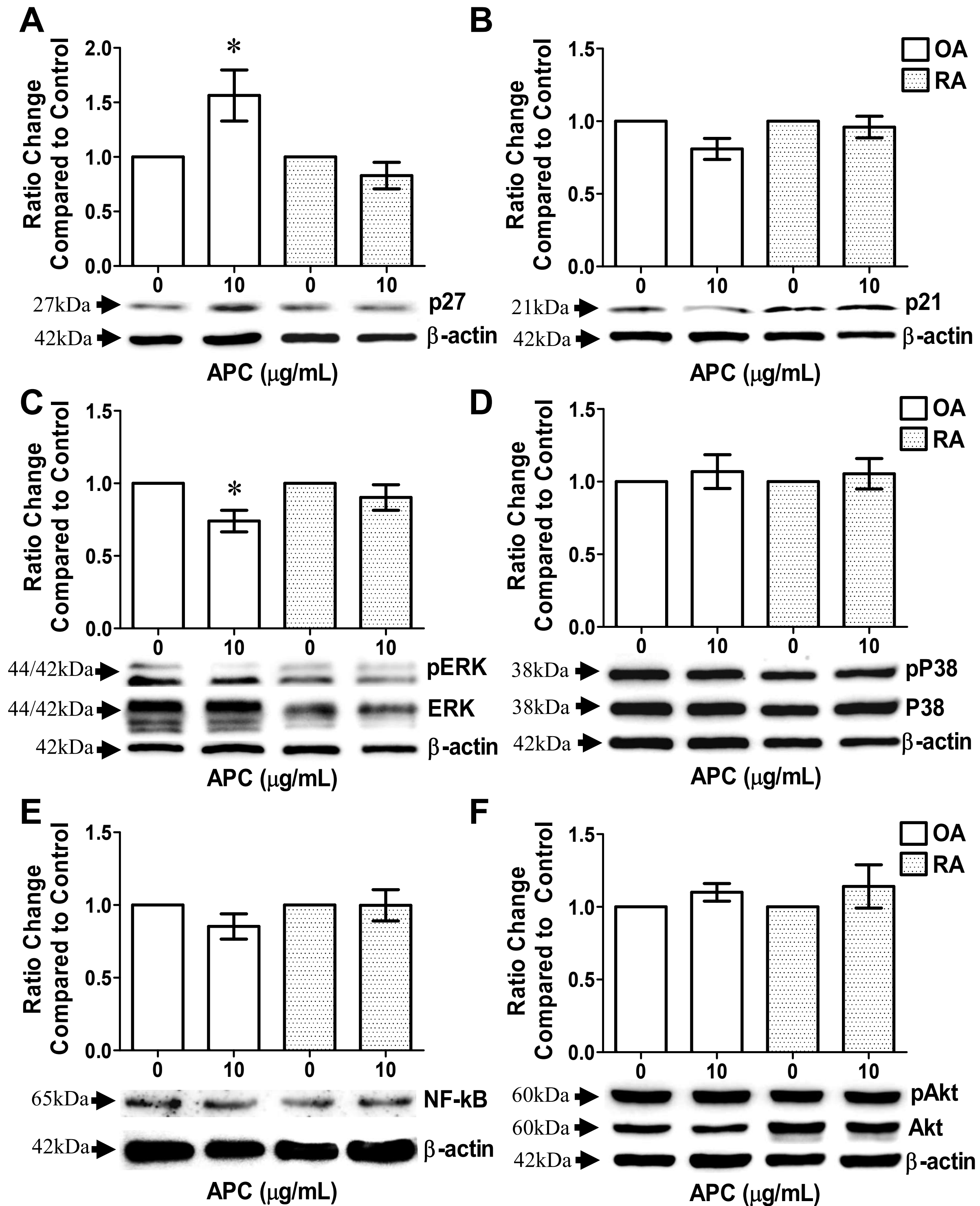

Figure 6-19 *Intracellular signalling in APC-treated HBDCs. OA and RA HBDCs were treated with APC and lysates were analysed by western blot. β-actin was used as a loading agent. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05, ** denotes P<0.01 between treatment and control.*

6.4.8 APC Reduced TNF-α Stimulated Cytokine Production in HBDCs

APC exerts anti-inflammatory effects through its suppression of pro-inflammatory cytokines such as TNF-α and IL-6 [270, 376, 500]. Here we investigated the effect of APC treatment on OA and RA HBDCs cytokine profiles including TNF-α, IL-1β, IL-6, and IL-17. First, we determined the basal levels of each cytokine secreted by untreated cells, and then measured the amount of each cytokine following TNF-α induction, with or without first treating with APC.

At baseline, there was no secretion of TNF-α by OA or RA cells. APC treatment induced TNF-α secretion in one set of OA HBDCs, but not in other OA HBDC sets (Figure 6-20). APC treatment did not induce TNF-α secretion in any of the RA cells (Figure 6-20).

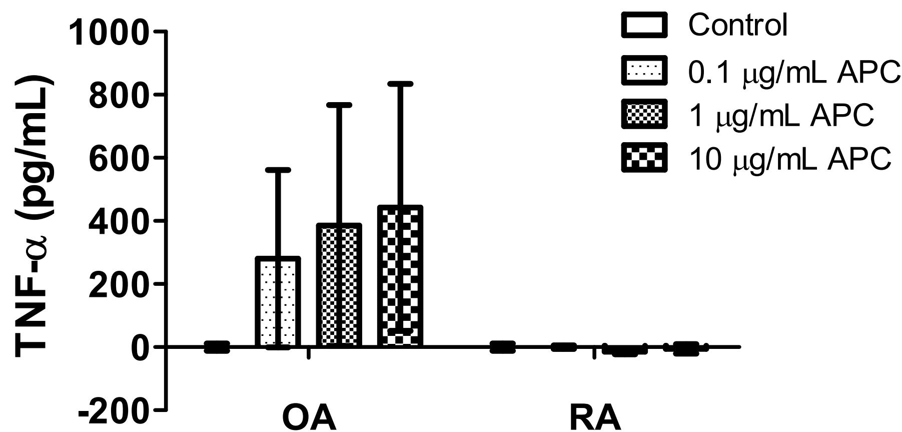

Figure 6-20 *TNF-α secretion by OA and RA HBDCs after APC treatment. Cells were cultured to confluence and incubated with 0.1, 1, or 10 μg/mL of APC over 24 h. Supernatants were then collected and assayed for TNF-α using ELISA. Data are represented as mean ± S.E (OA n=4 and RA n=3).*

TNF-α treatment did not stimulate IL-1β or IL-17 secretion in OA and RA HBDCs (Table 6-2, Table 6-3). OA and RA HBDCs secreted IL-6 at baseline and APC treatment did not alter

IL-6 production in OA or RA HBDCs when used alone (Figure 6-21). However, TNF-α treatment significantly stimulated IL-6 secretion by OA cells in a dose-dependent manner, with increases of 682%, 867%, or 953% in response to the 1, 10, or 100 ng/mL dosages, respectively ($P<0.05$, $P<0.01$, $P<0.01$; Figure 6-21A). APC treatment did not significantly decrease IL-6 production in response to TNF-α stimulation in OA HBDCs.

In RA HBDCs, TNF-α treatment significantly increased IL-6 production by 360%, 442%, or 452% at 1, 10, or 100 ng/mL, respectively ($P<0.01$ for all treatments; Figure 6-21B). APC treatment, when administered in combination with 10 or 100 ng/mL of TNF-α, significantly decreased IL-6 production by 32% or 29% ($P<0.01$, $P<0.05$) as compared to TNF-α stimulation alone.

These results show that APC may ameliorate the inflammatory profile of RA HBDCs through modulation of TNF-α-induced IL-6 production but not in OA HBDCs.

Table 6-2 *Average & (SEM) of cytokine levels of IL-1β and IL-17 after 10 µg/mL of APC treatment or 1, 10, 100 ng/mL of TNF-α in OA HDBCs (n=4).*

Cytokine	No treatment	APC 10 µg/mL	TNF-α 1 ng/mL	TNF-α 10 ng/mL	TNF-α 100 ng/mL	APC + TNF-α 1 ng/mL	APC + TNF-α 10 ng/mL	APC+ TNF-α 100 ng/mL
IL-1β (pg/mL)	0	0	0	0	0	0	0	0
IL-17 (pg/mL)	0	0	0	0	0	0	0	0
IL-6 (pg/mL)	193 (29)	228 (9)	1368 (182)	1707 (277)	1831 (474)	1227 (151)	1303 (145)	1311 (219)

Table 6-3 *Average & (SEM) of cytokine levels of IL-1β and IL-17 after 10 µg/mL of APC treatment or 1, 10, 100 ng/mL of TNF-α in RA HDBCs (n=3).*

Cytokine	No treatment	APC 10 µg/mL	TNF-α 1 ng/mL	TNF-α 10 ng/mL	TNF-α 100 ng/mL	APC + TNF-α 1 ng/mL	APC + TNF-α 10 ng/mL	APC+ TNF-α 100 ng/mL
IL-1β (pg/mL)	0	0	0	0	0	0	0	0
IL-17 (pg/mL)	0	0	0	0	0	0	0	0
IL-6 (pg/mL)	575 (178)	351 (61)	2467 (238)	3120 (115)	3178 (75)	1898 (135)	2127 (327)	2266 (228)

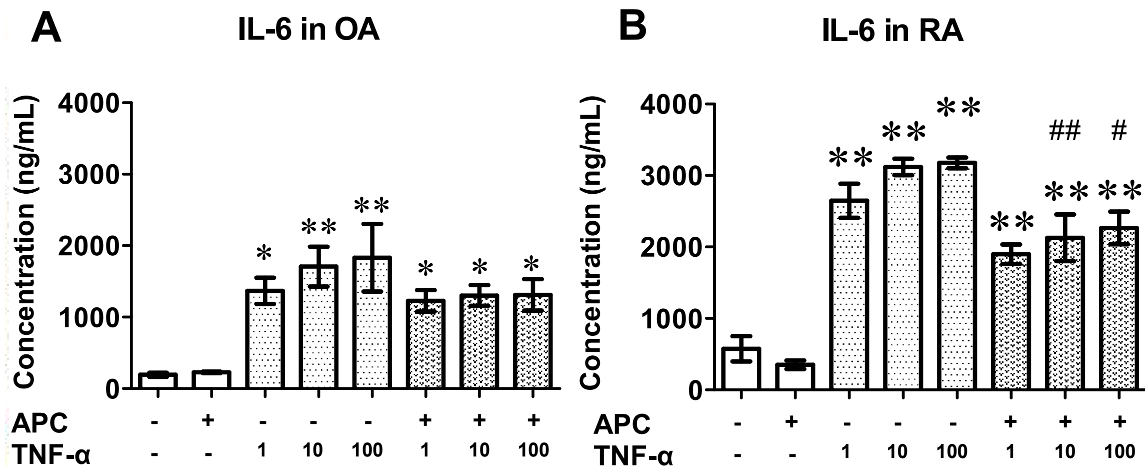

Figure 6-21 *IL-6 secretion by OA (A) and RA HBDCs after APC, TNF-α, or combined treatment (B). Cells were cultured to confluence and incubated with either 10 µg/mL of APC or 1, 10, or 100 ng/mL of TNF-α for 24 h or were treated with APC 30 min prior to TNF-α challenge. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05, ** denotes P<0.01 between treatment and control. # Denotes P<0.05, ## denotes P<0.01 between TNF-α control and respective APC + TNF-α treatment.*

6.4.9 APC Differentially Modulates MMP-2 and MMP-9 in HBDCs

APC has been shown to modulate MMP-2 and MMP-9 in synovial tissue [184]. Basal levels of MMP-2 and MMP-9 were determined by zymography in OA and RA HBDCs. Bands of pro- and active MMPs were confirmed against a positive control. OA HBDCs contained variable levels of pro-MMP-9, the precursor form of MMP-9. Levels of pro- or active MMP-9 levels in OA as compared to RA HBDCs was not significantly elevated (21%, *P*=0.4 and 8% *P*=0.39 Figure 6-22).The ratio of active MMP-9 to total MMP-9 (comprising both active and pro-forms) was not significantly different between OA and RA cells (6%, *P*= 0.54; Figure 6-22). Levels of pro-MMP-2, active MMP-2 were not significantly different between OA and RA HBDCs (11%, *P*=0.27 and 54%, *P*=0.15, respectively). The ratio of active to total MMP-2 was not significantly different between OA and RA cells (17%, *P*=0.11; Figure 6-22).

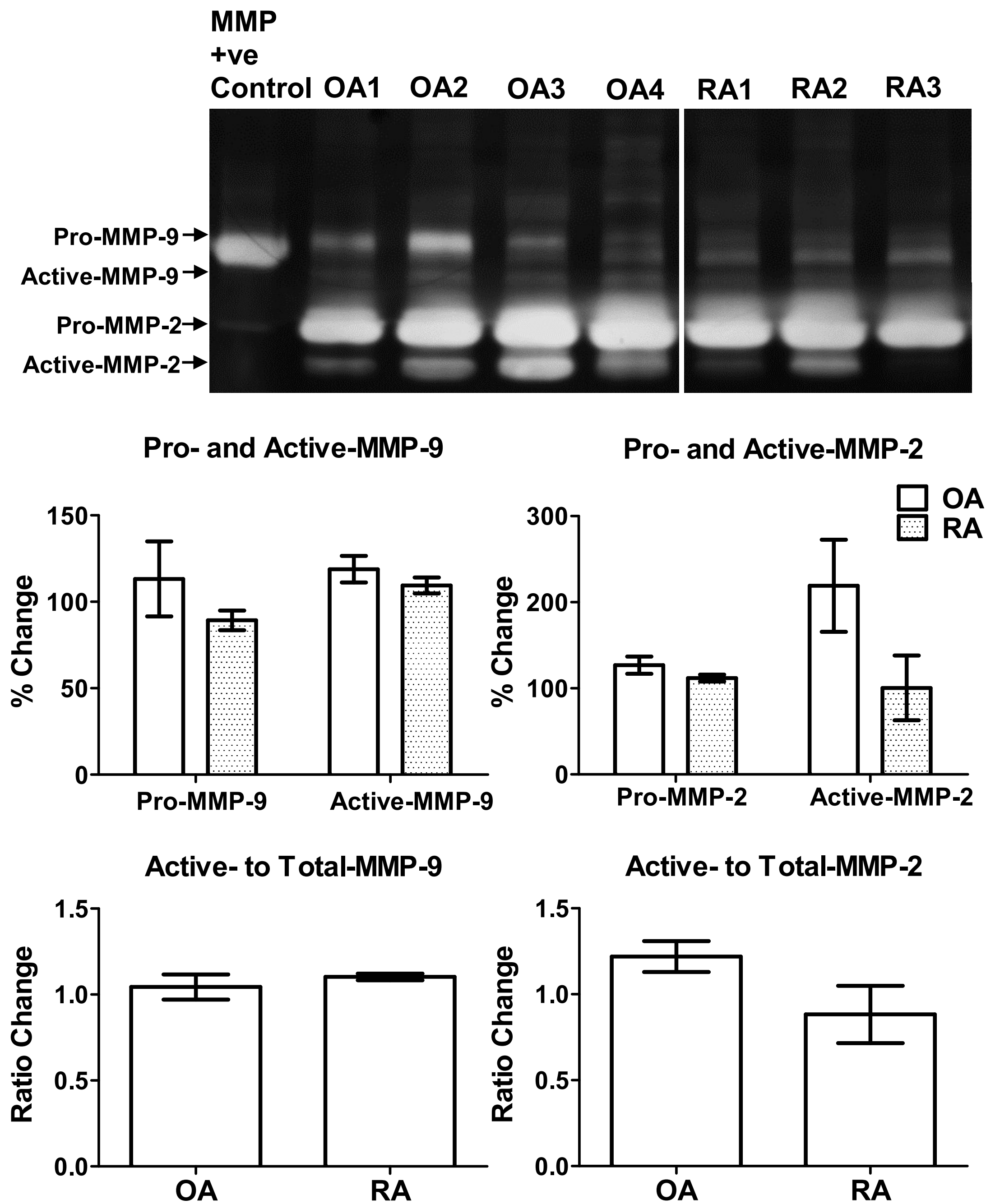

Figure 6-22 *MMP-2 and -9 production by OA and RA HBDCs. Supernatants from HBDCs were normalised to protein levels and run on a gelatin zymography to determine MMP-9 levels and MMP-2 levels. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05 between treatment and control.*

We further investigated the effect of APC, TNF-α, and combined treatment on MMP production in arthritic bone cells. APC alone had no effect on MMP-9 levels in OA HBDCs (Figure 6-23, 6-24). TNF-α treatment alone, 10 or 100 ng/mL, in OA cells significantly increased pro-MMP-9 production by 108%, 109% (both $P<0.05$), but did not affect active MMP-9 levels (Figure 6-23, 6-24). Treatment of HBDCs with APC 30 min prior to TNF-α challenge did not significantly affect MMP-9 levels (Figure 6-23, 6-24). In RA HBDCs, neither APC, TNF-α, nor combined treatment altered MMP-9 levels (Figure 6-23, 6-24).

APC treatment on OA cells significantly increased active to total MMP-2 by 19% ($P<0.05$; Figure 6-25) largely due to its enhancement of active MMP-2 levels by 73% ($P<0.05$; Figure 6-25). TNF-α treatment had no effect on MMP-2 in OA HBDCs. Combined APC and TNF-α treatment had no effect on pro-MMP-2 levels in OA cells (Figure 6-25). However, combined APC and TNF-α-treatment (10 or 100 ng/mL) significantly increased active MMP-2 by 98%-105%, (both $P<0.01$; Figure 6-25) as compared to respective TNF-α controls. This resulted in an increase of active to total MMP-2 in combined treatment cells by 18-26% as compared to respective TNF-α controls (all $P<0.01$; Figure 6-25).

In RA HBDCs, APC also stimulated active to total MMP-2 ratio by 17% ($P<0.05$) as compared to control. This was achieved by a 45% increase in MMP-2 activation from APC treatment ($P<0.05$; Figure 6-25). TNF-α treatment had no effect on MMP-2 (Figure 6-25). Combined APC and TNF-α treatment resulted in increased active MMP-2 by 60%-67% compared to respective TNF-α controls ($P<0.05$ for all; Figure 6-25). This caused an increase in the active to total MMP-2 ratios by 27%-28% in combined treatments as compared to TNF-α only treatments ($P<0.05$ for all; Figure 6-25).

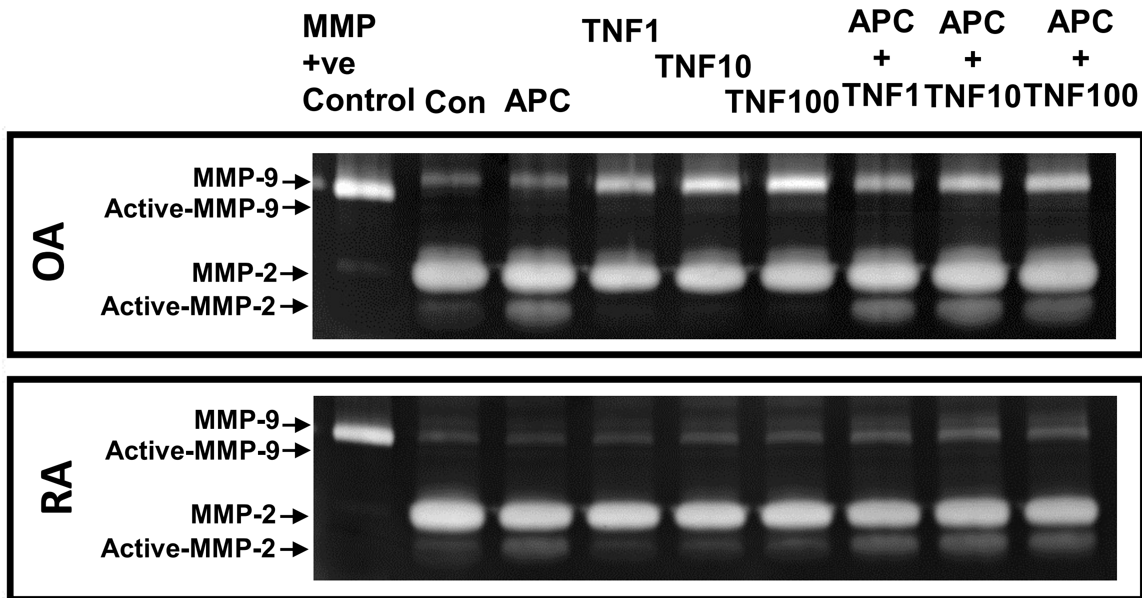

Figure 6-23 *Representative MMP-2 and -9 levels after APC treatment and TNF-α challenge in OA and RA HBDCs. OA HBDCs or RA HBDCs were cultured and treated with or without 10 µg/mL of APC for 30 min before challenge with 1, 10, or 100 ng/mL of TNF-α. Supernatants collected after 24 h and run on a gelatin zymography to determine pro- and active MMP-9 levels as well as pro- and active MMP-2 levels. Data from above zymographies are compiled into graphs in Figure 6-24 and 6-25. Control MMP bands do not precisely line up as MMP bands from different cell types can vary slightly in their migration on PAGE gels.*

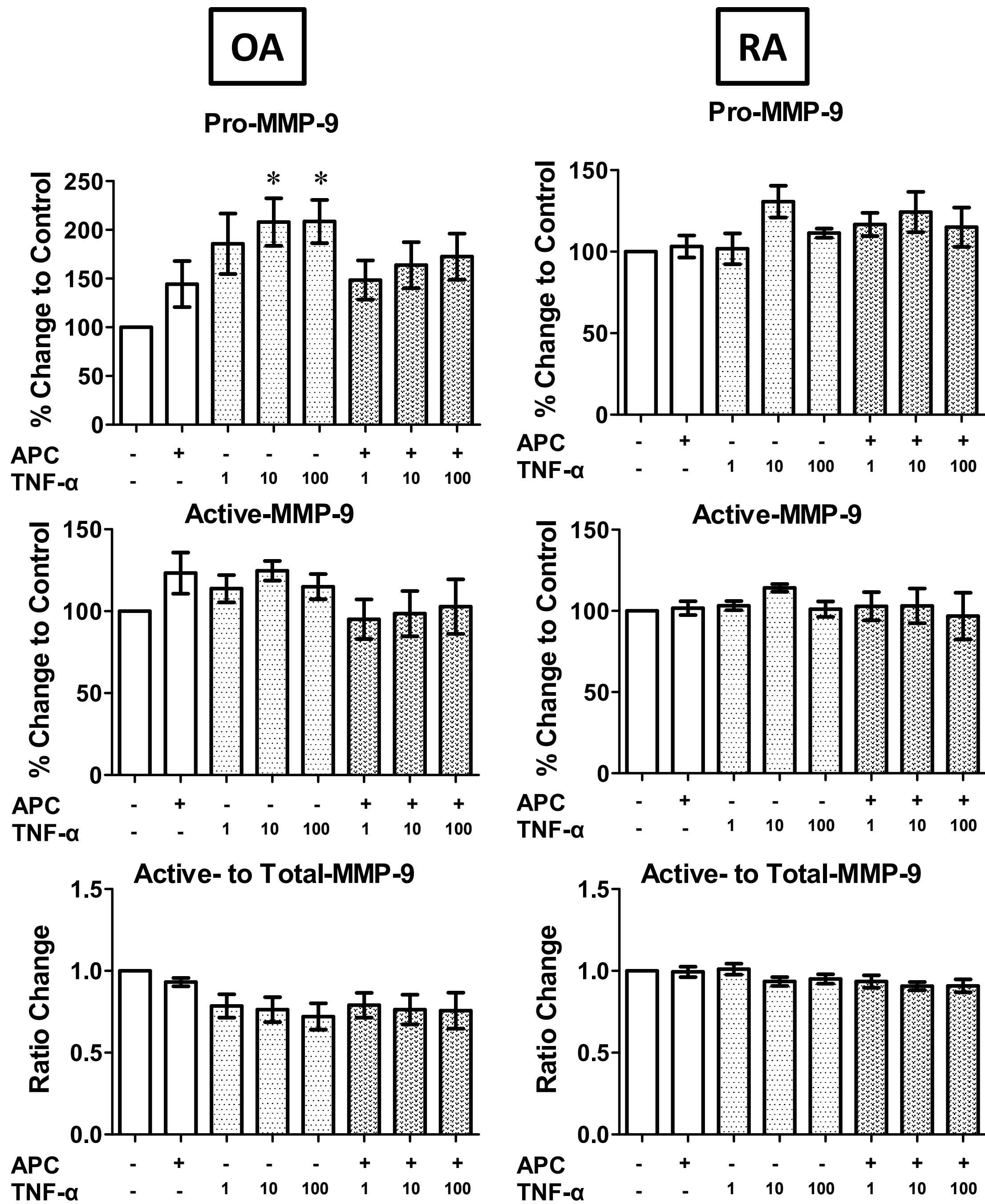

Figure 6-24 *MMP-9 levels after APC treatment and TNF-α challenge in OA and RA HBDCs. OA and RA HBDCs were treated with or without 10 µg/mL of APC before challenge with 1, 10, or 100 ng/mL of TNF-α. Supernatants collected after 24 h and run on a gelatin zymogram to determine pro-, active MMP-9 levels, and active to total MMP-9. Statistical analysis was conducted by one-way ANOVA and Newman-Keuls post-test. Data is presented as mean ± S.E (OA n=4, RA n=3). * Denotes P<0.05 between treatment and control.*

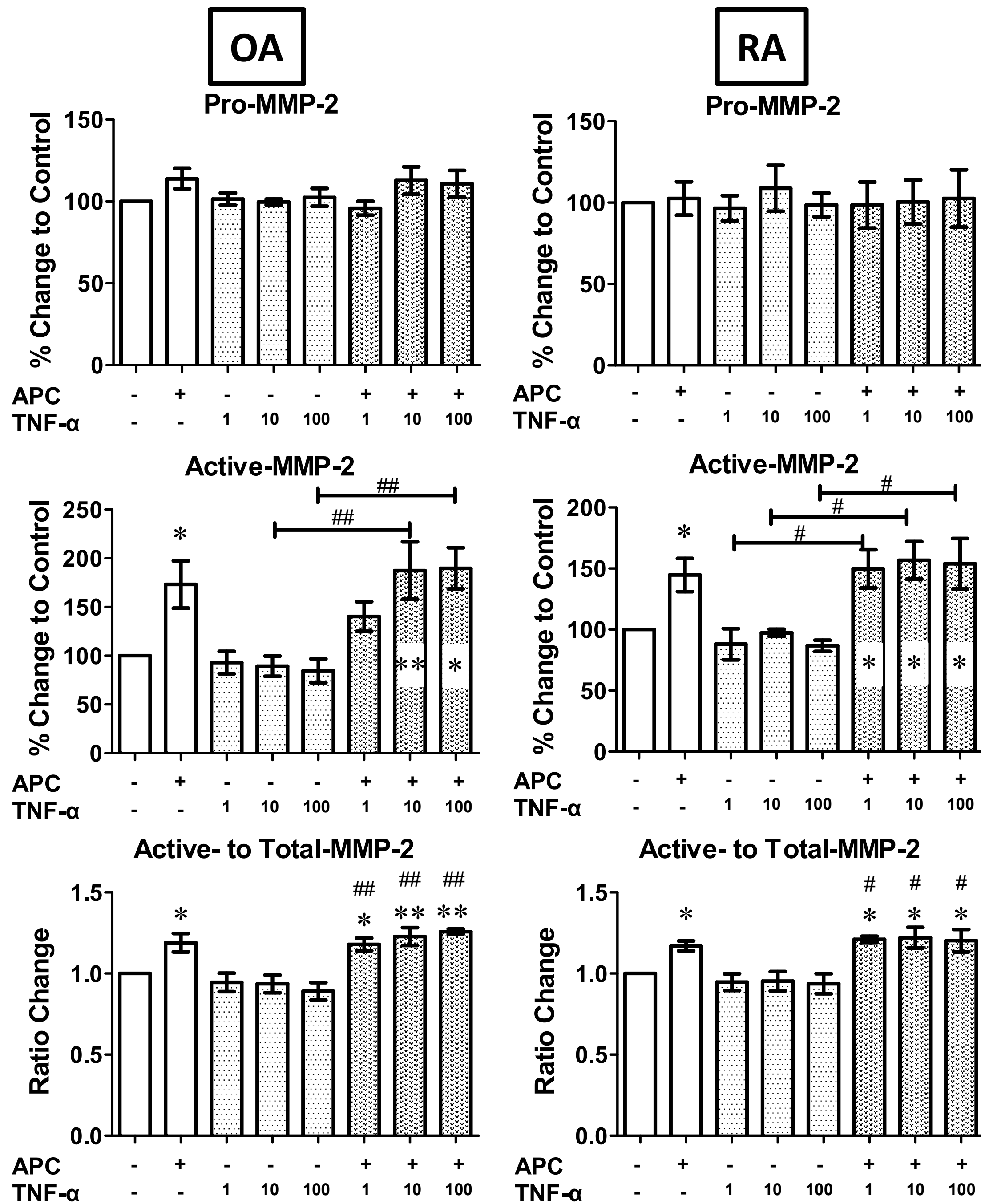

Figure 6-25 *MMP-2 levels after APC treatment and TNF-α challenge in OA and RA cells. OA (n=4) and RA (n=3) HBDCs were treated as per Figure 6-24. Supernatants collected after 24 h and run on a gelatin zymogram to determine pro- and active MMP-9 levels, and active to total MMP-9. Statistical analysis and data presented as per Figure 6-24. * Denotes P<0.05, ** denotes P<0.01 between treatment and control. # Denotes P<0.05, ## denotes P<0.01 between TNF-α control and APC + TNF-α treatment.*

6.5 Discussion

In this study, we sought to further determine whether APC plays a role in the viability and inflammation of bone cells derived from arthritic patients. Firstly, we demonstrated the expression of both APC and its receptors on the surface of subchondral bone and within primary OA and RA HBDCs. We then determined that APC treatment negatively impacted upon the viability of OA HBDCs via EPCR, PAR1, and PAR2 dependent pathways. Furthermore, we observed that APC down-regulated downstream ERK signalling activity and increased p27 expression in these cells. We also observed that APC treatment acted to counter TNF-α-induced IL-6 production by RA HBDCs, and enhanced activation of MMP-2 in both OA and RA derived cells. Overall, these findings illustrate the complex array of effects that APC treatment exerts upon OA and RA HBDCs.

6.5.1 OA and RA HBDCs Possess Different Characteristics

The expression of PC was previously reported in fracture haematomas [268], and in this study, PC expression was found on both subchondral bone surfaces and in HBDCs. The presence of PC on OA and RA bone surfaces suggested a possible role for APC in arthritic HBDCs. Isolation from bone tissue and not bone marrow reduces the potential for contamination of the osteoblastic cells [501]. Cultures of OA HBDCs were predominantly CFU-f negative, exhibited alkaline phosphatase expression, and were capable of differentiation upon combined treatment with ascorbic acid and β-glycerophosphate. Together, these observations indicate that the cell populations were highly enriched for cells of an osteoblastic cell lineage. In comparison, RA cells were adherent cells that were low in CFU-f colonies. RA cells also exhibited low alkaline phosphatase and did not generate calcified bone *in vitro* when differentiated. Our current observations are consistent with previous studies that demonstrated

that RA bone-derived cells exhibit low expression levels of the osteoblast marker alkaline phosphatase, and are also defective in bone formation [194].

6.5.2 Contrasting Effects of APC on HBDC Viability

Based upon the published data demonstrating APC's stimulatory effects upon MG-63 and normal osteoblast proliferation [268], we hypothesised that APC would also enhance the viability of OA and RA HBDCs. Unexpectedly, we found that APC actually down-regulated OA HBDCs viability. These data contrasts with the proven stimulatory role of APC on several different cells types including tenocytes, keratinocytes, endothelial cells, vascular smooth muscle cells, and neurons [259, 268, 282, 343, 371, 373]. However, we recently reported that APC can suppress proliferation in RA synovial fibroblasts [377] by acting i) through regulation of cell cycle proteins to suppress proliferation and ii) through suppression of inflammation, which in turn dampens synoviocyte proliferation [377]. Based on the findings that OA osteoblasts show excessive proliferation in response to chemokine and growth factor stimulation [502, 503], we postulate that APC acts on these cells to suppress excess OA HBDC proliferation.

OA and RA tissue demonstrate pathological changes including: extensive bone remodelling, osteophyte formation, and sclerosis in OA subchondral bone; and impaired osteoblast differentiation, hypomineralisation at sites of bone erosion and bone destruction in RA subchondral bone [194, 195, 210, 230, 493]. In our study, both OA and RA subchondral bone demonstrated histological features of remodelling and osteophyte formation, confirming these pathological changes in the subchondral bone environment. HBDCs derived from RA condyles demonstrated lower cellular viability than OA cells over 72 h, despite being seeded at the same cellular density. This was consistent with a previous publication that found a lower proliferative

capacity, reduced expression of osteoblast markers, and shorter telomere length in RA osteoblasts compared with OA osteoblasts [504].

We found that APC had no significant effect upon RA HBDC viability. This negative finding may be due the limitation of a small sample size in our study, and therefore, reduced power to detect relatively subtle effects. Furthermore, due to the insufficient availability of patient femoral condyles, two knuckles were included in order to maintain the size of the sample group. This could potentially alter the data, as the pathological processes in subchondral condyles may be distinct to those occurring in knuckle joints. Indeed, RA is known for primarily affecting the smaller joints including the metacarpals and proximal interphalangeal joints [177, 505, 506]. Here, we also found higher levels of proteoglycan loss in RA knuckles as compared to RA condyles suggesting greater joint damage in the knuckles.

Our viability results were consistent at 48 h and 72 h, however at 24 h there were some discrepancies between cell count and MTT reading. The latter measures cell viability by mitochondrial metabolism whereas the trypan blue cell dye exclusion assay determines viability through counting the number of viable and non-viable cells within the population. Although both assays are well-established measurements of cell turnover, they have different sensitivities owing to the different measurement parameters of each assay [390].

6.5.3 APC Regulates Signalling Molecules

APC increased the expression of the cyclin-dependent kinase inhibitor, p27, but had no impact upon p21 levels, and was found to decrease levels of phosphorylated ERK1/2 in OA HBDCs. This was consistent with APC-induced down-regulation of OA cell viability. Cell cycle modulator p27 binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4, thus

inhibiting cell cycle progression at G1 [507]. APC has been previously shown to up-regulate both p21 and p27 in RA synovial fibroblasts, where it concurrently down-regulates proliferation [377]. In normal dermal fibroblasts, APC acts in the complete opposite way to stimulate proliferation and down-regulate both p21 and p27 [377]. Thus, it appears that APC inhibits the growth of diseased cells that are rapidly growing, yet stimulates the growth of normal slow-growing cells. Modulation of p27 expression by APC was dependent upon ERK1/2 signalling in RA synovial fibroblasts [377]. ERK1/2 is well established as a stimulator of cell cycle progression from G0/1 to S phase [508, 509]. APC stimulates levels of pERK1/2 and promotes proliferation of normal human osteoblasts [268]. Conversely, the disruption of ERK signalling induces an anti-proliferative effect on human osteoblastic cell lines, which arrest in the G1 and G2/M cell cycle phases [510]. Although the results from our study demonstrate the involvement of p27 and pERK1/2 in APC signalling, we have not confirmed the involvement of ERK1/2 in p27 signalling.

In a similar manner, we also studied the effects of APC upon the intracellular proteins Akt, p38, and NF-κB. Akt signalling is involved in a variety of cellular functions including cell survival and proliferation [511, 512]. Akt knockout mice demonstrate reduced bone mineral density with decreased bone remodelling, and the Akt signalling pathway is required for BMP-induced osteoblast differentiation [513]. Although Akt is not required for the normal survival and proliferation of osteoblasts, it can be activated by stressors including hydrogen peroxidase [514]. APC-induced Akt protects both neurons and myocardium from ischemic damage and prevents noise-induced hearing loss in hair cells [266, 370, 515, 516]. In contrast, APC inhibits proliferation of both OA and RA synovial fibroblasts through the down-regulation of pAkt [377]. The MAPK p38 is up-regulated as a stress response to stimuli, such as inflammation, and it is known to play a major role in the development of arthritis [517]. In osteoblasts, p38

promotes osteoblast differentiation via the regulation of transcription factors such as *Runx2/Cfba1* and *Osterix* [421, 518]. It has been shown that APC-induces p38 activation in both keratinocytes and blood monocytes, and this leads to a wound healing phenotype and increased IL-10 production in each cell type respectively [260, 276]. In contrast, APC down-regulates p38 in tenocytes, RA monocytes, RA synovial fibroblasts, models of acute pancreatitis, septic shock and noise-induced hearing loss [184, 277, 292, 343, 377, 407, 516, 519]. APC does not modulate p38 expression in pulmonary neutrophils despite decreasing neutrophil chemotaxis in an acute lung inflammation model [72]. Based on the importance of Akt and p38 in osteoblasts and APC stimulation of these proteins in other cell types, we hypothesised that APC may modulate Akt and p38 in OA HBDCs. However, we found that APC had no effect on Akt or p38 activation. These studies suggest that APC acts differentially on Akt and p38 in different cell types with neither of these downstream pathways being required to mediate APC's effects upon osteoblast proliferation.

NF-κB interferes with BMP-2 and TGF-β signalling in osteoblasts and down-regulates osteoblast differentiation [520-522]. The inhibitory effect of TNF-α upon osteoblasts is mediated by NF-κB in both *in vitro* and *in vivo* models [196, 428, 523, 524]. APC inhibits NF-κB activation by lipopolysaccharide (LPS), high-mobility group box-1, and alendronate on keratinocytes, HUVECs, and MG-63 cells respectively [269, 282, 525]. APC treatment also reduces NF-κB in animal models of acute pancreatitis and neuronal excitotoxicity [526, 527]. We investigated the effect of APC on NF-κB and hypothesised that APC may suppress NF-κB activity. We found, contrary to our original hypothesis, that APC had no effect on NF-κB in OA or RA HBDCs. The main difference between APC action on NF-κB in other cells as compared to HBDCs lies in the presence of an inflammatory stimulus. In previous *in vitro* and *in vivo* studies, cells or animal models were first exposed to NF-κB enhancing agents prior to

APC addition. We speculate that if APC were applied after inflammatory provocation, that it would also suppress NF-κB.

6.5.4 APC Receptor Involvement

EPCR is present on normal osteoblasts and mediates APC's actions on these cells [268]. We identified EPCR expression on subchondral OA and RA bone surfaces, mainly in osteoblasts and bone lining cells. EPCR expression was also detected in OA and RA HBDCs by both immunostaining and RT-PCR. There was no visible difference in the intensity of EPCR staining between OA and RA on subchondral tissue surfaces. However, further investigation of EPCR expression on primary cells by the more sensitive ELISA technique determined that OA HBDCs expressed significantly less EPCR than RA HBDCs. We further investigated EPCR's role in culture using receptor antagonists; these data showed that EPCR was required for APC's actions on OA HBDCs.

PAR1 is expressed in most joint tissues including synovium, cartilage, bone and the respective cells in these tissues [380, 401, 528-530]. In our study, PAR1 was strongly expressed on bone surfaces, cuboidal cells and bone lining cells in both OA and RA tissue as well as HBDCs. On joint tissues, PAR1 primarily mediates the effects of thrombin. In RA synovium, PAR1 mediates the mitogenic actions of thrombin [528, 529]. In explanted OA cartilage, PAR1 mediates thrombin-induced cartilage damage [531]. Moreover, depletion of PAR1 is protective against cartilage damage in murine arthritis models [532]. In bone, PAR1 mediates the proliferative effect of thrombin, however, the role of PAR1 has not been identified in OA or RA osteoblasts [380, 384, 386]. We hypothesised that PAR1 would be required for APC's inhibitory effects on HBDCs and further determined the role of PAR1 using PAR1 antagonists, agonists and thrombin. We found that PAR1 is involved in APC-mediated suppression of OA

HBDCs. We further investigated the function of PAR1 through antagonist SCH79797 and agonist thrombin.

Similarly to our previous MG-63 study in Section 3.4.4, treatment with PAR1 antagonist SCH79797 alone suppressed HBDC viability. This is likely due to the importance of PAR1-mediated signals in maintaining normal cell growth. PAR1 is necessary for thrombin stimulation of osteoblastic proliferation and antibodies against PAR1 inhibit thrombin activation [380, 385, 401, 405]. SCH79797 is also anti-proliferative through the inhibition of ERK1/2 [404]. Thrombin treatment of osteoblasts also induces growth factors and cytokines including TGF-β, FGF-2, and VEGF in a PAR1-dependent manner [381]. Abolition of PAR1 may, therefore, eliminate endogenous thrombin stimulation of osteoblastic proliferation and signalling. Thrombin independent signalling of PAR1 by FVIIa and FXa has also been demonstrated in endothelial cells [329], although not on osteoblasts. Notably, intravenous dosage of FVII accumulates in bone, primarily in bone forming areas [533], although the function of FVII in bone is unknown. This is suggested to be due to the Gla protein interaction with bone [533]. However, whether these coagulation factors act on PAR1 in osteoblasts is still unknown.

Thrombin is a PAR1 agonist that stimulates MMP-induced cartilage degradation, pro-inflammatory cytokine release, and correlates with the severity of inflammation in arthritic joints [534, 535]. Thrombin is markedly elevated in the RA joint and plasma [536]. Previous reports have shown that thrombin stimulates normal osteoblast proliferation and prevents apoptosis [380, 382, 385, 387]. The current work is the first to report the effect of thrombin on OA or RA osteoblast growth and we found that thrombin had no effect on OA or RA HBDC viability. Interestingly, APC also works through the same PAR1 receptor, yet inhibits HBDC

viability. Although these results at first appear paradoxical, they may be explained by understanding the difference in PAR1 cleavage between thrombin and APC. Thrombin cleaves PAR1 at Arg41 which exposes a tethered ligand which then binds to an exosite of PAR1 to activate the receptor [340]. The peptide agonist used in this study mimics the amino acid structure of the peptide exposed after thrombin cleavage. In comparison, APC cleavage occurs at Arg46 which exposes a different tethered ligand that activates PAR1 via a different mechanism involving β-arrestin, and exerting different downstream effects [340]. This is consistent with evidence that PAR1 cleavage by thrombin or PAR1 agonists induce pro-inflammatory signalling whereas the cleavage of PAR1 by APC induces cytoprotective effects [340].

Similarly to PAR1, PAR2 plays an important role in mediating chronic inflammation in arthritis [537]. We found PAR2 in both OA and RA subchondral bone and HBDCs, but no clear difference was seen between PAR2 expression in OA and RA bone. Treatment with PAR2 agonist in monoarthritis prolongs joint swelling [538, 539]. Conversely, the deficiency of PAR2 in inflammatory arthritis mouse models reduces synovial thickness, cartilage damage and joint swelling [538, 539]. PAR2 inhibition is also known to reduce inflammatory signalling in OA synovial fibroblasts and inhibit RA synovial fibroblast proliferation, invasion, and inflammatory cytokine production [368], suggesting an inflammatory role for PAR2 [540-542]. We also demonstrate that APC can work through PAR1 and PAR2 in OA HBDCs suggesting an anti-inflammatory role for PAR2 in APC signalling.

6.5.5 APC Activation of MMP-2 and Suppression of TNF-α-Induced IL-6

APC is an anti-inflammatory agent in many diseases and we hypothesised that APC would down-regulate a spectrum of inflammatory cytokines with or without TNF-α challenge. Our present study showed that there was minimal production of IL-17 or IL-1 by HBDCs under basal conditions, which was consistent with a previous study that showed an absence of IL-1α, IL-1β expression by RA and OA osteoblasts [543]. There was no basal secretion of TNF-α in OA HBDCs. However, one set of HBDCs also markedly increased TNF-α in response to APC treatment. It is unknown whether the patient from whom the cells were derived had any other inflammatory conditions which could have affected this cytokine profile. Other OA and RA HBDCs expressed minimal amounts of TNF-α, consistent with previous literature [543]. Instead, cytokines (including TNF-α) are more commonly secreted by synovial cells to drive the inflammatory cascade in bone and cause impairment in osteoblasts [194, 544, 545]. The lack of TNF-a secretion from OA and RA HBDCs may be due to the small number of samples we collected, a greater number of samples may demonstrate different responses, and thus we cannot draw a solid conclusion from this data.

Similar to its action in RA and OA synovial cells, we found that TNF-α-stimulation markedly enhanced IL-6 levels in HBDCs, however, TNF-α did not stimulate IL-1β or IL-17. TNF-α and IL-6 are primary molecules through which bone destruction occurs [192, 546]. The stimulation of IL-6 by TNF-α in our RA cells further supports the concept that anti-TNF-α treatment can dampen the inflammatory cascade in RA bone. In OA, TNF-α is a potent driver of the inflammatory cascade [225, 547]. Both TNF-α levels and IL-1β levels are elevated in OA subchondral bone, and drive IL-6 production [225, 547]. The role of IL-6 in osteoblasts is controversial and the conflicting reports suggest that it depends upon the cells models used [195, 548, 549]. IL-6 studies *in vitro* show contradictory effects on alkaline phosphatase and

osteoblast differentiation with stimulation, inhibition or no effect depending on the cell type [550-557]. Furthermore, some studies have shown no effect of IL-6 on human osteoblast proliferation [558-560]. Whereas another study demonstrates a positive response from IL-6 on osteoblast proliferation [561]. Production and mRNA of IL-6 are higher in RA and OA osteoblasts as compared to normal osteoblasts [562]. A study has also found two sets of OA osteoblasts differentiated by their low and high level of IL-6 secretion, although this was not correlated with markers of bone cell activity [496]. From these complicated actions demonstrated by the literature, there is clearly still much more to be understood from the role of IL-6 in arthritic osteoblasts.

Our second hypothesis was confirmed when upon adding APC prior to TNF-α challenge, there was a marked decrease in TNF-α-induced IL-6, suggesting that APC can act to ameliorate the cytokine cascade [192]. This is consistent with APC's dampening effects on IL-6 in pancreatitis, sepsis, acid aspiration lung injury, and necrotising enterocolitis [563-566]. This reduction in IL-6 production by APC may be important in OA and RA subchondral bone, as IL-6 is a primary mediator of osteoclast differentiation and bone resorption [60, 202, 546, 567, 568]. The therapeutic significance of APC in arthritic bone can be assessed in preclinical models of OA and RA, and this is one potential future direction for this research.

A major limitation of this study is the fact that the HBDCs were derived from end stage disease joints, which possess a significantly different morphology and response to those of earlier stages of the disease. For example, it is known that the cytokine profiles of synovial cells from end-stage OA patients produce fewer cytokines in response to inflammatory IL-1α trigger as compared to normal synovial tissue explants [569]. A similar change can be found in RA where higher levels of cytokines, including IL-2, IL-4, and IL-17, are present in early stage disease

as compared to later disease [570]. These studies suggest that tissue from late stage arthritis is characterised by a blunted response to inflammation, thus extrapolation of the current results to early OA and RA disease is limited.

Cytokines including TNF-α can drive joint degradation in both RA and OA via their actions on MMPs [571, 572]. It has been previously reported that MMP-9 levels are detectable in RA synovial fibroblasts but not OA synovial fibroblasts [573], however, there is no published information on MMP-2 and MMP-9 levels in OA and RA osteoblasts. In this study, we found no significant difference in MMP-2 or MMP-9 between OA and RA HBDCs under basal conditions. TNF-α treatment significantly enhanced pro-MMP-9 production in OA HBDCs, a phenomenon previously observed in monocytes [574]. Interestingly, this pattern was not present in RA cells, which may reflect the patient demographics. For example, treatment with infliximab and golimumab have been shown to reduce MMP-9 levels in peripheral monocytes of RA patients and may affect the MMP profile in RA HBDCs [200, 575-577].

Elevated levels of APC in RA synovial fluid were previously found to correlate with MMP-2 levels, and this was confirmed in IHC studies of synovial sections [281]. APC differentially regulates MMP-2 and MMP-9 in skin keratinocytes, RA synovial fibroblasts and RA monocytes [184, 282], although in OA chondrocytes, APC activates both MMP-2 and MMP-9 [283]. Elevated levels of APC in RA synovial fluid correlate with elevated MMP-2 levels and this was confirmed in IHC studies of synovial sections [23]. Despite the evidence for APC-mediated MMPs in arthritic synovium, MMP-2 and MMP-9 have not previously been investigated in the context of bone [260, 282, 484, 578]. We hypothesised that APC would down-regulate TNF-α-induced pro-inflammatory MMP-9 and up-regulate anti-inflammatory MMP-2, similarly to its actions in synovial cells [184, 573]. However, we found that APC

treatment only attenuated pro-MMP-9 in OA HBDCs after TNF-α challenge. APC did not alter active MMP-9 levels in either cell type. However, the down-regulation of pro-MMP-9 levels may lead to a further decrease of active-MMP-9 levels by decreasing the available substrate for activation.

In contrast to its effects on MMP-9, APC markedly increased active MMP-2 in the presence or absence of TNF-α on both OA and RA HBDCs. This is similar to previous studies showing that APC stimulated the expression and activation of MMP-2 in RA synovial fibroblasts, endothelial cells, keratinocytes, and monocytes [184, 282, 578]. In monocytes and RA synovial fibroblasts, MMP-2 acts to inhibit inflammatory response and its suppression results in increased TNF-α and IL-17 [573]. Furthermore, deficiency of MMP-2 in mice results in severe clinical and histological arthritis [579]. The activation of MMP-2 by APC in all arthritic bone samples suggest that it may be involved in APC's dampening effect on inflammation. The effect of APC on other MMPs including MMP-1, MMP-3 and MMP-13 are suggested for future investigations as these MMPs are increasingly found to be implicated in OA pathogenesis [225].

6.6 Summary

APC is a potent stimulator of proliferation of many different cell types. It also acts to counter inflammation through the down-regulation of the cellular response to TNF-α and LPS. We have previously shown that APC increases the proliferative rate of MG-63 cells, and also their production of inflammatory cytokines. Here, we examined this response in primary subchondral HBDCs isolated from OA and RA patients. Primary cultures generated from both disease types yielded heterogeneous populations of cells which for the OA samples was enriched for pre-osteoblasts and osteoblasts. Investigation of PC and receptors found that they

were expressed on both OA and RA subchondral bone and also within primary cells. APC treatment was found to suppress OA HBDC viability, and this effect was mediated through the EPCR, PAR1, and PAR2 receptors. These effects coincided with activation of downstream p27 and suppression of pERK1/2. In contrast, treatment of RA and OA HBDCs with APC had no effect on NF-κB, p38, p21, or Akt. Notably, APC counteracted TNF-α to decrease IL-6 in RA HBDCs and stimulate MMP-2 activation in both OA and RA cells. In summary, we have shown that APC can suppress OA osteoblast viability and modulate inflammatory profiles in OA and RA cells via partly resolved signalling mechanisms.

CHAPTER 7:

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7 General Discussion and Future Directions

APC is a physiological circulating protein with potent anticoagulant and cytoprotective properties. A recombinant form of the protein has been used as a therapeutic agent to treat sepsis. The cytoprotective effects of APC in numerous tissue types have been widely published over the past 20 years. This includes research into the involvement of APC in diseases such as OA, RA, and tissue healing. The first report of APC in bone was in 2010, when Kurata *et al* detected the presence of PC in fracture haematomas and that APC stimulated human osteoblast proliferation [268]. These findings provided evidence for APC involvement in bone physiology and formed the basis for our investigation into the actions of APC on bone. We investigated the physiological effect of APC on osteoblasts as well as the therapeutic effects of APC on bone formation and fracture healing. Building upon evidence of APC in normal osteoblasts, we sought to further investigate the effect of APC on OA and RA HBDCs in the context of cellular viability and inflammation.

The major novel findings of this thesis include:

1) APC stimulates MG-63 viability through PARs;
2) APC augments BMP-2-induced ectopic bone formation, and does not affect osteoclasts numbers;
3) APC does not increase healing outcomes in a murine model of closed fracture healing;
4) APC reduces OA HBDC viability, ERK1/2 through canonical receptors; and
5) APC inhibits inflammation in RA and OA HBDCs; including suppression of TNF-α-stimulated inflammatory IL-6 in RA HBDCs.

The association between these findings in relation to current literature are discussed below.

7.1 Stimulation of Bone Anabolism

Osteoblasts are key moderators in bone formation and repair processes. Previous studies have determined that APC increases proliferation, cell viability, and markers of osteoblastic differentiation in MG-63 cells [268, 269]. Our studies found a contrastingly different effect from APC on MG-63 cells as compared to OA HBDCs. This difference may be attributed to several different reasons including the excessive the excessive proliferation of OA osteoblasts as discussed in Section 6.5, a lack of contact inhibition, the highly aberrant karyotype (Section 2.1.1), and the difference between a transformed cells, or the diseased phenotype of OA and RA osteoblasts [502, 503]. For example, MG-63 cells and OA osteoblasts respond differently to IL-1. MG-63 cells release high molecular weight isoforms of fibroblast growth factor (FGF)-2 in response to IL-1 [580]. These isoforms are the same isoforms that are secreted by primary osteoblasts from normal patients but different to the low molecular weight isoforms of FGF-2 released by osteoblasts from OA patients [580]. High and low molecular weight isoforms have opposing effects on bone, the low molecular weight isoform of FGF-2 increases bone mass, whereas the high molecular weight isoforms inhibit matrix deposition [581-583]. We postulate that APC may act differentially on FGF-2 isoforms to achieve opposing effects in MG-63 cells and OA osteoblasts.

Cumulating evidence in experimental, animal, and human studies have shown that APC is a potent therapeutic agent for soft tissue healing. APC induces migration and proliferation of keratinocytes, smooth muscle cells, and endothelial cells, which are essential for angiogenesis and wound healing *in vitro* and *in vivo* [259, 282, 371]. In small clinical studies, APC treatment in patients also improves healing of chronic leg ulcers, chronic wounds from orthopaedic surgery, and diabetic ulcers [290, 291, 309]. This APC-induced healing occurred despite

underlying vascular disease or recurrent infections. Bone healing is a specialised type of wound healing and thus shares a similar chain of events [584].

Both types of healing follow a sequence of dynamic events including haemostasis following injury, an inflammatory phase with cytokine production and immune cell infiltration into the injury site, the removal of damaged tissue, recruitment of progenitor cells, and the restoration of local vasculature [584-586]. This is followed by a proliferative phase, which involves predominantly fibroblasts, epithelial, and endothelial cells in soft tissue, and chondrocytes and fibroblasts in bone [70, 587]. These cells proliferate to fill up the wound space with a preliminary scaffold [70, 587]. A major difference between bone and soft tissue healing is the formation of a soft/hard callus in bone versus scar formation in soft tissue [584, 586]. However, both types of healing undergo further remodelling to improve wound strength which involves scar contraction in soft tissue or replacement of trabecular with compact bone [70, 588]. These similar mechanisms between soft tissue and bone repair as well as the strong evidence for APC as a soft-tissue healing agent inspired our work on bone healing.

While Kurata *et al* detected endogenous PC in fracture haematomas and demonstrated a protective effect of APC on osteoblasts [268], they did not further investigate the impact of APC on bone formation.

We found that APC not only stimulated osteoblastic cell line viability (Section 3.4.2) but also augmented BMP-2-induced bone formation *in vivo* (Section 4.4.1). This demonstrates an additive interaction between the two proteins. BMP-2 can also interact with factors in the coagulation system including Factor X (FX). The inhibition of FX leads to decreased levels of BMP-2 gene expression [589-592] and decreased osteoblast growth, metabolism, and alkaline

phosphatase production [591, 593]. BMP-2 binds to the matrix Gla protein [47], a domain which is present on FX and other coagulation factors including thrombin, FVII, FIX, and APC [245, 594]. However, it is unknown whether BMP-2 can interact with this site on APC or whether this site is required for the augmentation of ectopic bone formation by APC. We hypothesise that there is an interaction between APC and BMP-2 which may require the binding of BMP-2 to APC Gla domain. Further *in vitro* binding studies of APC and BMP-2 treatment as well as the use of anti-Gla-peptide antibodies could help determine whether BMP-2 binds to this Gla domain on APC.

APC has two main functions; as an anticoagulant and a cytoprotective agent. The effect of APC in stimulating bone formation may rely on its actions as a cytoprotective agent rather than its anticoagulant function. Further studies using modified APC with reduced anticoagulant actions, such as 3K3A-APC [595], will be useful to ascertain whether APC requires anticoagulant activity to enhance bone formation. This form of APC has already demonstrated safety in both preclinical and phase I clinical trials, with markedly reduced bleeding risk as compared to wild-type APC [257, 595]. Interestingly, the removal of anticoagulant function in 3K3A-APC demonstrates more neuroprotection against infarction and ischemia-induced apoptosis, as well as improved recovery from traumatic brain injury as compared to conventional APC [596-598]. Future studies may also need to consider species-specific effects, as murine APC is more protective than human APC in some mouse models. For example, murine 3K3A-APC administered at 4 h after embolic stroke in mice improved functional outcome and reduced by 80% the infarct volume 7 days after stroke, at 10-fold lower doses than human 3K3A-APC [599]. It is feasible that the use of murine 3K3A-APC in mouse studies will show improvements on bone formation and fracture healing over wild-type APC.

Elevated levels of pro-angiogenic factors, present in the fracture haematoma, enable mesenchymal and hematopoietic progenitor recruitment and differentiation into osteoblasts and osteoclasts [99, 115, 600-602]. Although APC did not affect osteoclast numbers in our study, the effect of APC on the osteoclast lineage is unknown. APC acts on monocytes, which are osteoclast precursors, to suppress production of, and response to, TNF-α through the inhibition of NF-κB [276, 277, 280, 353, 375, 603-612]. APC also blocks monocyte migration, chemokine release, proliferation, and endothelial binding, but enhances monocyte survival and microparticle release [276, 277, 280, 353, 375, 603-612]. Both TNF-α and intracellular NF-κB signalling are required for osteoclastogenesis from monocyte/macrophage precursors [613, 614], suggesting that the inhibitory actions of APC on monocytes may reduce osteoclast numbers. Therefore, we hypothesised that there would be a decrease in osteoclasts in response to APC, however, we found no change in the number of TRAP-positive cells in bone nodules. The effect of APC on osteoclast formation and activity is yet to be resolved.

The effect of APC on osteoclastogenesis could be further examined either directly or through osteoblast-to-osteoclast coupling. For the former, osteoclast precursors are treated with APC in the presence of osteoclastogenic agents including TNF-α, and the subsequent effect on NF-κB signalling and bone resorption measured. The coupling between osteoblasts and osteoclasts can be determined by first exploring the effect of APC on the RANK/RANKL/OPG axis in both cell types followed by an assessment of the cell communication using direct or indirect co-cultures in the presence of APC. This would provide a more comprehensive picture of how APC acts in bone.

Based on the previous literature and the results from the current study, we speculate that APC acts on a number of different cell progenitors and mature cells in the bone environment to

augment bone formation, as shown in Figure 7-1. These effects include protection of osteoblastic viability and stimulation of osteoblastic proliferation and differentiation, however it is unlikely that APC affects osteoclast numbers.

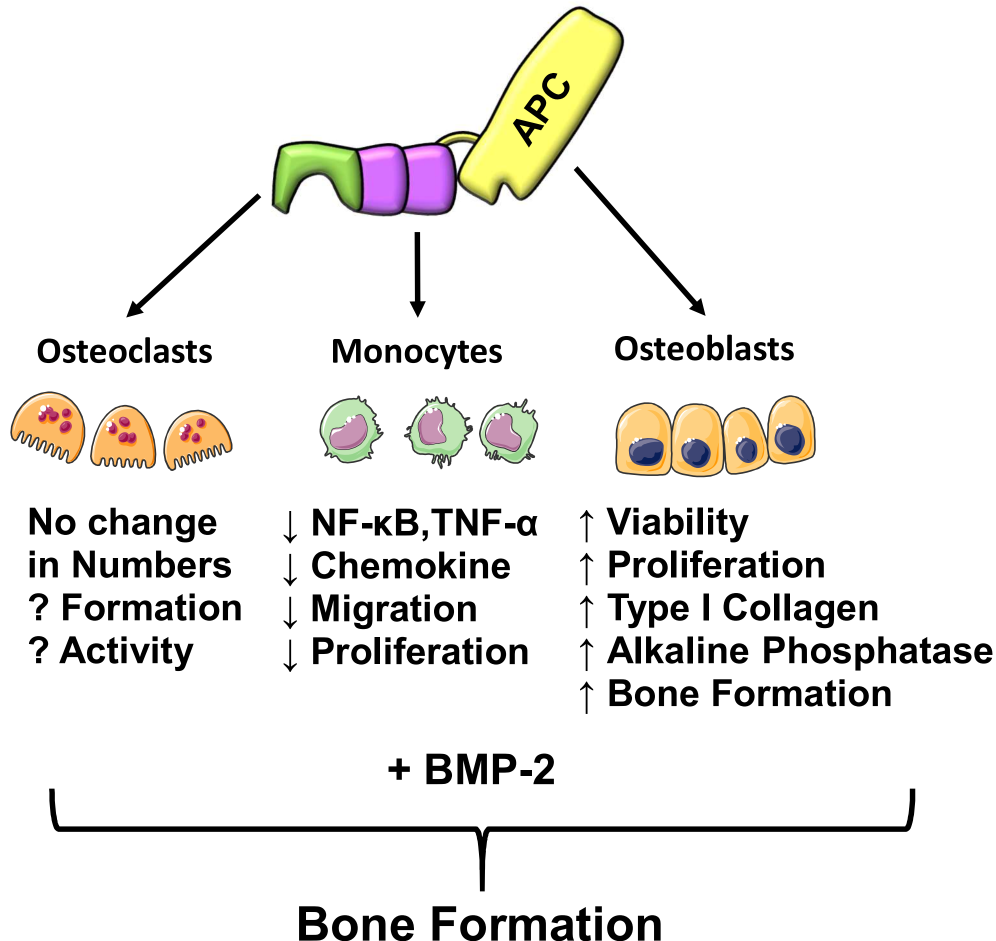

Figure 7-1 *Schematic showing our model of APC actions on osteoclasts, monocytes, and osteoblasts. APC does not affect osteoclast numbers in bone nodules, however, APC's action on osteoclastogenesis is unknown. In monocytes, APC decreases migration, proliferation, chemokine production, TNF-α production, and suppresses NF-κB. On osteoblasts, APC increases viability, proliferation, type I collagen, alkaline phosphatase, and bone formation. The combination of APC and BMP-2 enhances bone formation.*

7.2 Orthopaedic Application of APC

Similarities between fracture healing and soft-tissue/cutaneous wound healing [290, 291, 309, 584] led to our hypothesis that APC would enhance fracture repair. However, we found that APC had no effect on murine closed fracture repair as evidenced by a lack of increase in bone volume or tissue volume in fractures at day 21. This unexpected result may be explained by several reasons, as discussed in Section 5.5, including the use of human APC instead of mouse APC as described above, and the choice of closed fracture model. Closed fractures are less challenging to heal than open fractures as they have a shorter time to union and lower rates of non-union due to a more favourable biologic environment [95, 481]. APC may have therapeutic effects in a more traumatic fracture model, such as an open fracture.

An additional potential application for APC is osteonecrosis. Vascular disruption is postulated to be one of the primary mechanisms for the development of avascular necrosis of the femoral head [615-617]. Restoration of angiogenesis in the necrotic bone tissue accelerates the repair process [615, 616, 618]. Current trials using stem cell therapy in avascular necrosis of the femoral head aims to improve both osteogenesis and angiogenesis [618]. Similarly, the mechanism for bisphosphonate-related osteonecrosis of the jaw is also related to low bone turnover and disruption of angiogenesis [619, 620]. In previous studies, APC has been demonstrated to enhance angiogenesis and protect against selective bisphosphonate-induced osteoblast death [265, 269]. In our study, we demonstrate that APC can also enhance osteoblast activity. Thus, we propose that APC may be a useful therapeutic agent in both avascular and bisphosphonate-related osteonecrosis. However, the choice of bisphosphonates in conjunction with APC treatment is important as differential effects are found with pamidronate, zoledronate, and alendronate [269]. Whereas all three drugs induced MG-63 cell death in a dose- and time-dependent manner, pamidronate- and zoledronate-related cell death were

prevented by APC treatment, however, cell death induced by alendronate was accelerated by APC [269].

7.3 Mechanism of PAR Signalling

APC docking to EPCR and subsequent cleavage of PAR1 and/or PAR2 modulates the primary effects of APC [621]. The role of PAR1 in APC-mediated effects on osteoblasts, however, is unclear. Kurata *et al* indicated that APC does not act through PAR1 for its actions on normal human osteoblasts [268], whereas we demonstrated that PAR1 is required for both APC *in vitro* signalling in MG-63 and MC3T3-E1 cells and *in vivo* stimulation of BMP-2-induced bone formation. Similar to our results, PAR1 is known to mediate many of the APC-induced cytoprotective effects *in vitro* and *in vivo*, including inhibition of apoptosis, promotion of cellular proliferation, and related ERK signalling mechanisms in numerous cell types [248, 250, 260, 622, 623]. There are currently no reports that directly compare the difference in PAR1 signalling between osteoblast cell lines and primary osteoblast cells.

Although PAR1 mediated the enhancement of bone formation, osteoblast viability, and differentiation induced by APC, thrombin which is also a PAR1 agonist failed to exert a similar effect. How can two molecules which act on the same PAR1 receptor exert different effects? This may be partly explained by the requirement of cell membrane caveolae compartmentalisation in APC-induced PAR1 signalling, which is not necessary for thrombin signalling [267, 327, 354, 624, 625]. An alternative explanation was revealed in a recent paper which demonstrated novel cleavage of PAR1 by APC at a different site as compared to thrombin [340]. This paper demonstrated the difference between APC and thrombin signalling through the use of synthetic agonists peptides, TR47 and TRAP, which mimic the cleaved PAR1 N-terminus by APC at Arg46 and thrombin at Arg41, respectively [340]. The effect of

TR47, a 20-mer amino acid peptide, is similar to APC and demonstrates a biased signalling towards cytoprotective pathways but does not simulate the pro-inflammatory effects of thrombin [340]. TR47 stimulation of Akt and enhancement of endothelial barrier stabilisation *in vivo* suggests that this peptide could be a novel agonist that substitutes for APC to exert cytoprotective actions.

We found that EPCR, PAR1, and PAR2 were expressed on RA and OA HBDCs and subchondral sections of OA and RA patient samples, with the expression of PAR1 particularly prominent on bony surfaces in both OA and RA samples. Both PAR1 and PAR2 are involved in arthritic pathophysiology. PAR1 mediates thrombin-induced cytoprotection against oxidation in OA synoviocytes and thrombin-induced proliferation of RA synovial cells [283, 626, 627]. Ablation of PAR1 in mice significantly reduces the severity of joint inflammation and cytokine production in antigen-induced arthritis as compared to wildtype [532]. PAR2 expression is correlated with disease activity in both OA and RA synovium [541, 542, 628-630] and deficiency of PAR2 is protective against OA and RA-mediated joint damage [368, 388, 538, 539, 631-633]. Despite the involvement of PARs in inflammation and arthritis, only one paper has reported PARs in OA and RA osteoblasts. The authors found higher PAR2 expression in OA osteoblasts as compared to normal osteoblasts, and this was further enhanced in the presence of pro-inflammatory signals [630]. Activation of PAR2 by specific agonists stimulated OA osteoblast production of inflammatory cytokines and bone resorption factors [630]. To date, there have been no reports on APC-induced PAR signalling in OA or RA osteoblasts. We found that PAR1 and PAR2 mediated down-regulation of OA HBDC viability by APC (Section 6.4.6), confirming the unique actions of APC on PAR receptors and describing a new role for PAR signalling in OA osteoblastic cells.

7.4 Implications of Intracellular Signalling

ERK 1/2 is a primary signalling pathway by which APC induces its proliferative effects [247]. In normal osteoblasts, ERK1/2 is not only essential for proliferation but also plays an important role in osteoblast differentiation, as discussed in Section 3.5 [25, 31, 417, 510, 634]. In our study, APC differentially modulated ERK1/2 in OA HBDCs compared to MG-63 cells (Figure 7-2). ERK1/2 is likely to be involved in APC stimulation of MG-63 viability and differentiation as discussed in Section 3.5. However, in OA, ERK1/2 drives inflammatory signalling by mediating IL-1β- and S1P-induced cytokine and prostaglandin release by chondrocytes [635-637]. ERK1/2 is also involved in crosstalk between chondrocytes and osteoblasts in OA, where ERK1/2 activation is required for chondrocytic enhancement of osteoblastic differentiation and vice versa, for osteoblastic induction of hypertrophic changes in chondrocytes [638, 639]. Thus, overactivity of the subchondral bone is driven partly by ERK1/2 signalling. APC suppression of ERK1/2 in OA osteoblastic cells but may play an important role in down-regulating chondrocyte responses to osteoblast crosstalk.

In osteoblasts, MAPK p38 promotes osteoblast differentiation and mediates responses to inflammation in arthritis [518]. Stimulation of RA and OA synovial fibroblasts with TNF-α phosphorylates p38 and conversely, inhibition of p38 completely abolishes TNF-α-induced proliferation and IL-6 secretion in both OA and RA synovial cells [640]. Similarly to ERK1/2, p38 is involved in IL-1β- and S1P-mediated increase of prostaglandin and IL-6 in OA chondrocytes [635, 636]. In crosstalk between OA subchondral osteoblasts and articular chondrocytes, p38 down-regulation is required for osteoblast-induced hypertrophic changes in chondrocytes but is not involved in chondrocyte-induced osteoblastic differentiation [638, 639]. In RA, p38 contributes to the expression of pro-inflammatory cytokines, chemokines, and MMPs [517]. We have demonstrated that APC induces p38 in MG-63 cells, where it may

contribute to differentiation, but not in OA or RA HBDCs (Figure 7-2). The evidence for a pro-inflammatory role of p38 in arthritis and the lack of APC stimulation of p38 in OA or RA HBDCs is consistent with the anti-inflammatory APC actions we found on OA and RA HBDCs (Section 6.4.9-10), further providing evidence to support an anti-inflammatory role for APC in OA and RA. Activation of p38 in normal osteoblasts, however, promotes osteoblast differentiation as discussed in Section 3.5.

In normal bone physiology, Akt stimulates osteoblast proliferation and protects against apoptosis [424]. We have found that APC stimulates Akt in MG-63 cells, consistent with its stimulation of viability. In OA and RA, however, Akt plays a more complex role and mediates growth factor, cytokine signalling, and cell proliferation in synovial cells, chondrocytes, and osteoblasts. Akt is required for chemokine stimulation of IL-6 and IL-1β production in OA synovial fibroblasts [641-643], and important for cytokine production in RA blood mononuclear cells and survival of RA synovial fibroblasts [644, 645]. In osteoblasts, though, chemokines stimulate pAkt and proliferation in RA but not OA osteoblasts [646], suggesting a greater involvement of pAkt pathway in RA than OA cells. We found no effect of APC on any signalling protein in RA HBDCs, although it is unclear whether the sample size affected the results (as discussed in Section 6.5).

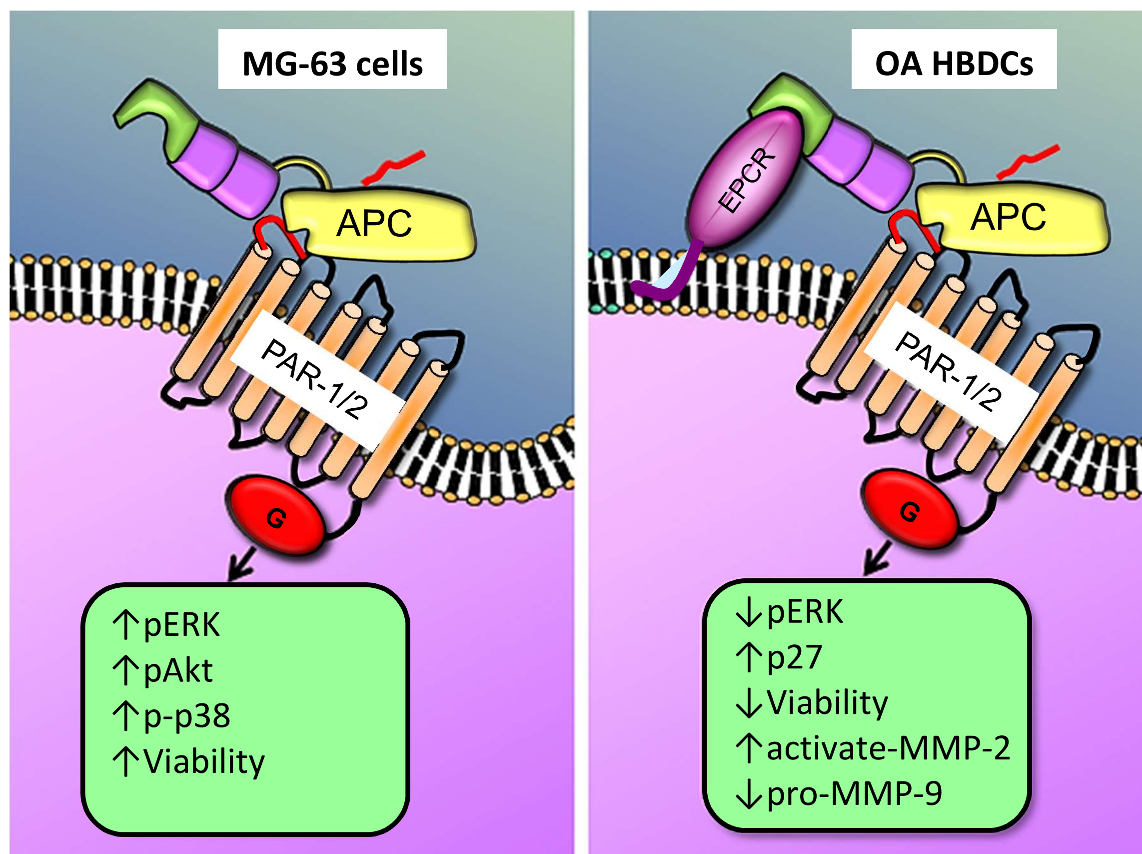

Figure 7-2 *Our summarised model contrasting APC's actions on MG-63 cells and OA HBDCs. APC stimulates pERK, pAkt, p-p38, and viability in MG-63 cells through PAR1 and PAR2. In contrast, APC inhibits pERK, viability, and pro-MMP-9 yet stimulates p27 and active-MMP-2 in OA HBDCs through EPCR, PAR1, and PAR2.*

7.5 Involvement in Arthritic Bone

The evidence from this thesis suggests a multi-faceted involvement of APC in arthritis. As APC down-regulated OA HBDC viability, APC treatment in OA may ameliorate high levels of bone turnover to decrease subchondral sclerosis and osteophyte formation. Investigation of APC in OA has been reported in cartilage *in vitro* [283, 284], however, no animal models studying APC in this disease have been published. Many models of OA are surgically-induced, which do not adequately reflect the development of spontaneous OA [647]. There are also spontaneous and genetic models, although these models do not represent the risk factors in

humans of trauma and obesity [648]. Models of mechanical loading better replicate the joint injury in humans as repetitive loading leads to osteophyte formation, synovial hyperplasia, fibrosis, and cruciate ligament pathology [649]. Mechanical loading models also demonstrate a transient elevation of TNF-α and IL-1β early in the disease with increased articular cartilage production of MMP-2 and MMP-9 [650, 651]. Compression of osteoblasts stimulates IL-6 production by these cells [652]. As Jackson *et al* reported that APC activates MMP-2 and MMP-9 in explanted OA cartilage, it will be important to determine whether this effect is maintained *in vivo* [283, 284]. We suggest that APC treatment of a cyclic loading model would be useful in the investigation of APC on OA bone as mechanical loading augments MMP-2, MMP-9, and IL-6 production and APC acts on these factors in OA HBDCs [651-653]. Subchondral sclerosis and osteophyte formation could be assessed histologically within joints and by bone scans.

In RA, there currently exists several animal models, each with their limitations. Collagen-induced arthritis (CIA) is a model which embodies tolerance and auto-antibodies of RA pathogenesis but it is heterogeneous, with varying levels of severity depending on the strain of mice [193]. Antigen-induced arthritis (AIA) is an inflammatory arthritis model which is initiated through injection of various antigens, commonly BSA, and this model facilitates research into T cell pathogenesis [654]. In both AIA and CIA, the primary pathogenic cytokine is IL-6, whereas in other models, including adjuvant-induced arthritis and streptococcal cell wall arthritis, levels of TNF-α, IL-6, and IL-1β play equally important roles [655]. As we have demonstrated that APC suppresses IL-6 levels in RA HBDCs, we suggest that any of these models, particularly AIA or CIA, would be suitable to assess the effects of APC on subchondral bone.

Although we have outlined appropriate choices of OA or RA animal models for testing the therapeutic benefit of APC, we acknowledge that these models may result in experimental bias due to the selection of pathways/pathology that would be particularly susceptible to APC. In order to reduce experimental bias, we also suggest that multiple models can be used to determine the effect of APC both OA and RA. These models should have both high and low predicted probability of involving APC-susceptible-pathways in order to provide a better representation of response in true disease.

7.6 Conclusion

The findings of this thesis extend upon existing research on APC in bone, with current literature demonstrating APC protection of viability in both normal human osteoblast and MG-63 as well as APC stimulation of MG-63 differentiation. We found that APC not only enhances the viability of osteoblastic cell lines but also promotes BMP-2-induced ectopic bone formation, highlighting a novel effect of APC on bone. This discovery opens up new realms of investigations pertaining to: mechanisms underlying APC and BMP-2 interaction; the potential effects of APC on osteoclast formation and activation; use of mutant forms of APC with reduced anticoagulant activity, and; application of APC in orthopaedic models. Although we hypothesised that APC would be beneficial in fracture healing, we were not able to demonstrate an effect on closed murine fracture healing. Nonetheless, we propose that APC may be beneficial in other orthopaedic models.

We demonstrated an anti-inflammatory role of APC on OA and RA osteoblastic cells through stimulation of active MMP-2 and in RA cells, suppression of TNF-α-induced IL-6. Interestingly, APC suppressed viability in OA cells, suggesting a different physiology for APC in these cells as compared to normal osteoblasts. This may be attributed to a number of

alterations in the signalling of OA including Akt, ERK1/2, and p38 pathways. The published divergent effects from APC on OA versus RA cartilage and synovial tissues is also clearly evident in our study using bone and osteoblastic cells.

Despite the variations in response to APC between osteoblastic cells of different origins, our study highlights the involvement of both PAR1 and PAR2 as primary receptors mediating the effects of APC.

The research conducted in this thesis provides the first evidence for a potential therapeutic effect of APC on normal and/or arthritic bone.

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