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Expression and Regulation of Bone Morphogenetic Protein Receptors in Human Alveolar Bone Cells

Thesis submitted to the University of London in fulfilment of the requirements for the degree of Doctor of Philosophy

By

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DECLARATION

I, Weerachai Singhatanadgit, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

(Weerachai Singhatanadgit)

ABSTRACT

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily of growth factors that stimulate osteoblast differentiation and function. They exert their biological activities through signal transduction via three specific serine/threonine kinase transmembrane receptors, designated types-IA, -IB and -II (BMPR-IA, -IB and -II). These BMP-specific receptors thus determine in part the sensitivity and responsiveness of target cells to the BMP and thereby the biological activity of the BMP. However, little is known about BMPR-IA, -IB and -II in bone. The present study was therefore carried out to examine the expression and regulation of the BMPR in primary human alveolar bone (AB) cells *in vitro*.

Cells were obtained from explants of human AB and exhibited a number of characteristic phenotypic features of osteoblasts. They were also found to express all three BMPR mRNA transcripts and proteins, each of which had a unique subcellular distribution. For example, in addition to its expected localisation at the plasma membrane, a major proportion of BMPR-IB was also observed in the cytoplasm and the nucleus. Moreover, the distribution of BMPR-IB was found to be highly regulated by TGF-β1, which caused a pronounced translocation of this receptor to the plasma membrane, resulting in a marked increase in BMP-2 binding and bone cell response.

The BMPR also appeared to be differentially controlled at the post-translational level by inflammatory cytokines, which were shown, for the first time, to cause shedding of the cell surface proteins and the concurrent generation of 'soluble' forms of the BMPR. IL-1β and TNF-α were found to significantly induce the shedding of soluble BMPR-IB specifically, thereby reducing BMPR-IB surface expression and diminishing BMP-2-induced AB cell functions, such as Smad1/5/8 phosphorylation, alkaline phosphatase (ALP) activity and osteocalcin (OC) expression. In contrast, the expression of BMPR-IB was found to be up-regulated by osteogenic growth factors including TGF-β1, FGF-2 and PDGF-AB, which enhanced BMP-2-induced AB cell functions. The biological importance of BMPR-IB in these cells was established using an RNA interference approach, which demonstrated that the expression of pivotal osteoblast-associated genes

ALP, OC, distal-less homeobox 5 (Dlx5) and core binding factor alpha1 (Cbfa1) was dependent on the BMPR-IB signalling pathway.

In conclusion, the activities of the BMPR-IA, -IB and -II genes in primary human AB cells were found to be controlled by a number of biological mediators. In addition, the expression of these receptors was also regulated at both the transcriptional and post-translational levels, with BMPR-IB being the most responsive receptor, at least *in vitro*. These findings suggest that BMPR-IB could thus be a possible therapeutic target for eliciting improved BMP-induced bone healing *in vivo*.

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LIST OF ABBREVIATIONS

AB	Alveolar bone
ActR	Activin receptor
ADAM	A disintegrin and a metalloprotease
AFI	Average fluorescence intensity
ALK	Activin receptor-like kinase
ALP	Alkaline phosphatase
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BGP	Bone Gla protein
Bis IX	Bisindolylmaleimide IX
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
Bp	Base pairs
BRK	Bone morphogenetic protein receptor kinase
BSA	Bovine serum albumin
BSP	Bone sialoprotein
Ca	Constitutively active
cAMP	Cyclic adenosine 3' 5'-monophosphate
Cbfa1	Core binding factor alpha-1
Cdc2	Cell division cycle 2
CDMP	Cartilage-derived morphogenetic protein
cDNA	Complementary DNA
C-ELISA	Cell-enzyme-linked immunosorbent assay
CFU-F	Colony forming unit fibroblasts
CFU-OB	Colony forming unit osteoblasts
Col I	Type-I collagen
COX	Cyclooxygenase
Ct	Threshold cycle
DAB	3,3'-Diaminobenzidine tetrachloride
Dex	Dexamethasone
DHT	5α-dihydrotestosterone
Dlx	Distal-less homeobox

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
Dn	Dominant-negative
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Dpp	Decapentaplegic
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EEA-1	Early endosomal antigen 1
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAM	Carboxyfluorescein
FCM	Flow cytometry
FCS	Fetal calf serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF	Growth-differentiation factor
GF	Gingival fibroblast
gp130	Glycoprotein 130
HA	Hydroxyapatite
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
ICC	Immunocytochemistry
Id	Inhibitor of differentiation protein
IGF	Insulin-like growth factor

IGFBP	Insulin-like growth factor binding protein
IGFBP-rP	Insulin-like growth factor binding protein-related protein
IGFR	Insuline-like growth factor receptor
IgG	Immunoglobulin G
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-6R	Interleukin-6 receptor
IRAK	IL-1 receptor-associated kinase
JAK	Janus kinase
JNK	c-Jun-NH2-terminal kinase
kDa	Kilodalton
KGF	Keratinocyte growth factor
LAMP-1	Lysosomal membrane glycoprotein 1
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCSF	Macrophage-colony stimulating factor
MMP	Matrix metalloprotease
M-MuLV	Moloney murine leukemia virus
MP	Metalloprotease
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
Msx	Muscle segment homeobox
MT-MMP	Membrane-type matrix metalloprotease
MW	Molecular weight
NF-κB	Nuclear factor kappa B
NGS	Normal goat serum
OC	Osteocalcin
OM	Osteogenic medium
ON	Osteonection
OP	Osteopontin
OPG	Osteoprotegerin
p.c.	Post coitum
PBS	Phosphate-buffered saline

PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDL	Periodontal ligament
PG	Prostaglandin
PGE	Prostaglandin-E
PI3K	Phosphatidylinositol 3-kinase
PKA	Proteinkiase-A
PKC	Protein kinase-C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenyl-methyl-sulfonyl fluoride
p-Smad	Phosphorylated Smad
PTH	Parathyroid hormone
PVDF	Polyvinylidene difluoride
Q-PCR	Quantitative real-time PCR
RA	Retinoic acid
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RAR	Retinoic acid receptor
rhBMP	Recombinant human bone morphogenetic protein
RISC	RNA-induced silencing complex
Rn	Normalised reported fluorescence
RNAi	RNA interference
rpm	Round per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
Runx2	Runt-related transcription factor-2
RXR	Retinoid X receptor
sBMPR	Soluble bone morphogenetic protein receptor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
sIL-6R	Soluble IL-6 receptor
siRNA	Small interfering RNA

Smurf	Smad ubiquitin regulatory factor
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-β
TGFβR	Transforming growth factor-β receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TM-MP	Transmembrane metalloprotease
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor-α
TRAF	TNF receptor-associated factor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
WB	Western blotting

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PUBLICATIONS

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PRESENTATIONS

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CHAPTER 1
INTRODUCTION

1.1. Fundamental biology of bone

1.1.1. Development of skeletal bone

The mammalian skeleton has three distinct origins: the first is the paraxial mesoderm, which gives rise to the axial skeleton; the second is the lateral plate mesoderm, which gives rise to the appendicular skeleton; and the third is the ectoderm, *i.e.,* the neural crest, which gives rise to the facial skeleton (Chung *et al.*, 2004). These bones thus have different ontogenic origins and are likely to have different genetic, biochemical and functional activities (Matsubara *et al.*, 2005).

Two mechanisms of bone formation have been observed during morphogenesis (Sommerfeldt and Rubin, 2001). One involves the differentiation of mesenchymal cells directly into osteoblasts, which then proceed to form mature bone. This intramembranous bone formation is found during skull development, and also in maxilla and mandibular morphogenesis. A second mechanism involves the differentiation of mesenchymal cells which proceeds via chondrocytes and forms the cartilaginous template of future bones. The hypertrophic cartilage is then replaced by osteoblasts, ultimately leading to ossification (endochondral ossification).

1.1.1.1. Structure and function of alveolar bone (AB)

A schematic figure of the structural feature of AB is shown in Figure 1.1. The maxilla and mandible of adult humans is subdivided into two portions: the AB that houses the roots of erupted teeth and the basal bone that is not involved in housing the roots. The AB consists of the thin part of the bone that forms the alveolar wall of the tooth socket, the inner and outer cortical plates and the spongy bone (cancellous bone). Within the cancellous bone are numerous marrow spaces, with smaller marrow spaces present in the cortical bone (Schroeder, 1986; Sodek and McKee, 2000).

During embryonic development, the intramembranous bone of the maxilla and mandible initially forms from osteoblasts arising from condensing mesenchyme in the facial region. As osteogenesis progresses, bone surfaces become lined by a contiguous layer of osteoblasts that continue to produce bone, which enlarges the dimensions of the trabeculae. Concomitant with the growth of this bone, new mesenchymal condensations arise in neighboring areas and produce new osteoblasts and new trabeculae, all of which will collectively form the fetal maxilla and mandible (Sodek and McKee, 2000). Although AB has specialized features relating to its functional properties, the cellular compartment and the composition of the extracellular matrix (ECM) of AB appear to be similar to other bone tissues, *e.g.,* skull and long bones (reviewed in detail in Section 1.1.2). The general functions of AB are to house the roots of teeth and to absorb and distribute occlusal pressures generated from tooth contact during mastication. Their most important and unique function is to anchor the roots of teeth to the maxilla and mandible, which form the primary support structure for teeth (Cho and Garant, 2000; Sodek and McKee, 2000).

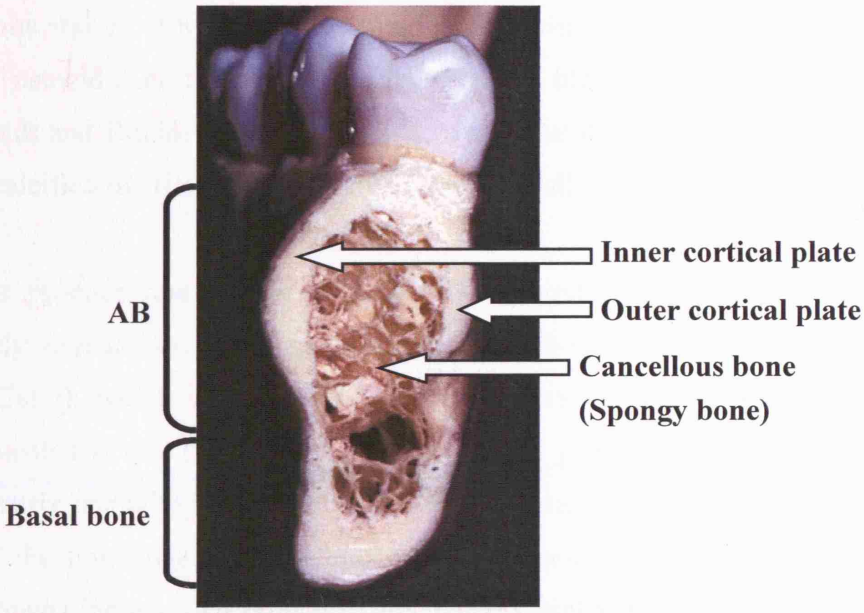


Figure 1.1. Unstained specimen of the mandible showing a cross section at the middle region of the mandible. The AB houses the roots of erupted teeth while the outer and inner cortical plates of compact bone are found on the right and left sides of the region of the cancellous bone, respectively. The basal bone is not involved in housing the roots. (Adapted from http://www.usc.edu/hsc/dental/ohisto/index.html)

1.1.2. Components of skeletal bone

The cells and the ECM are the two main components of bone. The three distinct cell types in bone are the matrix-producing osteoblast, the osteocyte and the bone-resorbing osteoclast. Osteoblasts are considered to derive from pluripotent mesenchymal stem cells whereas osteocytes can be viewed as highly specialized and fully differentiated mature osteoblasts (Aubin, 1998b). Osteoclasts are of hematopoietic lineage and their precursors are present in the monocytic fraction of bone marrow (Fujikawa *et al.*, 1996).

1.1.2.1. Osteoblasts

Morphologically, osteoblasts are cuboidal in shape and located at the bone surface, where they form a tight layer of cells (Sommerfeldt and Rubin, 2001). They are highly anchorage dependent and rely on extensive cell-matrix and cell-cell contacts via a variety of transmembrane proteins, such as integrins, connexins, cadherins and specific receptors, *e.g.,* for cytokines, hormones and growth factors, in order to maintain cellular function and responsiveness to metabolic and mechanical stimuli (Lecanda *et al.*, 1998; Ferrari *et al.*, 2000). The lifespan of an osteoblast has been reported to range between 3 days in young rabbits and up to 8 weeks in humans, during which time they produce 0.5-1.5 μm per day of osteoid (*i.e.,* the newly synthesised bone matrix prior to its mineralisation) (Sommerfeldt and Rubin, 2001). Eventually, some osteoblasts may become 'trapped' in their own calcified matrix, developing into phenotypically distinct osteocytes.

Osteoblasts produce and secrete proteins that comprise the bone matrix, which is subsequently mineralised. A major product of the bone-forming osteoblast is type I collagen (Col I), which forms pyridinoline cross-links that are unique to bone. Bone-forming osteoblasts also synthesise a number of other proteins that are incorporated into the bone matrix, including osteocalcin (OC) and osteonectin (ON), which constitute 40% to 50% of the noncollagenous proteins of bone (Manolagas, 2000). Other osteoblast-derived proteins include two proteoglycans, namely biglycan and decorin. A number of other glycoproteins are also produced by osteoblasts, such as osteopontin (OP), bone sialoprotein (BSP), fibronectin (FN) and vitronectin (VN), which are involved in cell adhesion and mineralisation of the bone matrix (Manolagas, 2000).

Osteoblasts are also essential for mineralisation, the process of deposition of hydroxyapatite (HA) within the bone matrix (Boskey, 1996; Boskey, 1998). Such cells are thought to regulate the local concentrations of calcium and phosphate in such a way as to promote the formation of HA. Osteoblasts express relatively high amounts of the enzyme alkaline phosphatase (ALP), which is localised mainly at the external surface of the plasma membrane and is thought to play a major role in bone mineralisation (Chentoufi *et al.*, 1993; Balcerzak *et al.*, 2003). Genetic deficiency of ALP leads to hypophosphatasia, a condition characterised by defective bone mineralisation (Whyte, 1994). Although the precise relationship between ALP and mineralisation remains unclear, ALP is considered to promote the formation of HA by hydrolysing pyrophosphates (inhibitors of HA formation), thus eliminating the inhibitory effect of pyrophosphate on the mineralisation process (Balcerzak *et al.*, 2003).

Osteoblasts are derived from multipotent mesenchymal stem cells (MSC) originating in the bone marrow, which also give rise to bone marrow stromal cells, chondrocytes, muscle cells and adipocytes, as shown in Figure 1.2 (Caplan and Bruder, 2001). Osteoblast progenitors can originate not only from stromal mesenchymal progenitors in the marrow, but also from pericytes (mesenchymal cells adherent to the endothelial layer of vessels) (Schor *et al.*, 1995).

Osteoblast differention *in vitro* is considered to be the process by which primitive stem cells progress to osteoprogenitor cells and then to fully functional osteoblasts. This process involves three stages: the proliferation stage; ECM production and maturation stage; and the mineralisation stage, with characteristic changes in gene expression during each stage (Stein GS *et al.*, 1996). The genes c-fos and c-myc are associated with the proliferation stage, while cyclin-B and -E are up-regulated post-proliferatively (Stein GS *et al.*, 1996). Differential expression of osteoblast-related genes occurs as the osteoprogenitor cells differentiate and the bone matrix matures and mineralises. For example, the ALP gene is first up-regulated but then down-regulated as mineralisation progresses, while OP reaches a peak prior to the expression of other matrix proteins such as BSP and OC (Aubin, 1998b). BSP is transiently expressed at very early stages and then increases again in differentiated osteoblasts, while OC is detected mainly during mineralisation (Aubin, 1998b). Thus, the expression of these genes is closely associated

with osteoblast differentiation and has been widely used as osteoblast-related markers, for example, ALP and OC being early and late markers of osteoblast differentiation, respectively (Aubin, 2001).

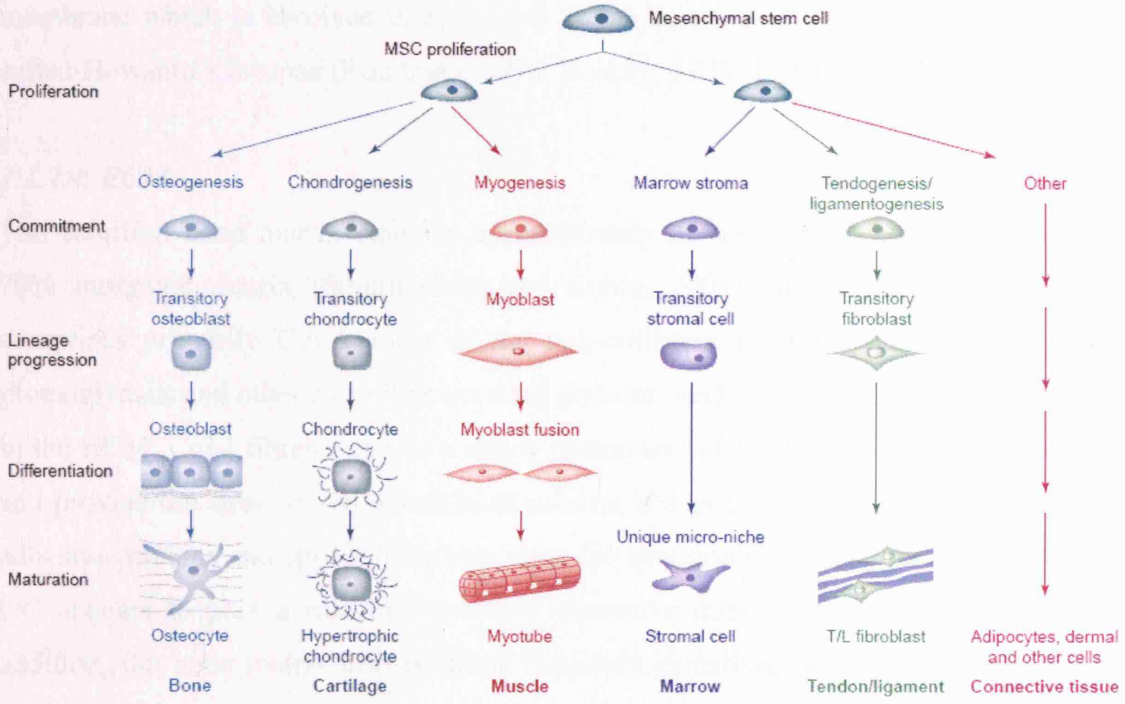


Figure 1.2. A schematic diagram depicting the stepwise cellular transitions from the putative MSC to highly differentiated phenotypes.
(Adapted from Caplan and Bruder, 2001)

1.1.2.3. Osteocytes

Derived from osteoblasts yet different in morphology and function, osteocytes are the most abundant cells in bone (Manolagas, 2000). They are smaller in size than osteoblasts, contain fewer ribosomes and endoplasmic reticulum (ER), have an increased nucleus to cytoplasm ratio and produce less matrix proteins. They have a high number of cytoplasmatic extensions, which connect with each other and with the bone-lining cells, creating a three-dimensional syncitium (Curtis *et al.*, 1985; Manolagas, 2000).

1.1.2.3. Osteoclasts

Osteoclasts are derived from hematopoietic stem cells and are highly multinucleated and polarized cells with several distinct ultrastructural features, including mitochondria, vacuoles and lysosomes (Teitelbaum, 2000). A most notable morphological feature of osteoclasts is a ruffled border, a complex system of finger-shaped projections of the membrane which is involved in resorption of the calcified bone matrix at specific sites called Howship's lacunae (Roodman, 1996; Boskey, 1998; Teitelbaum, 2000).

1.1.2.4. ECM

The calcified bone matrix contains approximately 25% organic matrix, 5% water and 70% inorganic matrix (Sommerfeldt and Rubin, 2001). While bone organic matrix comprises primarily Col I, most of the non-collagenous proteins in bone consist of proteoglycans and other osteoblast-secreted proteins, such as OP, BSP and OC, embedded in the ECM. Col I fibres serve as a major component of the fibrous framework of ECM and provide the structure on which bone mineral is deposited. Both OP and BSP are cell adhesive proteins and appear to be important for adhesion of osteoblasts and ECM, while OC appears to play a role in preventing excessive mineralisation (Mackie, 2003). In addition, the bone matrix also contains important signalling molecules, such as growth factors, cytokines and adhesion molecules (Sommerfeldt and Rubin, 2001;Mackie, 2003).

1.1.3. Mineralisation of bone matrix

The initiators of the mineralisation process are extracellular, small, round, lipid bilaminar vesicles secreted from osteoblasts at sites of initial mineralisation (Manolagas, 2000; Anderson, 2003). The first crystal of bone mineral is formed within these matrix vesicles, facilitated by the activity of ALP and calcium-binding molecules such as annexin I, which are concentrated at the matrix vesicle membrane. Subsequently, the preformed crystals are released through the matrix vesicle membrane and exposed to the extracellular environment containing sufficient calcium and phosphate ions to support continuing crystal growth, with the preformed crystals serving as 'seed' nuclei (Anderson, 1989; Anderson, 2003). During this process, collagen fibrils, FN, and glycoproteins such as ON determine the orientation and organisation of these bone mineral crystals (Manolagas, 2000). However, other glycoproteins, such as OC and phosphoproteins, appear to have a

regulatory role in preventing excessive mineralisation (Anderson, 1989; Ducy *et al.*, 1996).

1.1.4. Bone regeneration

The skeleton is a highly specialized and dynamic organ that undergoes continuous regeneration. During development and growth, the skeleton achieves its shape and size by removal of bone from one site and deposition of new bone at a different site, a process named "modelling". Once the skeleton has reached maturity, regeneration continues in the form of a periodic replacement of old bone with new bone at the same location. This process is called "remodelling" and is responsible for the regeneration of the adult skeleton (Manolagas, 2000). The purpose of remodelling in the adult skeleton is not entirely clear, although in bones that are load bearing, remodelling most likely serves to repair fatigue damage.

However, bone resorption and bone formation are not separate, independently regulated processes. Removal of old bone (resorption) is carried out by osteoclasts, whereas formation of new bone is a fundamental function of osteoblasts. In the uninjured adult skeleton in both cortical and cancellous bone, the osteoclasts and osteoblasts maintain a highly regulated spatial and temporal relationship with one another. Osteoclasts adhere to and resorb bone by acidification and proteolytic digestion, then leave the resorption site. Osteoblasts subsequently migrate and cover the excavated area and begin the process of new bone formation by secreting osteoid, which is eventually mineralised into new bone (Manolagas, 2000).

In fractured bone, the healing process restores the bone tissue to its original physical and mechanical features. It is well-established that bone healing occurs in three phases: the reactive (early inflammatory) phase; the reparative phase; and the remodelling phase (Kalfas, 2001). In the reactive phase, a hematoma develops within the fracture area during the first few hours, with infiltrating inflammatory cells and fibroblasts. This results in the formation of a granulation tissue (a fibrous connective tissue containing mainly fibroblasts and newly formed small blood vessels), vascular ingrowth and mesenchymal cell migration. During the reparative phase, fibroblasts produce an ECM supporting vascular ingrowth, with mesenchymal cells undergoing proliferation and differentiation

under control of local and systemic mediators. Progenitors differentiate into chondroblasts and osteoblasts, subsequently forming cartilage and "woven bone", which eventually ossifies and, during the remodelling phase, forms "compact bone" that closely duplicates the bone's original shape and strength (Ham and Harris, 1972; Kalfas, 2001). Thus, the differentiation of osteoprogenitor cells appears to be a crucial part in the healing of injured bone, a process which is now known to be tighly regulated by many soluble mediators.

1.2. Biological mediators and receptors involved in osteoblast differentiation and bone remodelling

Regulation of bone remodelling is under both local and systemic control. Local factors are operative in a paracrine and autocrine fashion, and osteoblasts, osteoclasts and inflammatory/immune cells function as both sources and targets of regulatory molecules. Numerous cytokines and growth factors have anabolic and/or catabolic effects on the development and differentiation of osteoblasts and thus bone formation (Aubin, 2001; Harada and Rodan, 2003). Among these bone-regulatory molecules are, for example, interleukins (IL)-1 and -6, tumor necrosis factor-α (TNF-α), prostaglandin E-2 (PGE-2), transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF), insulin-like growth factors (IGF), platelet-derived growth factors (PDGF), steroid hormones (*e.g.*, androgens and glucocorticoids) and retinoic acid (RA; the active metabolite of vitamin A). The abnormal production and/or activity of these molecules can lead to metabolic bone disorders or diseases such as osteoporosis, osteoarthritis or bone loss in periodontitis (Suda *et al.*, 1997; Mogi *et al.*, 2004).

1.2.1. IL-1

IL-1 is a multipotent cytokine, produced by hematopoietic and mesenchymal/osteoblastic cells (Haynes *et al.*, 1999), and comprises two individual peptides (IL-1α and IL-1β) which exert similar biological activities (Dinarello, 1989). Cellular responses elicited by IL-1 are known to be mediated through a type-I receptor (IL-1R-I) (Sims *et al.*, 1993), whereas the type-II receptor (IL-1R-II) acts as a non-signalling 'decoy' receptor for the cytokine (Colotta *et al.*, 1993). The intracellular signalling events downstream of the IL-1R are mediated by IL-1 receptor-associated kinase (IRAK), which phosphorylates

mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K) and ultimately activates a major cellular transcription factor, nuclear factor kappa B (NF-κB) (Dinarello, 1996; Auron, 1998).

In bone, although some studies have shown that both IL-1α and IL-1β are able to stimulate osteoblast differentiation and bone formation *in vitro* (Hanazawa *et al.*, 1986; Ohmori *et al.*, 1988), IL-1 has generally been widely recognised as a bone-resorbing agent (Canalis, 1986; Lorenzo *et al.*, 1987). Thus, depending on the differentiation stage of the cell, length of the culture period and concentration of the cytokine, IL-1α and IL-1β can inhibit Col I and OC expression, ALP activity and bone nodule formation *in vitro* (Stashenko *et al.*, 1987; Ellies and Aubin, 1990; Kim *et al.*, 2002; Tanabe *et al.*, 2004). In addition to the direct effect on osteoblasts, IL-1 has also been shown to induce the production of other inflammatory mediators which promote bone resorption, such as IL-6 (Chaudhary *et al.*, 1992), TNF-α (Wei *et al.*, 2005) and PGE-2 (Hughes *et al.*, 1999). Moreover, IL-1β has been shown to down-regulate the expression of BMP-2 mRNA in a human osteosarcoma cell line (Virdi *et al.*, 1998). These data suggest that IL-1β acts as a catabolic mediator in bone at least partly by suppression of osteoblast differentiation and bone formation.

1.2.2. IL-6

IL-6 is produced by osteoblasts and stromal cells (Holt *et al.*, 1996) and is a pivotal cytokine in osteoclastogenesis and bone resorption (Kotake *et al.*, 1996). IL-6 exerts its effect through a receptor complex consisting of the ligand binding subunit IL-6 receptor (IL-6R) and a signal-transducing molecule glycoprotein 130 (gp130). The ligand-receptor interaction results in the phosphorylation of the cytoplasmic tail of gp130, activating the 'signal transducer and activator of transcription' (STAT) family and also the MAPK pathway (Heinrich *et al.*, 1998). The effects of IL-6 are considered to be determined by either the expression of a functional cell surface IL-6R or the generation of a functionally active 'soluble' IL-6R (sIL-6R), which acts as an agonist for IL-6 (Vermes *et al.*, 2002). However, it has recently been reported that the cellular expression of IL-6R in osteoblast appears at a low level and the presence of sIL-6R is therefore crucial for the activation of the downstream effects of this cytokine (Franchimont *et al.*, 2005b).

IL-6 suppresses ALP activity, Col synthesis, OC mRNA expression and bone nodule formation by ostoblastic cells (Ishimi *et al.*, 1990; Fang and Hahn, 1991; Li and Stashenko, 1992; Hughes and Howells, 1993), and also down-regulates the expression of BMP-2 mRNA (Virdi *et al.*, 1998), suggesting that IL-6 not only affects bone remodelling through the formation of osteoclasts (Kotake *et al.*, 1996), but may also inhibit osteoblast differentiation and bone formation.

1.2.3. TNF-α

TNF-α is an inflammatory cytokine produced by macrophages and T-lymphocytes, and transduces intracellular signals via type-I and type-II TNF receptors (TNFR-I or p55 and TNFR-II or p75, respectively) (Nanes, 2003). Upon binding of a trimerized TNF protein to one of these two cell surface receptors, the initiation of signal transduction involves the activation of TNF receptor-associated factors (TRAF), TRAF-1 and TRAF-2, in osteoblasts, leading to the stimulation of MAPK and the activation and translocation of NF-κB into the nucleus, where it activates the transcription of many genes (Boyce *et al.*, 1999).

TNF-α is also produced and secreted by osteoblasts and can act in an autocrine manner to impair bone formation by inhibiting the differentiation of new osteoblasts from their progenitors and by suppressing mature osteoblast functions such as the production of OC (Nanes *et al.*, 1989; Nanes *et al.*, 1991; Shimizu *et al.*, 1998; Nanes, 2003). TNF-α has also been reported to ablate BMP-induced responses *in vitro*, for example by inhibiting BMP-2-stimulated ALP activity (Nakase *et al.*, 1997). Although it appears to have little, if any, direct effect on BMP-2, -4, or -6 (Gilbert *et al.*, 2000), TNF-α can inhibit osteoblast differentiation by suppression of IGF-1 and osteoblast-related transcription factors, such as core binding factor alpha1/runt-related transcription factor 2 (Cbfa1/Runx2) and osterix expression (Scharla *et al.*, 1994; Gilbert *et al.*, 2000; Gilbert *et al.*, 2002; Nanes, 2003). Moreover, an *in vivo* study has reported that the expression of BMP-2, -4, and -7 increased significantly during endochondral ossification in type-I TNF receptor-deficient (TNFR-I$^{-/-}$) mice, demonstrating the inhibitory effects of TNF-α on bone formation, presumably through a BMP-mediated effect (Lukic *et al.*, 2005). Furthermore, while exogenously administered BMP-4 has been shown to promote chondrogenesis in undifferentiated mouse chondrogenic cells (Ito *et al.*, 1999; Horiguchi

et al., 2000), it is notable that TNF-α nevertheless still blocked chondrogenesis despite the ability of this cytokine to up-regulate BMP-4 in these cells (Horiguchi *et al.*, 2000). This suggests that TNF-α might negatively regulate a pathway downstream to the BMP ligand, such as the BMPR, and thereby modulate BMP function.

1.2.4. Prostaglandins (PGs)

The PG family of bioactive mediators are closely involved in the regulation of bone metabolism, with PGE-2 generally regarded to be of major importance in bone remodelling (Takahashi *et al.*, 1988; Kawaguchi *et al.*, 1995). PGE-2 is generated from arachidonic acid in stromal cells as a result of cyclooxygenase 2 (COX-2) stimulation by bone-resorbing cytokines such as IL-1, IL-6 and TNF-α (Onoe *et al.*, 1996; Chen *et al.*, 1997; Tai *et al.*, 1997) as well as by PGE-2 itself (Fujita *et al.*, 2003). PGE-2 exerts a broad range of pathophysiological activities through its binding to PGE cell surface receptors, which are classified into four subtypes (EP_1, EP_2, EP_3, and EP_4). These interact with different GTP-binding proteins, ultimately transducing signalling via protein kinase C (PKC) and protein kinase A (PKA) (Coleman *et al.*, 1994; Li *et al.*, 2004b). The diversity of PGE-2 actions is ascribed to these PGE receptor subtypes coupled to these different signal transduction pathways.

Although some positive effects of PGE-2 on bone formation has been reported *in vitro* and *in vivo* (Jee and Ma, 1997; Kaneki *et al.*, 1999; Ramirez-Yanez *et al.*, 2004), PGE-2 is primarily involved in bone resorption by increasing osteoclast formation via suppression of osteoprotegerin (OPG) expression by osteoblasts (Suda *et al.*, 2004) and by inhibiting bone matrix expression and mineralisation (Kajii *et al.*, 1999). Exogenous PGE-2 has been shown to stimulate bone resorption *in vivo* (Kawaguchi *et al.*, 1995), and high doses of PGE-2 inhibit BMP-2-stimulated osteoblast differentiation *in vitro* (Takiguchi *et al.*, 1999), possibly by reduction of BMP/BMPR signalling.

1.2.5. TGF-β

TGF-β belongs to a family of structurally related growth factor (the TGF-β superfamily), which comprises TGF-β1 to TGF-β5, the BMP, activins, inhibins and Mullerian substance (Massague *et al.*, 1994). TGF-β is particularly enriched in bone, platelets and cartilage (Lieberman *et al.*, 2002) and elicits its biological activity by signal transduction

through a family of transmembrane protein serine/threonine kinases called the TGF-β receptor (TGFβR) family, which is structurally and functionally divided into type-I and type-II TGFβR (TGFβR-I and TGFβR-II, respectively) (Massague, 1998).

In vitro, TGF-β1 exhibits a biphasic effect on bone cell proliferation which is generally dependent on TGF-β1 concentration, cell density and differentiation stage of the cells. Thus, the stimulatory effect of TGF-β1 on DNA synthesis has been reported to decrease at high TGF-β1 concentrations, in sparse cultures and in cultures of more differentiated cells (Centrella and Canalis, 1985; Centrella *et al.*, 1987; Centrella *et al.*, 1998). However, in undifferentiated mesenchymal cells, TGF-β1 is considered to be a pleiotropic growth factor that stimulates proliferation (Janssens *et al.*, 2005). It has also been shown to promote bone formation via a chemotaxtic effect on osteoblasts, enhancement of osteoblast proliferation and stimulation of the early stage of osteoblast differentiation accompanied by increased production of ECM proteins *in vitro* (Lieberman *et al.*, 2002; Lieb *et al.*, 2004; Janssens *et al.*, 2005). Moreover, administration of TGF-β1 increases bone formation *in vivo* (Shigeno *et al.*, 2002; Szivek *et al.*, 2004; Srouji *et al.*, 2005).

1.2.6. BMPs

Among the TGF-β superfamily, the BMPs are the most potent growth factors in osteogenesis and the repair of osseous defects and fracture (Ducy and Karsenty, 2000; Groeneveld and Burger, 2000; Schilephake, 2002). They promote differentiation of mesenchymal stem cells into chondrocytes and osteoblasts, as well as osteoprogenitors into osteoblasts, and are considered to have clinical value for the treatment of orthopaedic and craniofacial conditions, including spinal fusion and bone fracture (Lieberman *et al.*, 2002; Boden *et al.*, 2002; Govender *et al.*, 2002). More detailed information about the BMPs is reviewed in Section 1.5.

1.2.7. FGFs

The FGFs, a family of 25 structurally related polypeptides which are secreted by macrophages, mesenchymal cells, osteoblasts and chondrocytes (Lieberman *et al.*, 2002; Katoh and Katoh, 2005), are known to play a critical role in limb initiation and patterning (Xu *et al.*, 1999) and in angiogenesis and mesenchymal cell mitogenesis (Friesel and

Maciag, 1995). The most abundant FGFs in normal adult tissue are the acidic FGF (FGF-1) and basic FGF (FGF-2), both of which promote growth and differentiation of various cell types including osteoblasts and chondrocytes. They elicit their functions via transduction of signals through four receptors (FGFR-I to FGFR-IV) that contain distinct membrane-spanning tyrosine kinases and play an important role in limb development and osteogenesis (Eswarakumar *et al.*, 2005).

FGF-2 is the most abundant in normal adult bone and is generally a more potent mitogen than FGF-1 (Canalis *et al.*, 1988). It enhances osteogenic differentiation of rat mesenchymal stem cells *in vitro* (Hanada *et al.*, 1997) and stimulates bone formation *in vivo* (Kato *et al.*, 1998; Power *et al.*, 2004), while low doses of FGF-2 have been reported to enhance BMP-2-induced ectopic bone formation (Takita *et al.*, 1997; Fujimura *et al.*, 2002), thus having a significant impact on the osteogenetic activity of BMPs in bone.

1.2.8. PDGFs

PDGF is a dimeric molecule consisting of disulfide-bonded A- and B-polypeptide chains and is a stimulator of growth and differentiation of connective tissue cells. Homodimeric (PDGF-AA, PDGF-BB) as well as heterodimeric (PDGF-AB) isoforms of this growth factor exert their effects on target cells by binding to two structurally related protein tyrosine kinase receptors (PDGFRα and PDGFRβ) (Heldin *et al.*, 1998).

PDGF-AA is secreted by cells of the osteoblastic lineage while PDGF-AB and PDGF-BB are systemically circulating isoforms released by platelets at injured sites of blood vessels (Canalis *et al.*, 1992). PDGF-AA acts as an autocrine factor for osteoblasts whereas PDGF-AB and PDGF-BB are paracrine factors which are not expressed by human osteoblasts although such cells can respond to exogenous PDGF-AB and PDGF-BB (Zhang *et al.*, 1991). In bone, PDGF is a potent mitogen for cells of the osteoblastic lineage, with PDGF-AA less potent than PDGF-AB and PDGF-BB (Canalis *et al.*, 1989; Canalis *et al.*, 1992; Centrella *et al.*, 1992). PDGF-AB has been shown to stimulate the proliferation of osteoblastic cells (Horner *et al.*, 1996) and periodontal ligament cells (Kawase *et al.*, 2005), and to significantly enhance calcium release in mouse calvaria cultures (Cochran *et al.*, 1993). The widespread expression of the PDGF A chain and its

receptor in rapidly forming human bone further indicates that this growth factor is likely to play an important part in osteogenesis in humans (Horner *et al.*, 1996).

1.2.9. IGFs

The IGF family of growth factors consists of two single chain peptide isoforms (IGF-1 and IGF-2) with approximately 40–50% homology between themselves and with insulin (Schilephake, 2002). Their biological activity is mediated through the type-I IGF receptor (IGFR-I), the IGFR-II and the insulin receptor (Butler and LeRoith, 2001). IGF binding proteins (IGFBP) transport and target the IGF to their respective receptors (Schilephake, 2002), and previous studies of osteoblast cell lines *in vitro* and osteogenesis *in vivo* have suggested that osteoblastic developmental sequences can be regulated by IGF-1 via these IGFBP (Thrailkill *et al.*, 1995; Wang *et al.*, 1995).

IGF-1 has been considered to play a pivotal part in skeletal development and in the repair and remodelling of the adult skeleton (Lieberman *et al.*, 2002). IGF-1 enhances bone formation via the up-regulation of osteoblast-related genes such as Cbfa1, ALP, BSP and OP (Strayhorn *et al.*, 1999; Okazaki *et al.*, 2003; Koch *et al.*, 2005). In addition, IGF-1 has been reported to up-regulate the osteoblast-related transcription factor, osterix, and act synergistically with BMP-2 on osterix expression (Celil and Campbell, 2005), suggesting the positive effect of IGF-1 in promoting the differentiation and function of osteoblasts. IGF-1 has been shown to be localised in healing fractures *in vivo* (Okazaki *et al.*, 2003), and the systemic application of IGF-1 increases markers of bone remodelling *in vivo*, such as serum OC and carboxyterminal propeptide of Col I (Bianda *et al.*, 1997), again indicating a stimulatory effect of IGF-1 in osteogenesis.

1.2.10. Androgens

Androgens are steriod hormones which are produced by both the testes and the adrenal glands in males and circulate bound mainly to albumin and the sex-hormone binding globulins. When it reaches peripheral tissues, it is converted by the enzyme 5α-reductase to the more potent 5α-dihydrotestosterone (DHT) (Hofbauer and Khosla, 1999). The biological activity of androgens is known to be mediated through the androgen receptor (AR), two isoforms of which have been identified (isoforms A and B) (Gao and McPhaul, 1998). Both AR are expressed in osteoblasts, osteoclasts and osteocytes, where they are

found primarily within the nucleus (Abu *et al.*, 1997). In contrast to the mechanism of action of growth factors, the androgens transverse the plasma membrane by diffusion and bind to AR localised in the peri-nuclear region. This ligand-receptor interaction subsequently induces a wide range of events, including a conformational change in the AR, dissociation from a variety of AR-associated proteins (*e.g.*, heat-shock proteins), phosphorylation of the AR, and dimerisation either with other steroid receptor monomers (heterodimerisation) or with AR monomers (homodimerisation) (Chang *et al.*, 1995). Most importantly, the activated ligand-receptor complex acts directly as a nuclear transcription factor, binding to androgen response elements in the promoter region of many different target genes and modulating gene transcription (Chang *et al.*, 1995).

Androgens have been reported to stimulate osteoblast proliferation (Kasperk *et al.*, 1997) and to enhance osteoblast differentiation and synthesis of osteoblast-related proteins such as Col I, ALP, ON and OC as well as mineralisation of the bone matrix (Kasperk *et al.*, 1997; Notelovitz, 2002). Androgens have also been shown to control the expression and activity of a number of bone-related cytokines and growth factors, including BMPs (Centrella *et al.*, 1994; Bellido *et al.*, 1995; Thomas *et al.*, 1998; Hofbauer and Khosla, 1999), suggesting that they are likely to play an important part in osteoblast differentiation and bone formation.

1.2.11. Glucocorticoids

Like androgens, glucocorticoids are also steroid hormones and bind to a cytosolic receptor, the newly formed receptor-ligand complex translocating to the nucleus and binding to glucocorticoid response elements in the promoter region of target genes (Canalis, 1996; Pemberton and Paschal, 2005). A number of synthetic glucocorticoids used clinically have profound effects on bone remodelling. These include hydrocortisone, the pharmaceutically prepared cortisol, and dexamethasone (Dex), whose potency is approximately 17-30 times that of hydrocortisone (Ishida and Heersche, 1998). These glucocorticoids have complex effects on bone formation and resorption, some resulting from their direct action on specific genes expressed by osteoblasts whereas others, as with androgens, may be indirect and mediated by locally produced growth factors and their binding proteins (Canalis, 1996). Physiological levels of glucocorticoids have been reported to have profound stimulatory effects on the differentiation and function of bone

cells (Canalis, 1996; Ishida and Heersche, 1998); for example, hydrocortisone at 0.2 μM stimulates osteoblast differentiation (Ireland *et al.*, 2004). Moreover, Bellows *et al.* (1987) reported that physiological doses of Dex and hydrocortisone increase the number and size of mineralised bone nodules. Notably, the osteogenic effects of BMP-2 on osteoblast functions have been shown to be potentiated by co-treatment or pre-treatment with glucocorticoid in rat calvarial osteoblasts and bone marrow stromal cells (Rickard *et al.*, 1994; Boden *et al.*, 1996), indicating that the glucocorticoid could increase the cellular responsiveness to the BMP possibly through increased expression of BMPR.

1.2.12. Retinoic acid (RA)

RA is the most active metabolite of vitamin A, an imbalance of which during embryogenesis profoundly influences the development of multiple organs including the skeleton (Weston *et al.*, 2003). RA has numerous isoforms generated by alternative splicing and acts via RA receptors (RAR) and retinoid X receptors (RXR). The RA-receptor complexes act directly as gene transcription factors via interactions with RA responsive elements and retinoid X responsive elements, respectively (Weston *et al.*, 2003).

RA is generally known to play a pivotal role in mesenchymal and chondrogenic cell differentiation during skeletogenesis (Weston *et al.*, 2003), acting as a morphogen in the developing limb bud (De Leenheer *et al.*, 1982) and enhancing chondrogenesis (Langille *et al.*, 1989) and BMP-2 stimulation of chondrocyte differentiation (Li *et al.*, 2003b). It has also been shown to induce expression of a major regulatory gene for osteoblast differentiation Cbfa1 and of the bone matrix-associated genes ALP, ON, OP, Col I and BMP-2 and -4 (Helvering *et al.*, 2000; Weston *et al.*, 2003), all of which are generally regarded as important target genes of the BMP/BMPR signalling pathway (Korchynskyi and ten Dijke, 2000; de Jong *et al.*, 2002; de Jong *et al.*, 2004; Takagi *et al.*, 2004).

1.3. Signal transduction by growth factors

Growth factors elicit profound effects on multiple types of cell by acting as ligands that bind to specific transmembrane receptors on target cells. The formation of the ligand-receptor complex induces a cascade of intracellular molecular reactions that are

ultimately transduced to the nucleus and produce major changes in gene activity and thus the biological response to the exogenous ligand (Johnson and Vaillancourt, 1994). Ligand binding is often, but not always, highly specific, and can range from simple interaction of one specific growth factor with a single receptor, to a complex process with one or more ligands binding to receptor complexes containing multiple polypeptides (Trippel *et al.*, 1996; Barnes *et al.*, 1999).

As shown in Figure 1.3, once the binding of the ligand to an extracellular domain of the transmembrane receptor is established, changes occur in the conformation of the intracellular domain. These lead to a series of phosphorylation reactions involving a cascade of intracellular protein kinases and result ultimately in the activation of transcription factors that bind to specific sequences of the promoter regions of many genes, regulating their transcription into mRNA and thence translation into protein (Trippel *et al.*, 1996; Barnes *et al.*, 1999). Generally, the intracellular signalling cascade activates a wide range of genes and, as a result, the ligand may generate multiple effects, even within a single cell type. However, although each family of growth factors has its own corresponding family of transmembrane receptors with marked differences in structure, many of the key links in the gene-activating chain of reactions are shared by these families. Thus, binding of different growth factors to their respective receptors can contribute to similar cellular responses, such as cell proliferation and differentiation (Pearson *et al.*, 2001; Lee *et al.*, 2002; Celil and Campbell, 2005). Notably, growth factors also exhibit pleiotropic activity, exerting a wide variety of effects even in the same type of cell at different stages of development (Trippel *et al.*, 1996; Barnes *et al.*, 1999).

Although the mechanisms of signal transduction by growth factors and their receptors have been widely studied in many types of cell, there is still only limited understanding of their expression and function in the regulation of bone growth, differentiation and repair processes. A better understanding of the expression and function of the growth factor receptors would undoubtedly be of great benefit for optimising the clinical application of their corresponding growth factors.

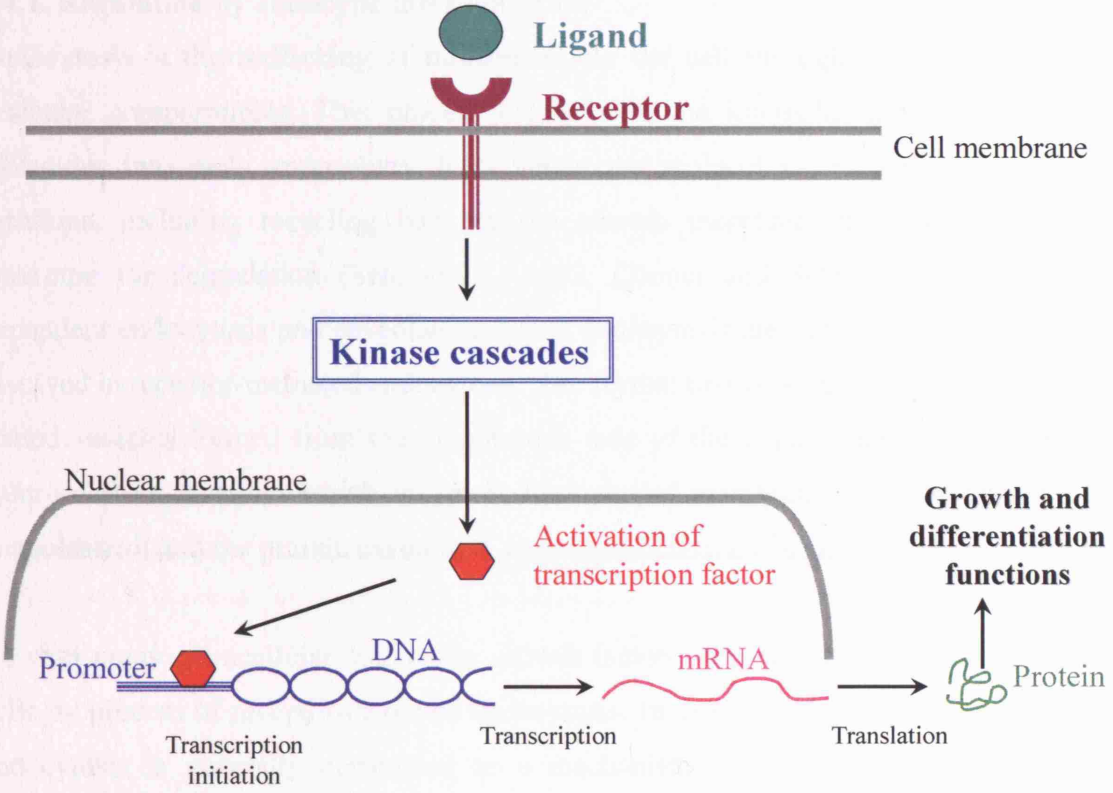


Figure 1.3. Schematic diagram of signal transduction by growth factors. A growth factor ligand in the extracellular environment binds to the extracellular domain of its cognate transmembrane receptor. This ligand-receptor interaction activates the intracellular kinase domain of the receptor, subsequently initiating a series of phosphorylation steps (kinase cascade) in the cytoplasm. These are translocated to the nucleus and culminate with the activation of transcription factors that bind to the promoter region of target genes and control mRNA transcription.

1.4. Regulation of cell surface receptors

Growth factors and many other soluble mediators exert their biological function through binding to the extracellular domain of transmembrane receptors, resulting in the activation of specific intracellular signal transducing pathways which subsequently control the expression of target genes in the nucleus. Regulation of these cell surface receptors is therefore of paramount importance for the functional efficacy of the corresponding mediators.

1.4.1. Regulation by endocytic internalisation

Endocytosis is the trafficking of molecules into the cell through a complex series of vesicular compartments. This process begins with the internalisation of extracellular molecules into early endosomes, from where the molecules are 'sorted' to multiple locations, including recycling back to the plasma membrane or trafficking to the lysosome for degradation (Seto *et al.*, 2002; Conner and Schmid, 2003). Clathrin-dependent endocytosis and caveolae-mediated endocytosis are the two main mechanisms involved in receptor-mediated endocytosis. The former involves the formation of clathrin-coated vesicles formed from the cytoplasmic side of the plasma membrane, while the latter involves caveolae, which are small, flask-shaped membrane invaginations enriched in cholesterol and the protein caveolin (Conner and Schmid, 2003).

As with many extracellular molecules, growth factor-receptor complexes also enter into cells by process of receptor-mediated endocytosis. In growth factor signalling processes, endocytosis is generally considered as a mechanism by which receptors are down-regulated, and the cells desensitized to signalling molecules by trafficking the receptors to the lysosomes for degradation. However, the receptor can sometimes become dissociated from the growth factor ligand in early endosomes, allowing for rapid recycling of the (ligand-free) receptor to the cell surface and increasing receptor availability for further signalling (French *et al.*, 1995; Seto *et al.*, 2002). However, in the absence the respective ligand, cell surface receptors are generally only slowly internalised and then rapidly recycled back to the plasma membrane, resulting in their accumulation at the cell surface (Seto *et al.*, 2002).

There is increasing evidence of the importance of endocytosis in a number of growth factor receptor signalling pathways, such as for TGF-β (Zwaagstra *et al.*, 1999; Zwaagstra *et al.*, 2001), BMP (Jortikka *et al.*, 1997; Nohe *et al.*, 2005; Hartung *et al.*, 2006), PDGF (Chiarugi *et al.*, 2002; Wang *et al.*, 2003), FGF (Citores *et al.*, 2001) (including keratinocyte growth factor (KGF) (Marchese *et al.*, 1998; Belleudi *et al.*, 2002) which is FGF-7), IGF (Furlanetto, 1988; Braulke, 1999), and vascular endothelial growth factor (VEGF) (Wang *et al.*, 2002). Thus, it has been shown that endocytic internalisation of TGFβR-I and -II occurs via a process involving clathrin-dependent endocytosis, in which both heteromeric and homomeric TGFβR complexes undergo ligand-dependent

internalisation (Anders *et al.*, 1997), after which, if the ligand dissociates from the complex, the receptors are constitutively recycled to the cell surface. However, if the ligand remains associated with the complex, the TGFβR is targeted for lysosomal degradation (Anders *et al.*, 1997). As with TGFβR, BMPR on the surface of myoblasts have been shown to be down-regulated by the ligand, with the BMP-BMPR complex undergoing rapid internalisation and the internalised BMPR recycled to the plasma membrane (Jortikka *et al.*, 1997). More recently, it was reported that endocytic internalisation of BMPR-IB and -II can occur via a clathrin-mediated process, resulting in an activation of Smad-dependent BMP signalling (Hartung *et al.*, 2006), whereas only BMPR-II undergoes caveolae-mediated endocytosis (Nohe *et al.*, 2005). Hartung *et al.* (2006) further showed that the association of BMPR complexes with caveolae, via direct binding of BMPR-II and caveolae, leads to Smad-independent BMP signalling. Thus, endocytosis appears to be a crucial process regulating intracellular signallings initiated from cell surface receptors, including that from the BMPR.

1.4.2. Regulation by shedding

Shedding of 'soluble' peptide fragments of transmembrane cell surface receptors has also been established as a mechanism by which a wide range of cell surface proteins are functionally down-regulated (Lum *et al.*, 1999; Philip *et al.*, 1999; Hanneken, 2001; Dello and Rovida, 2002; Garton *et al.*, 2003; Hu *et al.*, 2004). Moreover, the concomitantly increased level of such 'soluble' receptors, comprising the ligand-binding domain, can compete with cell-associated receptors for binding of extracellular mediators (Dello and Rovida, 2002). Thus, shedding is functionally equivalent to post-transcriptional alternative splicing of mRNA transcripts, which can also generate soluble forms of cell surface molecules (Heaney and Golde, 1998; Meissner *et al.*, 2001) such as the soluble receptor for IL-6 (Jones *et al.*, 2001). The process of shedding can thus result in a marked decrease in cell surface expression of a wide range of transmembrane proteins, including cytokine and growth factor receptors (Dello and Rovida, 2002).

Although shedding occurs at a low, constitutive level (*i.e.*, 'basal' shedding) in non-stimulated cells, it can be activated by several mechanisms. For example, synthetic compounds such as phorbol esters are able to activate PKC, which induces the shedding process (Arribas and Borroto, 2002; Dello and Rovida, 2002). Thus, soon after phorbol

ester treatment, cells can shed the ectodomains of a considerable proportion of cell surface molecules (Arribas and Borroto, 2002). Ca^{++}-ionophores are also common inducers of shedding (Dello and Rovida, 2002). Both phorbol esters and Ca^{++}-ionophores differentially activate a number of transmission pathways, such as PKC, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK (Vecchi *et al.*, 1996; Rizoli *et al.*, 1999; Umata *et al.*, 2001; Dello and Rovida, 2002). This leads to the activation of the disintegrin/Zn-metalloprotease (MP) or a disintegrin and a metalloprotease (ADAM) family that can induce shedding of transmembrane proteins by their proteolytic action (Blobel, 2002; Dello and Rovida, 2002). Moreover, other specific families of MP, such as the matrix MP (MMP), the membrane-type MMP (MT-MMP) and transmembrane MP (TM-MP), have also been shown to induce proteolytic shedding of plasma membrane-associated proteins (Arribas *et al.*, 1996; Arribas and Borroto, 2002; Blobel, 2002; Dello and Rovida, 2002).

Processes involving receptor shedding have previously been shown to be of major importance in a number of biological events. For example, shedding of TNF-α, TNFR-I and TNFR-II crucially contributes to the regulation of TNF-α-induced responses both *in vitro* and *in vivo* (Black *et al.*, 1997; Pinckard *et al.*, 1997). Moreover, a number of the transmembrane growth factor receptors, including epidermal growth factor receptor (EGFR), FGFR PDGF and TGFβR, have previously been reported to be regulated by proteolytic shedding (Tiesman and Hart, 1993; Philip *et al.*, 1999; Yabkowitz *et al.*, 1999; Hanneken, 2001; Hu *et al.*, 2004). Thus, regulation of cell surface growth factor receptors by ectodomain shedding is clearly of biological importance in controlling cell responsiveness to external stimuli, although it is not yet known whether BMPR can be modulated by this type of regulatory process.

1.5. Bone morphogenetic proteins (BMPs)

1.5.1. Identification and classification

Urist (1965) reported that implantation of decalcified bone matrix into muscle induced new ectopic bone formation associated with endochondral bone formation. The factor contained in the decalcified bone matrix was subsequently identified and named bone morphogenetic proteins (BMPs) (Urist and Strates, 1971). In 1988, Wozney *et al.* first cloned four cDNA sequences for human BMPs, *i.e.,* BMP-1, BMP-2A (BMP-2), BMP-2B (BMP-4) and BMP-3. Except for BMP-1, which is type I procollagen C-proteinase (Kessler *et al.*, 1996), the BMPs are subfamily members of the TGF-β superfamily (Figure 1.4) with, in humans, approximately 20 different BMPs derived from distinct gene sequences which share a high degree of homology (Reddi, 1998; ten Dijke *et al.*, 2003b).

All BMPs are synthesised as precursor proteins, the mature BMP deriving from the carboxyterminal region by proteolytic cleavage (Massague, 1990; Wozney *et al.*, 1990). All secreted BMPs are dimeric, each monomer containing seven cysteine residues, six of which build a cysteine knot and the seventh involved in dimerisation with the second monomeric peptide (Scheufler *et al.*, 1999).

Members of the BMP family can be subdivided into several groups based on the amino acid sequence similarity of the mature ligand (Kawabata *et al.*, 1998; Ducy and Karsenty, 2000). BMP-2 and BMP-4 form one subgroup (BMP-2/4 group). BMP-5, BMP-6, BMP-7 (also termed osteogenic protein-1) and BMP-8 form another subgroup (BMP-7 group). Growth-differentiation factor-5 (GDF-5, also termed cartilage-derived morphogenetic protein-1, CDMP-1), GDF-6 (CDMP-2 or BMP-13) and GDF-7 (BMP-12) form a third group (GDF-5 group).

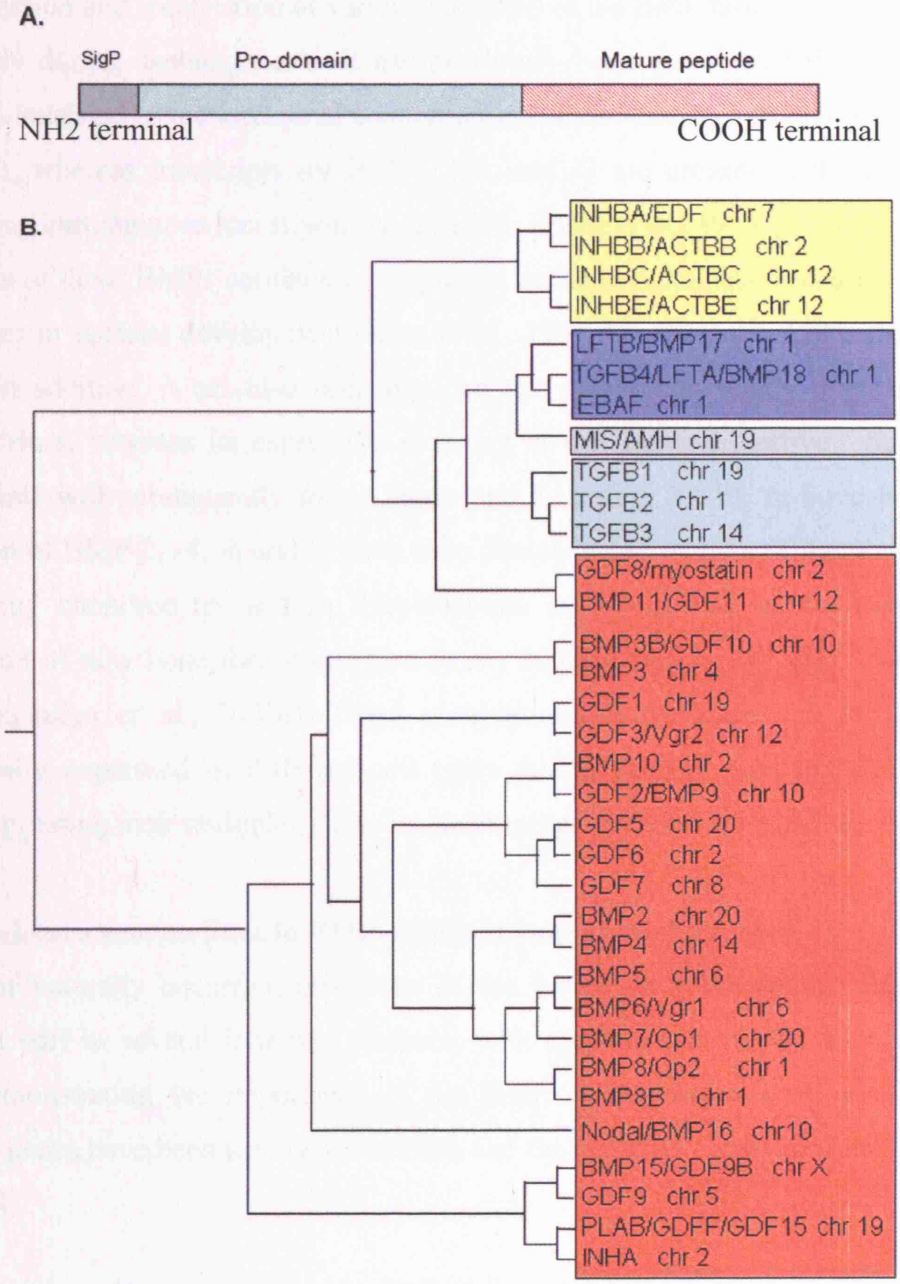


Figure 1.4. TGF-β superfamily. A: Schematic structure of a TGF-β superfamily member. Signal peptide (SigP), pro-domain, and mature peptide are indicated. B: Members in the human TGF-β superfamily. The human chromosomal location of each gene is indicated. The dendrograms indicate the relative level of amino acid sequence similarity of the mature ligand between members in TGF-β superfamily.
(Adapted from ten Dijke *et al.*, 2003a)

1.5.2. Skeletal expression of BMPs

The expression and localisation of various members of the BMP family have been studied extensively during skeletogenesis. It has previously been shown that the transcript for BMP-5 is localised to mesenchymal condensations before cartilage development (King *et al.*, 1994), whereas transcripts for BMP-2, -4, and -7 are present in the mesenchyme surrounding cartilaginous loci (Lyons *et al.*, 1995; Rosen *et al.*, 1996a). The expression of transcripts of these BMPs continue to be present at the perichondrium and periosteum at later stages in skeletal development (King *et al.*, 1994; Lyons *et al.*, 1995; Rosen *et al.*, 1996a). In addition, it has also been reported that GDF-5 is weakly expressed in the perichondrium, whereas its expression is strong at the interface between cartilage loci where joints will subsequently form (Storm and Kingsley, 1999). In bone healing, the expression of BMP-2, -4, -6 and -7 have been demonstrated in cells of the periosteum, in proliferating chondrocytes and in fibroblast-like cells involved in the initiation and progression of new bone formation (Cho *et al.*, 2002; Kloen *et al.*, 2002; Kloen *et al.*, 2003; Kugimiya *et al.*, 2005a). These observations demonstrate that the BMPs are differentially expressed in different cell types during skeletal development and bone repair, suggesting their multiple effects in skeletogenesis and bone regeneration.

1.5.3. Skeletal abnormalities in BMP-deficient animals and humans

Studies of naturally occurring mutations of the BMPs have shown that they play an important part in several inherited diseases, with mutations in the BMP-5 and GDF-5 genes demonstrating the importance of the BMP in the skeleton while other BMP-encoding genes have been inactivated in mice and the resulting defects reported, as shown in Table 1.

Table 1. Abnormalities in BMP-deficient animals and humans

Genes	Phenotypes
BMP-2	Embryonical lethality before the onset of skeletogenesis due to failure of mesoderm induction (Zhang and Bradley, 1996).
BMP-4	Embryonical lethality before the onset of skeletogenesis due to failure of mesoderm induction (Winnier *et al.*, 1995).
BMP-5	Short ear mice characterised by a reduction in the rate of cartilage formation (Kingsley *et al.*, 1992) and a minimal increase in growth plate height and growth rate (Mikic *et al.*, 1996).
BMP-6	BMP-6-deficient mice are viable with a mild delay of sternum ossification (Solloway *et al.*, 1998).
BMP-7	BMP-7-deficient mice have patterning abnormalities of the ribs and a preaxial polydactyly in the limbs, and they die shortly after birth due to severe renal failure and eye defects (Luo *et al.*, 1995).
GDF-5	Brachypodism in mice resulting in a reduction in length of several long bones and the replacement of two bones in most digits by a single skeleton compartment (Storm *et al.*, 1994). Acromesomelic chondrodysplasia (Hunter-Thompson type) in humans, characterised by abnormalities of the limbs and limb joints which are phenotypically similar to murine brachypodism (Thomas *et al.*, 1996).

1.5.4. Functional roles of BMPs

The BMPs have been shown to regulate cell differentiation in cartilage and bone during skeletal development (King *et al.*, 1994; Lyons *et al.*, 1995; Rosen *et al.*, 1996a; Wan and Cao, 2005). They are also known to be the most effective growth factors in osteogenesis and the repair of osseous defects and fracture (Ducy and Karsenty, 2000; Groeneveld and Burger, 2000; Schilephake, 2002), attributed mainly to their stimulation of osteoblast differentiation (ten Dijke *et al.*, 2003a; Chen *et al.*, 2004b). Thus, BMP-2 and -7 induce osteoblast differentiation of the multipotent mesenchymal cell line C3H10T1/2 (Yamaguchi *et al.*, 2000), while BMP-2 stimulates ALP activity and parathyroid hormone (PTH)-dependent cyclic adenosine 3' 5'-monophosphate (cAMP) production in the osteoprogenitor cell line ROB-C26 (Yamaguchi *et al.*, 1991), in the clonal osteoblastic

cell line MC3T3-E1 (Takuwa *et al.*, 1991; Nakase *et al.*, 1997) and in primary osteoblastic cells isolated from human bones (Kawasaki *et al.*, 1998). Moreover, BMP-2 has been shown to have a marked stimulatory effect on the mRNA levels of ALP, Col I, OP, BSP and OC (Lecanda *et al.*, 1997). BMP-7 stimulates osteoblast differentiation in the osteoblastic cell line ROS17/2.8, by increasing synthesis of Col I and OC, ALP activity and PTH responsiveness (Maliakal *et al.*, 1994). Thus, the BMPs appear to be capable of promoting osteoprogenitor cells to differentiate into more mature osteoblasts.

BMP activity appears to be required not only for initiation and early osteoblast differentiation but also in later stages of osteogenesis such as matrix mineralisation (Wada *et al.*, 1998; van der Horst *et al.*, 2002), and possibly in regulating the life span of osteoblasts, whereas BMP-2 and -4 have been found to inhibit TNF-mediated apopotosis (Chen *et al.*, 2001). These *in vitro* findings suggest that the BMP might also accelerate new bone formation *in vivo*.

Recombinant human proteins of several BMPs (rhBMPs) are active in chemotaxis, mitogenesis and differentiation *in vitro* (Sampath *et al.*, 1992; Fiedler *et al.*, 2002; Nakashima and Reddi, 2003) and, moreover, also have the ability to induce ectopic bone formation *in vivo* due to their unique and important role in osteogenic transdifferentiation of myogenic cells (Urist, 1965; Wozney *et al.*, 1988; Katagiri *et al.*, 1994; Akiyama *et al.*, 1997; Katagiri *et al.*, 1997; Namiki *et al.*, 1997; Kawasaki *et al.*, 1998). Thus, in animal models, rhBMP are able to promote new bone formation during healing of bone defects (Cheung and Zheng, 2006; Saito *et al.*, 2006) and fusion of damaged spines (Boden *et al.*, 1999; Vaccaro *et al.*, 2002). The rhBMP have also been used successfully in animal experimental models to accelerate osteogenesis around endosseous implants (Bessho *et al.*, 1999; Cochran *et al.*, 2000) as well as to promote wound healing and bone formation in periodontal defects (King *et al.*, 1997; Saito *et al.*, 2003). BMP gene transfer has also recently been used to stimulate bone healing of osseous defects *in vivo* (Park *et al.*, 2003; Kawai *et al.*, 2005).

The results of experimental models have led to the use of rhBMP-2 and rhBMP-7 in human clinical trials. The first study of osteoinduction by rhBMP in humans was reported by Boden *et al.* (2000), with subsequent studies also indicating that rhBMP-2 and

rhBMP-7 can promote spinal fusion in humans (Boden *et al.*, 2002; Vaccaro *et al.*, 2003; Mummaneni *et al.*, 2004; Vaccaro *et al.*, 2004; Vaccaro *et al.*, 2005) and can accelerate bone healing of long bone fractures (Geesink *et al.*, 1999; Friedlaender *et al.*, 2001; Govender *et al.*, 2002). Moreover, rhBMP-2 has been used for local ridge augmentation procedures (Cochran *et al.*, 2000), including guided bone regeneration therapy for intra-oral implants in human AB sites (Cochran *et al.*, 2000; Jung *et al.*, 2003).

Since the identification of BMP as an osteogenic factor in demineralised bone, the Food and Drug Administration (FDA) has approved the use of rhBMP-7 for long bone defects and of rhBMP-2 in a collagen carrier within a cage for anterior lumbar interbody fusions (Rengachary, 2002; Khan and Lane, 2004). However, the clinical outcome of rhBMP treatment in patients has not been demonstrated to be significantly more effective than autogenous bone grafts (Boden, 2001), and further studies are therefore still required to examine the mechanisms that could enhance the biological activity of rhBMP in bone regeneration.

Taken together, there is much evidence demonstrating that the BMPs have a potent stimulatory effect on bone formation both *in vitro* and *in vivo*. As with other growth factors, the BMPs elicit their biological function by signal transduction through their cognate BMPR. The expression and regulation of these receptors are thus central to the action of the BMP.

1.6. BMP receptors (BMPR)

1.6.1. Identification and classification

BMPR are transmembrane serine/threonine kinases which are members of the TGFβR superfamily (Massague, 1998) (Figure 1.5). Despite the growing number of BMP ligands, there are only three type-I and three type-II receptors known to transduce the BMP signal in mammalian cells (Yamashita *et al.*, 1996; Nohe *et al.*, 2004). The type-I receptors include BMPR-IA (activin receptor-like kinase 3; ALK-3), BMPR-IB (ALK-6) and ALK-2 (activin receptor type-I; ActR-I) (Koenig *et al.*, 1994; ten Dijke *et al.*, 1994a; ten Dijke *et al.*, 1994b). BMPR-II, ActR-II and -IIB comprise the type-II receptors (Liu *et al.*,

1995; Rosenzweig *et al.*, 1995; Yamashita *et al.*, 1995; Yamashita *et al.*, 1996), in addition to an alternative mRNA splice variant of BMPR-II which lacks most of the C-terminal cytoplasmic tail (Beppu *et al.*, 1997). However, only BMPR-IA, BMPR-IB and BMPR-II are considered to be the BMP-specific receptors (Yamashita *et al.*, 1996). A summary of the mammalian BMPR is shown in Table 2.

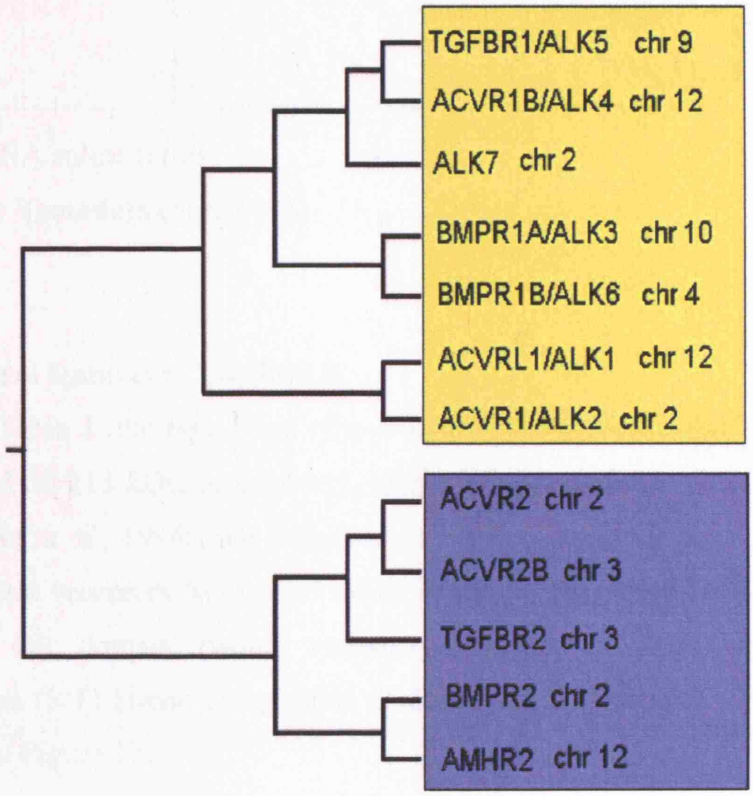


Figure 1.5. The human TGF-β superfamily type-I and type-II receptors. The human chromosomal location of each gene is also indicated. TGFBR = TGFβR; ACVR = activin receptor; ALK = activin-like kinase; BMPR = BMP receptor; AMHR = anti-Mullerian hormone receptor. The dendrograms indicate the relative level of amino acid sequence similarlity in the kinase domain.
(Adapted from ten Dijke *et al.*, 2003a)

Table 2. Type-I and -II BMPR in mammals

Designation	Alternative designations	Number of amino acids
Type-I receptors		
BMPR-IA	ALK-3, BRK-1	532
BMPR-IB	ALK-6, BRK-2	502
ActR-I	ALK-2	509
Type-II receptors		
BMPR-II	BRK-3	*530, 1038
ActR-II		513
ActR-IIB		*504, 512, 528, 536

*Different mRNA splice forms

(Adapted from Yamashita *et al.*, 1996)

1.6.2. Structural features of the BMPR

As shown in Table 2, the type-I and type-II BMPR are glycoproteins of approximately 50-55 kDa and 50-115 kDa, respectively, with core polypeptides of 502 to 1038 amino acids (ten Dijke *et al.*, 1994a; ten Dijke *et al.*, 1994b; Yamashita *et al.*, 1996; Beppu *et al.*, 1997). These receptors have three major domains: the amino-terminal extracellular domain; the GS domain (which contains a unique SGSGSG motif); and the serine/threonine (S/T) kinase cytoplasmic domain (carboxy-terminal) (Massague, 1998), as illustrated in Figure 1.6.

1.6.2.1. Extracellular domain

The extracellular region is relatively short (approximately 150 amino acids) and N-glycosylated (Yamashita *et al.*, 1996; Peri *et al.*, 2003). In the extracellular domain, human BMPR-IB shows 42% amino acid homology with human BMPR-IA (Ide *et al.*, 1997a). BMPs bind to their cognate receptors via BMP binding epitopes within the BMPR extracellular domain (Sebald *et al.*, 2004).

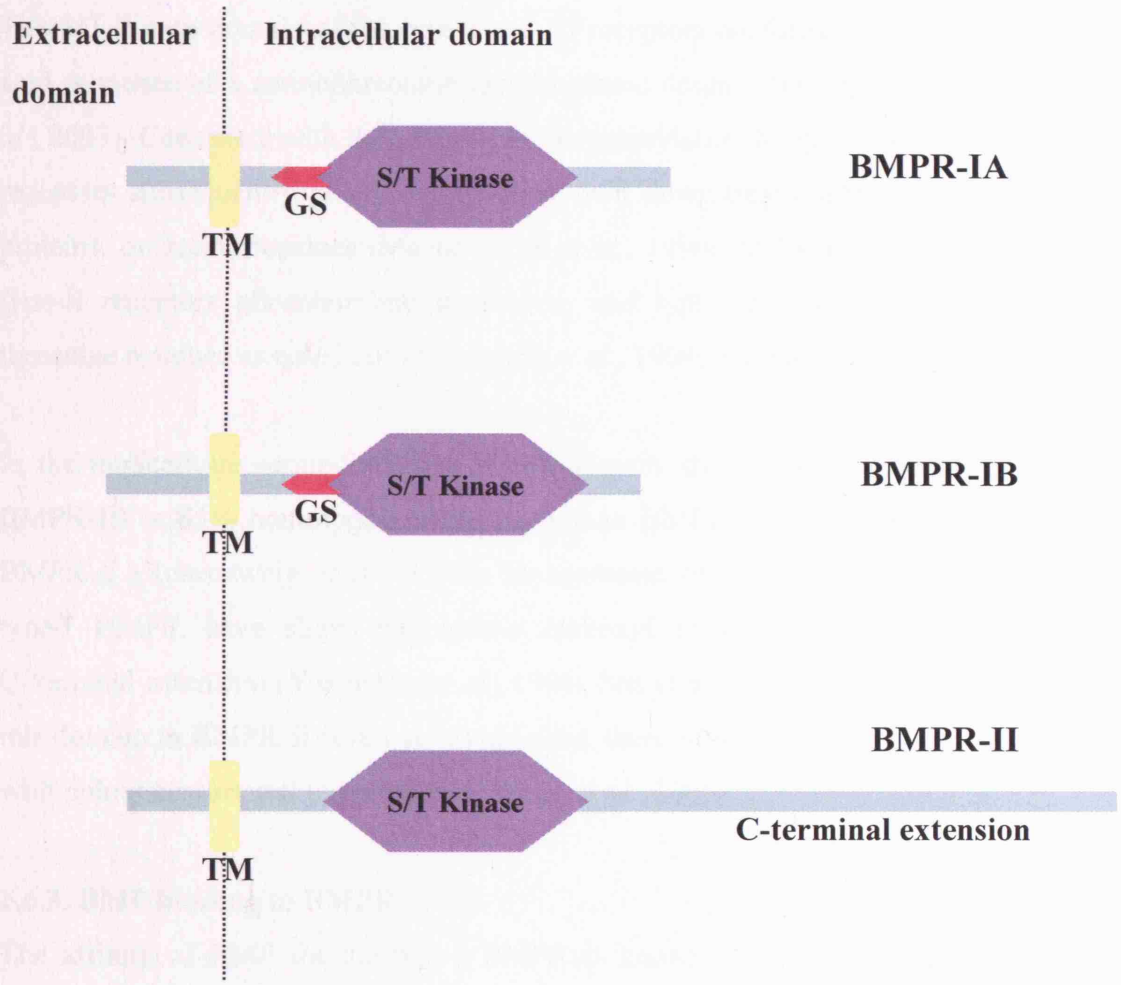


Figure 1.6. Domain structures of BMPR-IA, -IB and -II. TM: transmembrane region; GS: GS domain; S/T Kinase: serine/threonine kinase domain.

1.6.2.2. GS domain

A unique feature of type-I receptors is a highly conserved 30-amino acid region located immediately upstream of the S/T kinase domain (Figure 1.6). This region is called the GS domain because it is serine- and glycine-rich and contains a unique SGSGSG motif (Yamashita *et al.*, 1996; Peri *et al.*, 2003). Phosphorylation of the serine and threonine residues in the GS domain of the type-I receptor by the type-II receptor is required for the activation of subsequent downstream signalling events by the type-I receptor (Wrana *et al.*, 1994; Yamashita *et al.*, 1996; Massague, 1998).

1.6.2.3. Kinase domain

The S/T kinase domain of the type-I and -II receptors conforms to the canonical amino acid sequence of a serine/threonine protein kinase domain (Franzen *et al.*, 1993; Peri *et al.*, 2003). Consistent with this, following phosphorylation by type-II receptors, the type-I receptors subsequently phosphorylate their own downstream substrates, such as Smad proteins, on serine residues (Macias-Silva *et al.*, 1996; Abdollah *et al.*, 1997), whereas type-II receptors phosphorylate themselves and type-I receptors on both serine and threonine residues as noted above (Bassing *et al.*, 1994; Yamashita *et al.*, 1996).

In the intracellular serine-threonine kinase domain, the amino acid sequence of human BMPR-IB is 85% homologous with the human BMPR-IA, but only 40% with human BMPR-II (Rosenzweig *et al.*, 1995). Downstream of the the kinase domain, the two type-I BMPR have short cytoplasmic carboxyl tails, while BMPR-II has a long C-terminal extension (Yamashita *et al.*, 1996; Peri *et al.*, 2003). Although the function of this domain in BMPR-II is not yet established, there is evidence that it may be associated with pulmonary arterial hypertension (Wong *et al.*, 2005).

1.6.3. BMP binding to BMPR

The affinity of BMP for the type-I BMPR is known to be much greater than for the type-II BMPR (ten Dijke *et al.*, 1994a; Rosenzweig *et al.*, 1995), and type-I BMPR has been shown to increase the binding affinity of type-II BMPR for the BMP ligand (Liu *et al.*, 1995). However, it has also been reported that a BMP dimer can bind with high affinity to a heteromeric complex consisting of at least one type-I receptor and one type-II receptor (Kawabata *et al.*, 1998; Nohe *et al.*, 2002). Moreover, the combinatorial nature of these receptors depends to some extent on the BMP ligands. Thus, in BMPR-transfected COS-1 cells, BMP-2 was found to be strongly bound to BMPR-II when complexed with BMPR-IB but less when complexed with BMPR-IA (Liu *et al.*, 1995). BMP-4 preferentially binds to both BMPR-IA and -IB, while BMP-2, BMP-7 and GDF-5 all bind with higher affinity to BMPR-IB than to BMPR-IA (ten Dijke *et al.*, 1994a; Yamashita *et al.*, 1995; Nishitoh *et al.*, 1996; Macias-Silva *et al.*, 1998; Gilboa *et al.*, 2000; Nohe *et al.*, 2004), again demonstrating that the different BMPs bind with different affinities to the BMPR complexes.

The oligomerisation pattern of the BMPR to from these complexes is known to be very different from other TGF-β-type receptors, being far more flexible to modulation by ligand (Gilboa *et al.*, 2000). Multiple BMPR oligomers are present at the cell surface prior to ligand binding, comprising heterodimers (BMPR-IA/BMPR-II and BMPR-IB/BMPR-II) as well as homodimers (BMPR-IA/BMPR-IA, BMPR-IB/BMPR-IB, BMPR-II/BMPR-II and BMPR-IA/BMPR-IB), with the former the most prominent (Gilboa *et al.*, 2000). These preformed heterodimeric complexes are, within the TGFβR superfamily, unique for the BMPR. However, BMP-2 binding significantly increases both hetero- and homo-oligomerisation (except for the BMPR-II homo-oligomer, which binds ligand poorly in the absence of type-I BMPR) (Gilboa *et al.*, 2000). Thus, two modes of BMPR hetero-oligomerisation appear to occur: one prior to ligand binding, and the other which is ligand-mediated (Gilboa *et al.*, 2000; Nohe *et al.*, 2002). In addition, Nohe, *et al.* (2002) have demonstrated that the dimeric ligand, in this case BMP-2, has two mechanisms for binding to the receptors, as shown in Figure 1.7. It can bind to a 'preformed heteromeric complex' consisting of at least one type-I and one type-II receptor, resulting in the activation of the Smad-dependent signalling pathway. The other mechanism involves binding to a homodimer consisting of either the high-affinity receptors BMPR-IA or BMPR-IB and then recruiting BMPR-II into a heteromeric complex of BMPR-IA/BMPR-II or BMPR-IB/BMPR-II ('BMP-induced signalling complex'), leading to activation of the Smad-independent signalling pathway, *e.g.*, p38 MAPK pathway. (Nohe *et al.*, 2002) (Figure 1.7), as detailed in Section 1.6.4.

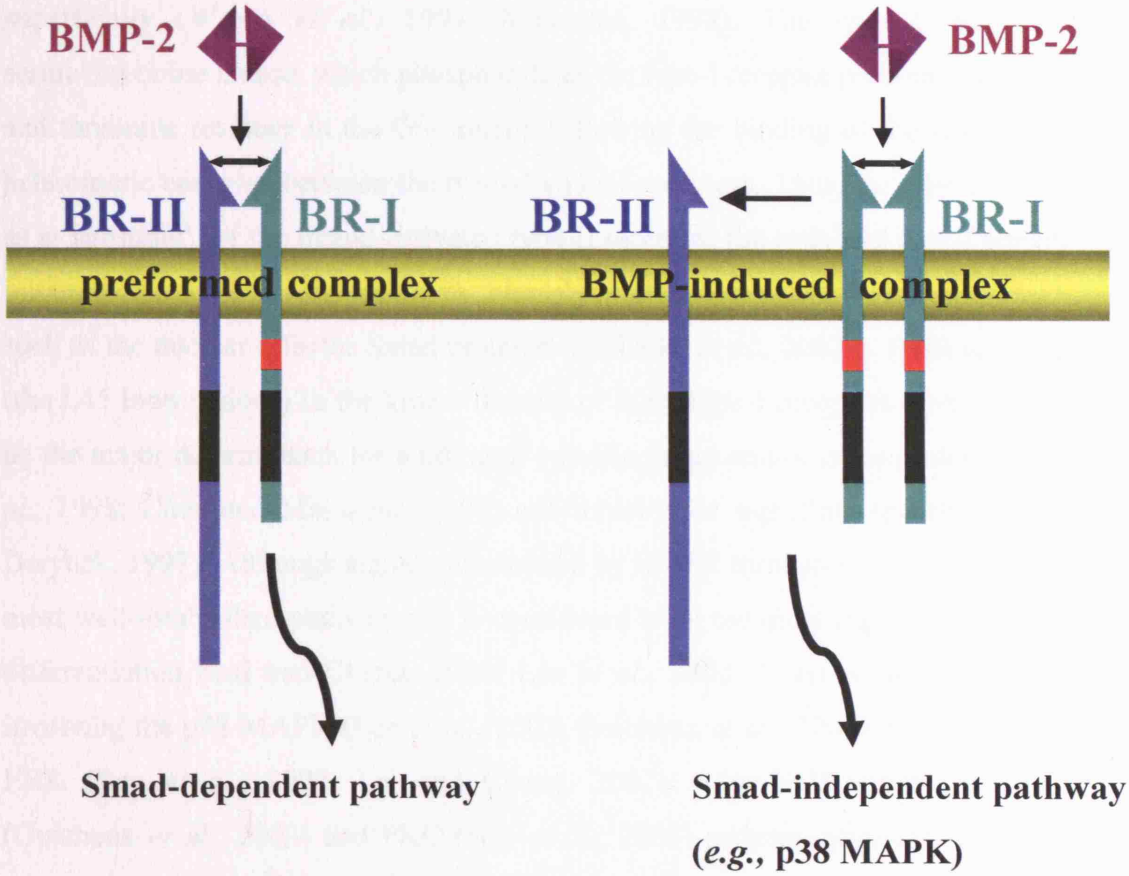


Figure 1.7. Schematic model of BMP-2 signal transduction through preformed BMPR complexes and BMP-2-induced signalling complexes. Binding of BMP-2 to preformed receptor complexes, composed of monomeric type-I and type-II BMPR, possibly heterodimeric complexes, initiates the Smad pathway. A second pathway, which is a Smad-independent pathway, is initiated through a BMP-2-induced oligomerisation of the BMPR. BR-I: type-I BMPR; BR-II: type-II BMPR.
(Adapted from Nohe *et al.*, 2002)

1.6.4. BMPR activation and signal transduction

The mechanism of BMPR activation appears to be similar to that of the TGFβR superfamily (Wrana *et al.*, 1994; Massague, 1998). The type-II receptor has a serine/threonine kinase, which phosphorylates the type-I receptor predominantly on serine and threonine residues in the GS-domain following the binding of the BMP ligand to a heteromeric complex between the types-I and -II receptors. Thus, the type-I receptor acts as a 'substrate' for the ligand-activated type-II receptor, the activated type-I receptor then initiating intracellular signalling by phosphorylating specific downstream components such as the nuclear effector Smad proteins (ten Dijke *et al.*, 2003b). Particular sequences (the L45 loop regions) in the kinase domain of these type-I receptors have been found to be the major determinants for binding of specific Smad and/or other proteins (Persson *et al.*, 1998; Chen and Massague, 1999) and thereby for signalling specificity (Feng and Derynck, 1997). Although signal transduction by BMPR through the Smad pathway is the most well-established pathway and is considered to be the most significant for osteoblast differentiation (Lai and Cheng, 2002; Lee *et al.*, 2002; Nohe *et al.*, 2004), pathways involving the p38 MAPK (Lee *et al.*, 2002; Guicheux *et al.*, 2003), Ras (Mulder, 2000), ERK (Lou *et al.*, 2000; Lai and Cheng, 2002), c-Jun-NH2-terminal kinase (JNK) (Guicheux *et al.*, 2003) and PKC (Hay *et al.*, 2004) proteins have also been shown to contribute to BMP/BMPR signal transduction.

The Smads are signal-transducing molecules in the TGFβR signalling (Heldin *et al.*, 1997; Kawabata *et al.*, 1998). At present, eight mammalian Smad proteins (Smad1 through Smad8) have been identified and classified into three subgroups according to their structure and function (Heldin *et al.*, 1997; Kawabata *et al.*, 1998). The first subgroup, receptor-activated Smad (R-Smad) is activated following receptor activation and comprises Smad1, Smad2, Smad3, Smad5 and Smad8, with Smad1/5/8 being involved in BMPR signalling (Liu *et al.*, 1996; Yamamoto *et al.*, 1997; Kawai *et al.*, 2000). The second, the common mediator Smad (Co-Smad) consists of Smad4, which interacts with phosphorylated R-Smad, forming a heterodimeric complex which is translocated into the nucleus (Zhang *et al.*, 1996; Kawabata *et al.*, 1998). The third subgroup, the inhibitory Smad (I-Smad) comprises Smad6 and Smad7, which inhibit signalling by the TGFβR superfamily (Hayashi *et al.*, 1997; Imamura *et al.*, 1997).

Triggered by BMP ligand binding, the activated BMPR in turn phosphorylate the intracellular signalling intermediates Smad1/5/8, which then associate with Smad4 and are translocated to the nucleus, as noted above, in which they regulate the transcription of target genes either through direct binding to DNA or in association with other DNA-binding proteins (Heldin *et al.*, 1997; Massague, 1998). A summary of Smad-mediated BMPR signal transduction is shown in Figure 1.8.

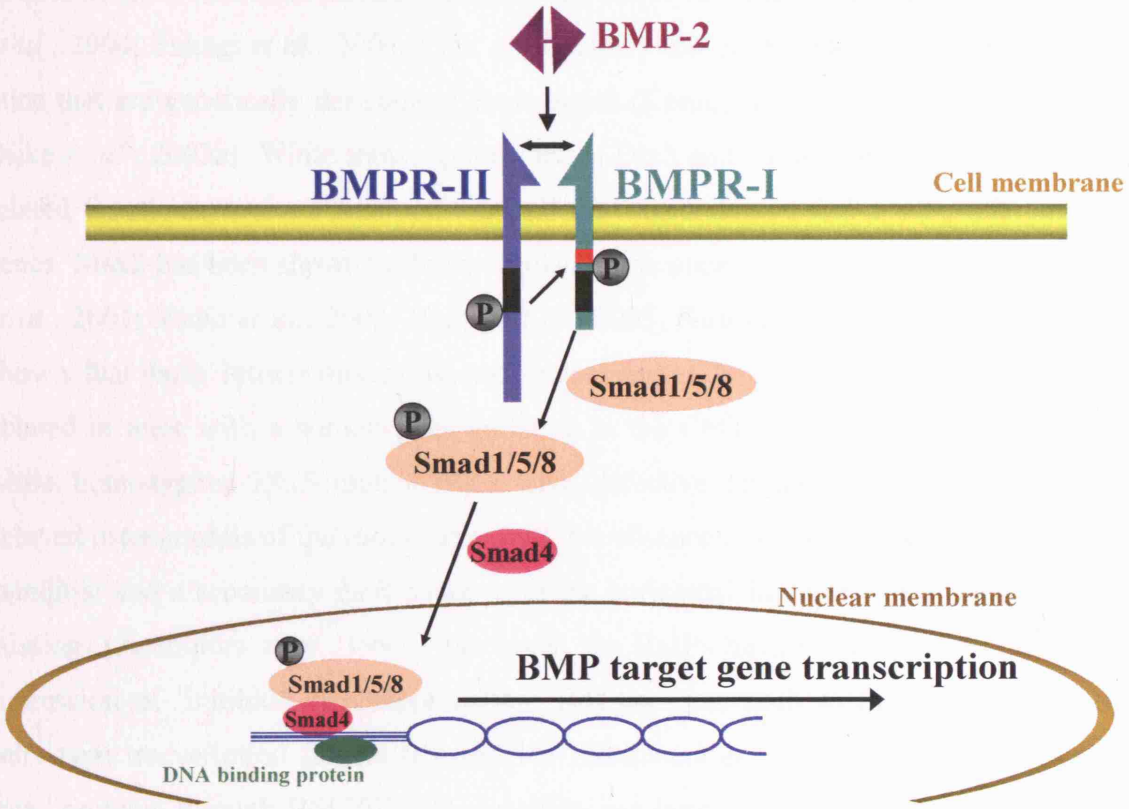


Figure 1.8. A schematic diagram showing the mechanism of BMP signalling through BMPR and Smad. The BMPs bind to the BMPR heteromeric complex (BMPR-I and BMPR-II) on the target cells, leading to phosphorylation of the BMPR-I. The consequently phosphorylated Smad1/5/8 further transduce the BMP signal by interaction with Smad4 and translocation to the nucleus. P: phosphorylated form.

1.6.5. Osteoblast differentiation genes activated by BMP/BMPR signalling

A number of studies have shown that the BMP/BMPR signal induces the differentiation of mesenchymal stromal cells and osteoprogenitors into cells with more mature osteoblast phenotypes and simultaneously inhibits their differentiation into other cell lineages by causing changes in gene expression (Korchynskyi and ten Dijke, 2000; ten Dijke *et al.*, 2003a; ten Dijke *et al.*, 2003b; Miyazono *et al.*, 2005). Many BMP/BMPR-induced transcription factors, such as distal-less homeobox 5 (Dlx5), Cbfa1/Runx2, jun B proto-oncogene (JunB), osterix and muscle segment homeobox 2 (Msx2), have been identified in both *in vitro* osteoblast differentiation (Ducy *et al.*, 1997; de Jong *et al.*, 2002; de Jong *et al.*, 2004; Takagi *et al.*, 2004; Celil *et al.*, 2005) and *in vivo* skeletal abnormalities of mice that are genetically deficient in these genes (Korchynskyi and ten Dijke, 2000; ten Dijke *et al.*, 2003a). While transcription factors Dlx5 and Cbfa1 stimulate osteogenesis-related functions, such as ALP activity and the expression of Col I, OP, BSP and OC genes, Msx2 has been shown to down-regulate these genes (Ducy *et al.*, 1997; Shirakabe *et al.*, 2001; Tadic *et al.*, 2002; Barnes *et al.*, 2003; Kim *et al.*, 2004). It has also been shown that both intramembranous and endochondral bone formation are completely ablated in mice with a homozygous mutation in the Cbfa1 gene (Komori *et al.*, 1997), while homozygous Dlx5 mutant mice have defective craniofacial development (*i.e.*, delayed osteogenesis of the roof of the skull, the absence of the coronoid processes of the mandible and a secondary cleft palate with the horizontal laminae of the palatine bones missing) (Acampora *et al.*, 1999). Moreover, the BMPs have been shown to induce the expression of 'inhibitor of differentiation' (Id) proteins, such as the basic helix-loop-helix-type transcription factors Id1 and Id3 (Hollnagel *et al.*, 1999). The induction of these proteins through BMP/BMPR signalling has been suggested to prompt osteoblast differentiation indirectly by blocking the differentiation of mesenchymal stem cells toward the adipocyte and myoblast lineages (Jen *et al.*, 1992; Moldes *et al.*, 1997).

A number of ECM proteins, including ALP, Col I, OP, BSP and OC, are also known to be potently induced through the BMP/BMPR pathway (Korchynskyi and ten Dijke, 2000; de Jong *et al.*, 2002; de Jong *et al.*, 2004; Takagi *et al.*, 2004). These proteins play a central part in bone matrix mineralisation and bone formation, as described in Section 1.1.

1.6.6. Regulation of BMPR signalling

1.6.6.1. Extracellular regulation

BMPR signalling can be negatively modulated by a group of extracellular secreted polypeptide antagonists which bind to the ligand BMP and thus block binding to the BMPR. Such antagonists, which thereby control the level of BMP available for activation of the cognate receptors, include noggin, chordin, follistatin and the follistatin-related gene, ventroptin, twisted gastrulation, and the Dan/cerberus family of genes, which is comprised of the head inducer cerberus, the tumor suppressor Dan, gremlin and its rat homolog drm, the protein related to Dan and cerberus, caronte, dante and sclerostin (Balemans and Van Hul, 2002; Canalis *et al.*, 2003). Most of these have been shown to be capable of binding to BMP-2, BMP-4 and BMP-7 (Balemans and Van Hul, 2002).

Noggin, a well-known BMP antagonist which is produced by osteoblastic cells in response to BMP-2 stimulation, has been shown to decrease the stimulatory effects of BMP on DNA and Col synthesis and ALP activity in cultured rat osteoblasts (Gazzerro *et al.*, 1998). In addition, primary stromal cells from transgenic mice overexpressing noggin display impaired differentiation when compared with cells from wild-type animals and do not express OC mRNA, suggesting that noggin arrests the differentiation of stromal cells and prevents cellular maturation (Gazzerro *et al.*, 2003).

1.6.6.2. Intracellular regulation

As well as the extracellular proteins which directly affect BMP as noted above, a number of intracellular molecules have also been shown to modulate BMPR signalling (Imamura *et al.*, 1997; Zhu *et al.*, 1999; von Bubnoff and Cho, 2001; Murakami *et al.*, 2003). For example, in the absence of the BMP ligand, Smad6 is located predominantly in the nucleus, but is exported rapidly into the cytoplasm after ligand stimulation (Nakayama *et al.*, 1998). It is known that Smad6 antagonises BMPR signalling by interacting with activated BMPR-IB, preventing access of Smad1 to BMPR-IB and therby interfering with Smad1 phosphorylation by BMPR-IB (Imamura *et al.*, 1997). Smad6 can also inhibit BMPR signalling by competing with Smad4 for heteromeric complex formation with activated Smad1 (Hata *et al.*, 1998; Itoh *et al.*, 2001). Moreover, the expression of Smad6 itself is induced by various extracellular stimuli, such as BMP-2 and -7 (Takase *et al.*,

1998; Li *et al.*, 2003a), suggesting that as with noggin, Smad6 may be involved in a negative feedback regulation of BMPR signalling.

An additional mechanism has also been described in which Smad6 interacts with Smad ubiquitin regulatory factor 1 (Smurf1), which targets Smad proteins for proteosomal and lysosomal degradation (Lo and Massague, 1999; Zhu *et al.*, 1999; Lin *et al.*, 2000). In addition, Smurf1 can interact with activated type-I BMPR through Smad6 and subsequently induce ubiquitin-dependent degradation of the type-I BMPR, resulting in down-regulation of type-I BMPR (Murakami *et al.*, 2003).

Thus, several mechanisms have now been described which demonstrate that BMPR signalling is negatively controlled by both extracellular and intracellular modulators. However, thus far little is known about the positive regulation of BMPR expression and function in bone cells.

1.6.7. Differential expression of BMPR

During organogenesis in mouse embryos, BMPR-IA mRNA is expressed almost ubiquitously, whereas BMPR-IB mRNA has a more restricted expression profile, being observed in mesenchymal precartilage condensations, epithelium and blood vessels (Dewulf *et al.*, 1995). In adult mouse tissues, Dewulf *et al.* (1995) have reported a relatively high level of two BMPR-IA transcripts (6.2 and 3.8 kilobases) in the heart, brain, lung, liver, skeletal muscle, and kidney. In contrast, mouse BMPR-IB mRNA was detected only in the brain and lung, and only one BMPR-IB transcript of 7.5 kilobases reported (ten Dijke *et al.*, 1994a). For BMPR-II, four distinct transcripts of 10, 9.5, 6 and 5 kilobases have been reported in mice to be highly expressed in heart, brain, lung and skeletal muscle, with the larger transcripts expressed more abundantly in these tissues (Beppu *et al.*, 1997).

In developing chicken limb, BMPR-IB and BMPR-II are co-expressed in condensing pre-cartilaginous cells, while BMPR-IA is only weakly detected in the limb mesenchymal cells (Kawakami *et al.*, 1996). BMPR-II is also expressed in the apical ectodermal ridge and interdigital limb mesenchymal cells whereas BMPR-IB is strongly expressed in the posterior-distal region of the limb bud (Kawakami *et al.*, 1996). The spatial and temporal

expression and the co-expression patterns of the BMPR-IB and -II, which constitute signalling receptor complexes for BMP as noted above, indicate that these receptors have an important role in limb development.

While the expression of BMPR-IA, -IB and -II transcripts has been extensively studied *in vivo*, much less information is available on the expression of their corresponding proteins, which are the functionally indispensable signal transducing compartments for the BMP. In extra-osseous sites in adult mice, BMPR-IA and -II but not the BMPR-IB proteins are readily detected in muscle by Western blot analysis (Nakamura *et al.*, 2003). Using antibodies specific for BMPR-IA and -IB, Ishiodou *et al.* (1995) reported the expression of type-I BMPR during embryonic bone development and fracture healing and found both BMPR-IA and -IB expressed in condensing mesenchymal cells at 13.5 days post coitum (p.c.). At 15.5 days p.c., expression of BMPR-IB but not of BMPR-IA was observed in the cells in perichondrium of developing cartilage whereas both receptors were subsequently observed in chondrocytes and in osteoblasts. In normal rat adult bone, expression of BMPR-IA but not of BMPR-IB was observed in osteoblasts in the periosteum, but after femoral fracture expression of BMPR-IB was up-regulated in cells at the proliferating osteogenic layer of the periosteum, in fibroblast-like spindle cells and chondrocytes in endochondral ossification sites and in osteoblasts in the newly formed trabecular bone (Ishidou *et al.*, 1995). These findings show that BMPR-IB as well as BMPR-IA are differentially expressed in developing cartilage and bone as well as in fracture repair, further suggesting that these receptors are of importance both in bone morphogenesis and bone repair processes.

In humans, the BMPR-IA, -IB and -II proteins have been shown to be expressed in a number of cell types including fibroblasts, chondroblasts, osteoblasts, osteoclasts and endothelial cells in areas of healing fractured bone and new blood vessel formation, respectively (Kloen *et al.*, 2003). These BMPR proteins have also been observed in fracture non-unions, with a trend toward decreased expression of BMPR-IB as compared with BMPR-IA and BMPR-II in fibrous tissue at non-union sites (Kloen *et al.*, 2002), again indicating the differential expression and regulation of these BMPR during fracture healing *in vivo*.

BMPR expression in bone cells *in vitro* was first reported by Malpe *et al.* (1994), who identified binding sites for BMP-7 in the human osteosarcoma cell lines TE85 and SaOS2 and in primary human bone cells derived from skull and in stromal cells from human vertebrae. A number of subsequent studies have shown that BMPR-IA is expressed in the osteoblastic cell line MC3T3-E1, osteosarcoma cell lines, C2C12 cells, human foreskin fibroblasts and MvlLu mink lung epithelial cells (ten Dijke *et al.*, 1994b; Akiyama *et al.*, 1997; Gobbi *et al.*, 2002), whereas the expression of BMPR-IB has been identified in rat osteoprogenitor-like ROB-C26 osteoblast cells, human gingival fibroblasts, human prostate cancer cells and glioma cell lines (Nishitoh *et al.*, 1996; Ide *et al.*, 1997a; Hillmann *et al.*, 2002). BMPR-II has also been demonstrated in COS-1 cells, glioblastoma cells, Mv1Lu cells, ROB-C26 cells and epithelial cells (Rosenzweig *et al.*, 1995; Nishitoh *et al.*, 1996).

Although the expression of type-I and-II BMPR in non-human pre-osteoblastic C2C12, 2T3, MC3T3-E1 and KS483 cells has previously been reported (Namiki *et al.*, 1997; Wada *et al.*, 1998; van der Horst *et al.*, 2002), less information is available about these receptors in primary human bone-derived cells. Moreover, although different osteoblastic cell lines have been shown to differentially express different BMPR, little is known about the expression pattern and regulation of the BMPR in cells derived from normal human bone.

1.6.8. Diseases in naturally occurring-mutant BMPR

A number of studies of inherited diseases demonstrate that mutations of BMPR can have serious pathological consequences. Mutations of the BMPR-IA gene have been shown to cause familial juvenile polyposis, an autosomal dominant gastrointestinal polyposis syndrome in which patients are at risk of developing gastrointestinal cancers (Howe *et al.*, 2001; Zhou *et al.*, 2001), while a mutation in the BMPR-IB gene in humans causes brachydactyly type A2, an autosomal dominant hand malformation characterised by shortening and lateral deviation of the index fingers and, to a variable degree, shortening and deviation of the first and second toes (Lehmann *et al.*, 2003). The skeletal phenotype of these patients is similar to that of the acromesomelic chondrodysplasias of Grebe, Hunter-Thompson and DuPan disorders caused by mutations in the gene coding for GDF-5 (Lehmann *et al.*, 2003), which binds to BMPR-IB specifically (Nishitoh *et al.*,

1996). It has also been reported that mutations in the BMPR-IB gene in humans can result in severe defects in limb formation, including aplasia of the fibula, brachydactyly, ulnar deviation of the hands, fusion of the carpal/tarsal bones and genital anomalies with hypoplasia of the uterus and ovarian dysfunction (Demirhan *et al.*, 2005). Mutations in the BMPR-II gene can cause primary pulmonary hypertension (Newman *et al.*, 2001), again demonstrating the functional importance of the BMPR *in vivo*.

1.6.9. Functional role of the BMPR

Null mutation of the BMPR-IA gene in mice (homozygous for the BMPR-IA null allele) has been shown to result in embryonic lethality by embryonic day 9.5 (E9.5), although morphological defects are first detected at E7.5 and no mesoderm forms in the mutant embryos, indicating that BMPR-IA is essential for the inductive events that lead to the formation of mesoderm during gastrulation (Mishina *et al.*, 1995). Moreover, Mishima *et al.* (2004) generated BMPR-IA conditional null mice (heterozygous for the BMPR-IA null allele), which were viable. These mutant mice had irregular calcification, low bone mass and severely reduced osteoblast function (Mishina *et al.*, 2004), demonstrating that BMPR-IA is also important for postnatal bone formation. Mice lacking BMPR-IB have been shown to be viable, despite marked reduction in the proliferation and differentiation of prechondrogenic cells in the phalangeal region. In adult BMPR-IB mutant mice, the proximal interphalangeal joint is absent and the phalanges replaced by a single rudimentary element, and the metacarpal and metatarsal bones are also underdeveloped (Yi *et al.*, 2000). Such mice also display appendicular defects which resemble those observed in GDF-5 null mice, and since GDF-5 binds BMPR-IB with high affinity (Nishitoh *et al.*, 1996) and plays a critical role in cartilage and bone formation (Storm and Kingsley, 1999; Baur *et al.*, 2000), these findings suggest the possibility that BMPR-IB has an important role in skeletal formation *in vivo*. In BMPR-IB and BMP-7 double mutant mice, severe appendicular skeletal defects have been observed in the forelimbs and hind limbs, with severely deformed ulna and radius (Yi *et al.*, 2000), again indicating that BMPR-IB is likely to be involved in cartilage and bone formation *in vivo*.

The functional role of the BMPR has also been studied using dominant-negative (dn) and constitutively active (ca) forms of BMPR genes. For example, chondrogenesis of limb mesenchymal cells in chicken has been shown to be markedly inhibited by dnBMPR-IB

and dnBMPR-II, but not by dnBMPR-IA. In addition, the limb bone pattern in the posterior-distal region was disturbed by co-expressing the dnBMPR-IB and dnBMPR-II in the whole limb bud, thus providing evidence that BMPR-IB/-II signals are essential for chondrogenesis as well as limb bone patterning *in vivo* (Kawakami *et al.*, 1996). Furthermore, it has been reported that BMPR-IB expression in the embryonic limb is necessary for the initial steps of *in vivo* chondrogenesis, while during later chondrogenesis BMPR-IA is specifically expressed in prehypertrophic chondrocytes and regulates chondrocyte differentiation (Zou *et al.*, 1997). Volk *et al.* (2000) reported that caBMPR-IB increased Col X mRNA and ALP activity in embryonic chick sternum while caBMPR-IA had much less effect. Moreover, dnBMPR-IB blocked BMP-induced hypertrophic chondrocytes more effectively than dnBMPR-IA (Volk *et al.*, 2000), further demonstrating the importance of BMPR-IB signalling in cartilage formation.

In mesenchymal cells, BMP-2 signalling via BMPR-IA was found to inhibit myogenic differentiation and induce osteoblast differentiation in C2C12 myoblasts (Namiki *et al.*, 1997), while caBMPR-IB-transfected C2C12 cells differentiated into ALP- and OC-positive cells but myogenin- and myogenic creatine kinase-negative cells (Akiyama *et al.*, 1997). Korchynskyi *et al.* (2003) further showed that either of the two caBMPR-I induced elevated levels of Smad1/5/8 phosphorylation and ALP activity in C2C12 cells, further suggesting an important role for both BMPR-IA and -IB in osteoblast differentiation. In preosteoblastic MC3T3-E1 cells, BMP-2-induced osteoblast differentiation has been shown to depend, at least in part, on the expression of BMPR-IA since cells transfected with an inactive truncated BMPR-IA did not respond to exogenously added BMP-2 and did not differentiate into nodule-forming osteoblasts (Suzawa *et al.*, 1999). In addition, BMPR-IA was shown to be responsible for the initiation of osteogenic and chondrogenic differentiation of the mesenchymal precursor cell line C3H10T1/2, since forced expression of dnBMPR-IA but not dnBMPR-IB significantly suppressed BMP-2-induced levels of ALP activity and chondrogenic matrix formation (Kaps *et al.*, 2004). In contrast, overexpression of an inactive truncated BMPR-IB has been shown to ablate BMP-2-induced osteoblast differentiation in 2T3 and C2C12 cells, concomitant with supression of Cbfa1, ALP and OC mRNA expression (Namiki *et al.*, 1997; Chen *et al.*, 1998). BMPR-IB signalling also promotes osteoblast apoptosis through PKC-mediated up-

regulation of caspase activity (Hay *et al.*, 2004), while BMPR-II is involved in BMP-7-induced osteoblast differentiation (Yeh *et al.*, 1998).

In summary, these observations indicate that the BMPR are essential components in the initiation and regulation of osteoblast differentiation, although definitive information about the precise role of each of the BMPR remains incomplete.

1.6.10. Regulation of BMPR

Previous studies have reported that the expression of the BMPR can be controlled by a number of growth factors and hormones. Thus, while TGF-β1 was found to up-regulate BMPR-IA, -IB and -II transcripts during rabbit periosteal chondrogenesis *in vitro* (Sanyal *et al.*, 2002), BMPR-IB mRNA and protein were found to be induced by low doses of FGF-2 when applied to back muscles of adult mice *in vivo* and in muscle-derived primary culture cells *in vitro*, with the levels of BMPR-IA and -II remaining unaffected (Nakamura *et al.*, 2005a). In addition, androgen has been shown to up-regulate BMPR-IB but not BMPR-IA transcripts in prostate cancer cells (Ide *et al.*, 1997a; Ide *et al.*, 1997b), while BMPR-IA gene expression was induced by a PTH-related peptide in C3H10T1/2 cells (Chan *et al.*, 2003) and by growth hormone in developing rat periodontium *in vivo* (Li *et al.*, 2001).

The expression of these BMPR has also been reported to be differentially controlled by their ligand BMP, for example, BMP-2 induction of BMPR-IA and -II but not BMPR-IB transcripts in murine myoblastic cells *in vitro* and skeletal muscle tissue *in vivo* (Akiyama *et al.*, 1997; Nakamura *et al.*, 2003; Nakamura *et al.*, 2005b). Further, a study of rat intervertebral disc cells has demonstrated that BMPR-IB and -II mRNA expression are up-regulated but BMPR-IA mRNA decreased by BMP-2 (Li *et al.*, 2004a), while BMP-2 did not appear to have any significant effect on BMPR-IA transcripts in cultured rat periosteal cells during *in vitro* chondrogenesis (Hanada *et al.*, 2001). However, regulation of the BMPR by the ligand BMP-2 in human bone cells specifically is not yet known. BMP-7 treatment has also been shown to stimulate expression of BMPR-IA and BMPR-II mRNA, but appears to have little effect on BMPR-IB mRNA expression in cultured rat medial collateral ligament cells (Tsai *et al.*, 2003), whereas it significantly up-regulates BMPR-IA and BMPR-II mRNA expression, and down-regulates BMPR-IB mRNA in

fetal rat calvaria cells (Yeh *et al.*, 2000). In marked contrast, the expression of BMPR-IA, -IB and -II transcripts in cultured murine C2C12 cells has been reported to be down-regulated by treatment of the cartilage-derived morphogenetic protein-1, -2, and -3, which are members of the BMP family (Yeh *et al.*, 2005).

Thus, BMPR expression appears to be differentially controlled by a number of soluble mediators in a number of tissues and cells. However, the mechanism(s) which regulate the expression of the BMPR and control the BMP/BMPR signalling pathways in normal human bone are still unclear.

1.7. Statement of problem

The bone remodelling process involves the coordinated activity of osteoclasts, which break down the bone matrix, and osteoblasts, which produce and deposit the bone matrix, calcium, phosphorous and other minerals to form new bone (Manolagas, 2000). The balance between these activities determines the mass and density of bone and bone healing. Many diseases of bone including osteoporosis (a common age-related phenomenon in post-menopausal women in which bone mass is greatly reduced), osteogenesis imperfecta (also known as brittle-bone disease), osteoarthritis and periodontitis (which involves severe AB resorption and subsequent loss of affected teeth), are likely to be caused by the dysregulation of osteoblast and osteoclast activity (Suda *et al.*, 1997; Manolagas, 2000; Mogi *et al.*, 2004). Understanding the molecular mechanisms that underlie osteogenic processes by which new bone is formed is thus of critical importance.

A number of growth factors have been shown to have a fundamental role in osteogenesis by promoting cellular proliferation of undifferentiated mesenchymal cells and inducing differentiation of committed osteoprogenitor cells toward cells of the osteoblastic lineage (Lieberman *et al.*, 2002). However, the BMPs are the only growth factors with morphogenic and osteoinductive properties that, on their own, can induce osteoblastic differentiation of uncommitted mesenchymal cells (Rosen *et al.*, 1996b; van der Horst *et al.*, 2002; Nakashima and Reddi, 2003), and are therefore generally regarded as the most potent osteogenic growth factors for enhancing bone repair and regeneration. Despite the FDA approval of the use of the rhBMP, the use of these factors has nevertheless had only

limited success in several *in vivo* studies (Bowers *et al.*, 1991; Wikesjo *et al.*, 1999; Karrholm *et al.*, 2006). Moreover, clinical trials have indicated that the rhBMP have no greater success than autogenous bone grafting in orthopedic surgery (Boden, 2001). Such findings indicate the need for further research into mechanisms which could enhance the value of the BMP as a therapeutic tool, for example by positive modulation of signalling via the BMPR to increase the responsiveness of target cells to these factors.

The expression of the specific BMPR and their distribution and regulation in bone cells is thus central to understanding how to improve BMP-mediated bone healing and regenerative processes. However, detailed studies on the regulation of BMPR expression in human osteogenic cells are limited, and have hitherto been investigated mainly at the transcriptional level (*i.e.,* modulation of BMPR mRNA). Thus, the mechanisms which control the expression and localisation of the corresponding BMPR proteins, the functional forms of BMPR that interact directly with the BMP at the cell surface, are still unclear. Since the response of cells to the BMP is at least partly dependent on the BMPR, as shown by the lack of BMP effect in the absence of BMPR expression *in vivo* (Hamdy *et al.*, 2003), the regulation of these receptors in primary human bone cells is therefore fundamental to improving bone healing processes and identifying potential new targets for therapeutic intervention.

1.8. Hypothesis of this study

Cell surface receptors are the major components which, together with their respective ligands, determine the signal transduction processes that modulate cell activation and function. The type and level of receptors expressed at the plasma membrane are therefore of utmost importance for determining the responsiveness of the cell to the ligand (Yamashita *et al.*, 1995). The sensitivity of bone cells to the BMP thus depends at least in part on the expression of the BMPR and its functional activity in BMP/BMPR signal transduction.

The hypothesis of the present study is that the regulation of BMPR expression, localisation and function can be controlled by a number of biologically active mediators, including inflammatory cytokines, growth factors and hormones, and is therefore central to the response of primary human bone cells to the BMP and thus BMP activity.

1.9. Aims of this study

The present study was carried out to examine:

(i) the expression and cellular localisation of BMPR-IA, -IB and -II in primary human AB cells;

(ii) the regulation of these BMPR by inflammatory cytokines, growth factors, and hormones; and

(iii) the effects of these factors on the biological functions of the BMPR (*e.g.*, Smad1/5/8 phosphorylation, bone-related gene expression and ALP activity).

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1. Isolation and maintenance of primary human AB cells

2.1.1. Collection of bone samples

Human cortico-lamellar bone fragments were obtained from the maxilla of three patients undergoing extractions of third molar teeth at the Eastman Dental Hospital, from a region of the bone distal to the periodontal ligament and cementum. This ensured that the cells were of bone cell lineage only. The participants signed informed consent to the use of these tissues, in accordance with the protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital. These bone fragments were immediately placed in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Life Technologies Ltd, Paisley, UK) containing 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Somerset, UK) (10% FCS-DMEM) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine and 25 μg/ml amphotericin B (all from Gibco).

2.1.2. Isolation by primary explantation

The three AB cell samples (from three patients) were obtained from the explants using a previously reported technique (Salih *et al.*, 2001; Pradel *et al.*, 2005), with minor modifications. Briefly, the bone tissue was washed extensively with phosphate-buffered saline (PBS) (Gibco) to remove blood and debris, cut into pieces approximately 1-2 mm^3 and placed in 24-well tissue culture plates (Falcon, Becton Dickinson, Cowley, UK) without medium for 15 min at 37°C in a humidified atmosphere of 5% CO_2 in air, to enable the explants to adhere to the plates. Subsequently, 10% FCS-DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine and 25 μg/ml of amphotericin B was added and the explants cultured until the outgrowth of adherent cells reached confluence.

2.1.3. Passage of cells

Once the cultures reached confluence in regions adjacent to the explants (after approximately 21 days), the medium was aspirated and the cells washed twice with PBS. The cells were then treated with trypsin-EDTA (0.25% trypsin in 1 mM EDTA) (Gibco) at 37°C for 5 min, followed by vigorous shaking. Standard culture medium (10%FCS-DMEM supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 2 mM L-glutamine) was then added to neutralise the trypsin, the detached cells collected in a sterile Falcon tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the cell pellet re-suspended in culture medium and re-cultured in 25 cm^2 tissue culture flasks (Nunc, Leicestershire, UK) at a density of $1x10^4$ cells/cm^2. A total number of 20 flasks were obtained from each of the three different AB cell samples. These 'first passage' (P1) cultures were allowed to grow for approximately 3 days until they become confluent, after which the total number of $5x10^6$ cells from 19 flasks (of each AB sample) were frozen in liquid nitrogen, as detailed in Section 2.1.5. The remaining cells were re-cultured at a dilution of 1:4 to expand each sample and the P2 cultures reached confluence after 3-4 days in culture.

2.1.4. Culture of cells

The confluent outgrowth cells were harvested by incubating with trypsin-EDTA, centrifuged and then re-cultured as adherent monolayers as described above (Section 2.1.3). Cells between P3 and P5 were used in the experiments.

2.1.5. Cryopreservation and recovery of cells

An aliquot of cells was cryopreserved at early passages for use in future experiments. After trypsinising the cells, the cell pellet was re-suspended in a 'freezing medium' consisting of full culture medium and 10% dimethyl sulfoxide (DMSO) (Sigma, Dorset, UK). For each of the three AB cell samples, 25 aliquots (containing approximately $2x10^5$ cells per aliquot) were placed into cryotubes (Nunc) and a slow freezing procedure was carried out by storing the cryotubes in a pre-freezing container (Marathon, London, UK) at -70°C for 24 h, before transferring to liquid nitrogen for long-term storage.

To recover the cells, the cryotubes were placed in a water bath at 37°C and the thawed cell suspension rapidly added to pre-warmed culture medium in a sterile Falcon tube and

centrifuged at 1000 rpm for 5 min. The supernatant was aspirated and the cell pellet re-suspended in a tissue culture flask and cultured as described.

2.1.6. Cell viability

The cultured cells were examined daily by phase-contrast light microscopy to assess cell growth and any possible microbial contamination. The viability of the cells was determined at the time of each passage by trypan blue exclusion. Briefly, the cell pellet obtained after trypsinisation was re-suspended in PBS and 10 µl mixed with an equal volume of 1% trypan blue (Sigma) for 5 min. The number of cells in 10 µl of this mixture was counted under the phase-contrast light microscope, using a haemocytometer (Bright-Line™ Hemacytometer; Sigma). Viable cells appeared bright and colourless, while dead cells were dark blue.

2.2. Immunocytochemistry (ICC)

2.2.1. Principle of ICC

ICC enables the identification and localisation of specific antigens within a cell sample using specific antibodies and an enzyme-based detection system whose substrates produce an insoluble coloured reaction product. This technique was used to examine the presence and cellular distribution of the three BMPR proteins in the AB cells.

2.2.2. ICC protocol for the BMPR proteins

Immunocytochemical analysis of the BMPR proteins was carried out using the Avidin-Biotin Complex method. Briefly, exponentially growing monolayers of cells were obtained by seeding the cells on sterile plastic coverslips (Thermanox®, NUNC™; Naperville, IL, USA) at a density of $5x10^3$ cells/cm^2 and incubating for 48 h. After washing three times with PBS, they were fixed with 1% paraformaldehyde (Merck, Poole, UK) in PBS for 30 min, followed by incubating with 0.1% saponin (Sigma) in PBS, for 30 min at room temperature (RT), to permeabilise the cells in order to allow antibody penetration for the detection of intracellular as well as cell surface proteins. The cells were treated with 20% normal rabbit serum when using a secondary rabbit anti-goat immunoglobulin G (IgG) antibody, or normal goat serum (NGS) when using a secondary

goat anti-mouse IgG antibody (Sigma) in PBS containing 0.1% saponin for 30 min to block non-specific binding of antibody. They were then washed three times with PBS and incubated for 1 h at RT with primary goat polyclonal anti-BMPR-IA antibody or primary mouse monoclonal antibodies (mAbs) against human BMPR-IB and -II (R&D systems, Abingdon, UK), diluted 1:100 in PBS containing 2% NGS and 0.1% saponin. NGS or non-specific mouse IgG isotypes IgG2a and IgG2b (DAKO, High Wycombe, UK) were used instead of the primary antibodies for BMPR-IA, -IB and -II, respectively, as the negative controls. They were then incubated with biotinylated rabbit anti-goat IgG secondary antibody (DAKO) or biotinylated goat anti-mouse IgG secondary antibody (Sigma), diluted 1:500 in PBS containing 2% NGS and 0.1% saponin, for 1 h at RT. Streptavidin-horseradish peroxidase (HRP) (Sigma) was then added (1:200) for 30 min at RT and the brown coloured insoluble enzyme reaction product, corresponding to the presence of the protein, was developed using 3,3'-diaminobenzidine tetrachloride (DAB) (Sigma) for 10 min. Counter-staining was carried out using Mayer's hematoxylin (Merck) for 1 min. After washing twice with tap water, the slides were dehydrated in ascending concentrations of alcohol (70, 90 and 100%) for 1 min each, then xylene for 5 min, air-dried at RT and mounted in DePex (Merck). The stained cells were examined under light microscope Olympus BX 50 (Olympus UK, Middlesex, UK) and photographed with Nikon Coolpix 4500 digital camera (Best Scientific, Wiltshire, UK).

2.3. Flow cytometry (FCM)

2.3.1. Principles of FCM

FCM is a technique for measuring certain physical and chemical features of individual cells in a cell suspension as it passes through a fixed laser beam and is based on the angular reflection of an incident laser light (Carter and Ormerod, 2000; Shapiro, 2003). Light reflected by the cells at low angle (< 2°) is detected in the forward direction along the axis of the incident light and this "forward scatter" (FSC) is considered to be proportional to the relative size of the cell (Carter and Ormerod, 2000). Light reflected at more than 2° is detected at 90° or more to the light axis and is referred to as orthogonal or "side scatter" (SSC), which is considered to be proportional to the cytoplasmic granularity or internal complexity of the cell (Carter and Ormerod, 2000) (Figure 2.1).

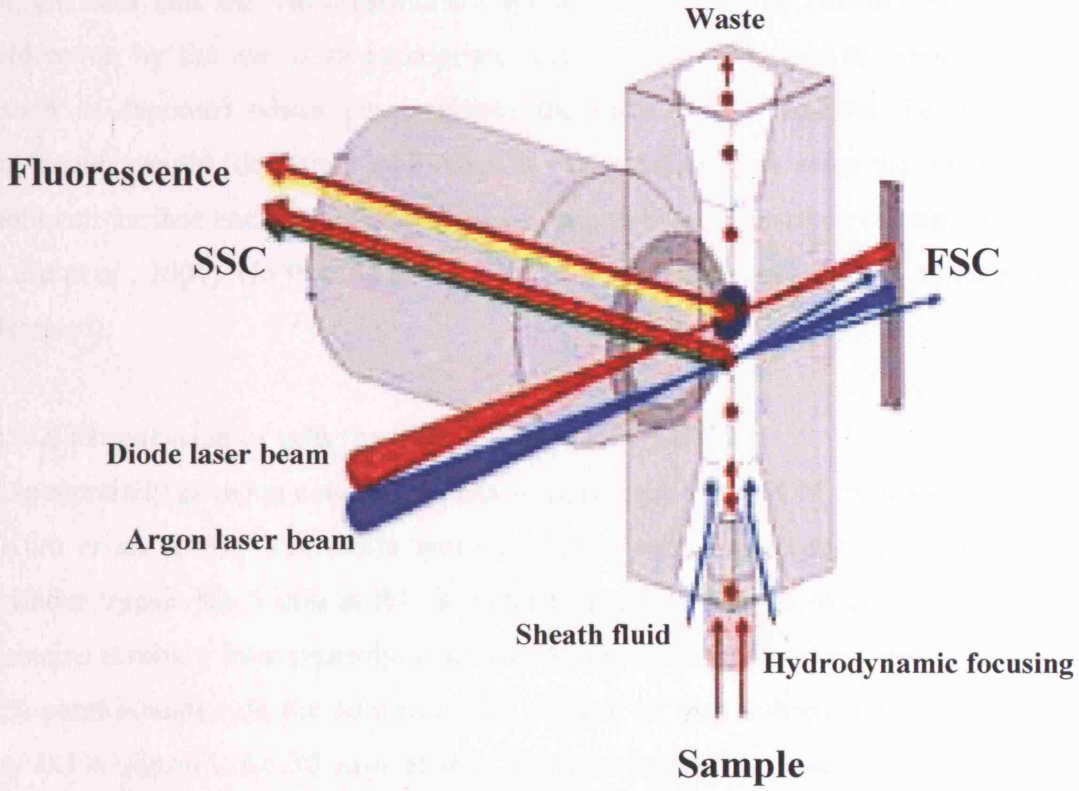


Figure 2.1. Schematic illustration of FCM. A Flow cytometer uses the principle of hydrodynamic focusing for presenting particles to a laser (or any other light source). The sample is injected into the centre of a sheath flow and each single particle in the suspension flows through a cuvette in a narrow stream. As each passes the laser beam, the forward scatter, side scatter and fluorescent emission signals generated by the individual particles are generated, detected and then displayed for analysis.
(Adapted from http://www.partec.de/partec/flowcytometry.html)

Using this procedure, the relative levels of cell-associated proteins can also be measured by FCM, by first treating the cells with a fluorochrome 'tag' or fluorescent-labelled antibody which is specific for the target protein (Carter and Ormerod, 2000). When laser light is applied to the cell, the fluorochrome absorbs energy from the laser and subsequently releases this energy by emitting fluorescence at a particular wavelength. The intensity of this emitted fluorescence is proportional to the amount of fluorochrome-labelled antibody, which is in turn proportional to the level of protein. A FACScan flow cytometer (Becton Dickinson) employing an argon-ion laser for excitation was used for

FCM in this study. The electronic system of the instrument was optimised for collection of the data and the same instrument settings were used to perform all experiments. Moreover, by the use of an appropriate concentration of a specific non-ionic detergent (such as saponin) which permeabilises the cells and so enables the entry of high molecular weight 'detecting' antibodies, it was possible to measure the relative levels of both cell surface and total cellular protein, as previously described (Sumner *et al.*, 1991; Kuru *et al.*, 2001). (In the absence of detergent treatment, only the cell surface protein is detected).

2.3.2. Preparation of cells for BMPR analysis by FCM

Exponentially growing cultures of cells were prepared for FCM as described previously (Kuru *et al.*, 2001). The cells were detached using a scraper and 20 mM EDTA without trypsin for 5 min at RT, in order to prevent the loss of cell surface-associated proteins resulting from proteolytic activity of this enzyme. The cells were then fixed with 1% paraformaldehyde for 30 min at RT, without or with subsequently permeabilising by 0.1% saponin for 30 min at RT. The permeabilising procedure thus allows both surface and intracellular (*i.e.*, the total cellular) protein to be measured, as noted above. Cells were washed with PBS containing 2% FCS (FCS-PBS) followed by centrifugation at 1000 rpm for 5 min after each step described below. The cells were treated with 10% normal donkey serum when using a secondary donkey anti-goat IgG antibody, or with NGS when using a secondary goat anti-mouse IgG antibody (Sigma), without or with 0.1% saponin, for 30 min to block non-specific binding of antibody. Following centrifugation, $1x10^5$ cells were reacted, for 60 min at RT, with goat polyclonal antibody against human BMPR-IA (1:20) and mouse mAbs against human BMPR-IB and -II (1:25), diluted in FCS-PBS, without or with 0.1% saponin, for measuring the cell surface or total protein level, respectively. NGS or non-specific mouse IgG isotypes IgG2a and IgG2b were used instead of the primary antibodies for BMPR-IA, -IB and -II, respectively, as negative controls. The specific reactivity of the antibodies with their corresponding proteins had been tested and confirmed by the supplier. Fluorescein isothiocyanate (FITC)-labelled secondary donkey anti-goat IgG (Serotec, Oxford, UK) or goat anti-mouse IgG (DAKO) antibodies diluted 1:20 in FCS-PBS, without or with 0.1% saponin, were then added for 60 min at RT (in some experiments, Alexa Fluor® 488-labelled secondary antibody (Molecular Probes, Paisley, UK) diluted 1:200 in FCS-PBS,

60 min at RT, was used instead of FITC-labelled secondary antibodies in order to enhance the fluorescent signals). After centrifugation, the cells were re-suspended in 400 µl of FCS-PBS and the fluorescence intensity of $1x10^4$ individual cells was measured using a flow cytometer (FACScan; Becton-Dickinson, Cowley, UK).

Cells were introduced through an injection port in the FACScan at a rate of 60 µl per min. As the cells passed through the flow chamber, each was subjected to the incident laser beam and subsequently the emitted light corresponding to the FSC, SSC and fluorescence intensity were collected as electronic signals and transmitted to the attached computer. Analysis of the stored data was performed using the CELLQuest Software programme (Becton Dickinson).

The values are presented as arbitrary units of fluorescence intensity, which depend on the electronic input and output settings of the cytometer, which were kept constant in all the experiments, as noted above. The fluorescence values used were the geometric means of the cell population and are shown as the average fluorescence intensity (AFI) of 10000 individual cells. For each antibody, the arbitrary AFI value obtained is proportional to the level of expression of the specific protein. Thus, differences in AFI between samples after reaction with a specific antibody reflect differences in the relative levels of expression of that particular protein by each of the different samples. However, AFI values obtained using different antibodies cannot be compared with each other as each antibody has a different reactivity with its respective protein. Surface and total cellular protein levels were obtained from the AFI of the non-permeabilised and permeabilised samples, respectively, and the level of intracellular protein calculated from the difference between these two AFI values.

2.4. Immunogold electron microscopy (EM)

2.4.1. Principle of immunogold EM

Immunogold EM is a technique which utilises a transmission EM (TEM) to elucidate the precise ultrastructural location and relative semi-quantification of antigens in cell or tissue samples. This is achieved by a process known as immunogold labelling, in which

careful sample preparation is essential for preserving both the integrity of the sample and antigenicity of the protein. Subsequently, sections are incubated with specific antibodies against the protein of interest. Since these detecting antibodies are not normally visible in the TEM, the presence of the corresponding protein is made electron-dense and thus EM-visible by incubating with a second antibody conjugated to a small gold particle, which is electron dense. The sections are then stained with uranyl acetate and lead citrate to enhance the contrast between intracellular structures of differing electron density. Despite the labour-intensive effort required for this work, immunogold EM has a significant number of advantages over light microscopic procedures for investigating specific subcellular structures and the precise localisation of proteins of interest.

2.4.2. Immunogold EM protocol for BMPR-IB protein

For immunogold localisation of BMPR-IB, the cells were detached by incubating in 20 mM EDTA at RT for 5 min, after which they were gently scraped from the culture flasks. Cell pellets were then prepared, fixed with 0.5% glutaraldehyde at 4°C for 30 min, dehydrated in cold ethanol and embedded at -20°C in LR White resin (Agar Scientific, Essex, UK). Ultra-thin sections (approximately 0.1 μm thick) collected on specimen grids (Agar Scientific) were rehydrated with distilled water and then treated with 10% NGS in 20 mM Tris buffer, pH 7.4, for 30 min at RT to block non-specific binding, followed by three washes with 20 mM Tris buffer (wash buffer). The sections were subsequently incubated with mouse monoclonal anti-BMPR-IB antibody (diluted 1:20 in 2% NGS in wash buffer) for 1 h at RT. Non-specific mouse IgG isotype IgG2a was used instead of the primary antibody for BMPR-IB, as the negative control. After three rinses with wash buffer, the sections were incubated with 10 nm gold-conjugated secondary goat antibody against mouse IgG (Agar Scientific), diluted 1:100 in 2% NGS in wash buffer for 1 h at RT. The sections were then stained with uranyl acetate (1 min) and lead citrate (1 min) and the presence of BMPR-IB, indicated by small, round black gold particles, was visualised and photographed in a TEM (JEOL 100 CX II; Jeol Ltd, Herts, UK).

To identify specific cellular compartments, double-labelling immunogold EM was carried out. The early endosomal antigen 1 (EEA-1), a marker of early endosomes (Zhang *et al.*, 2004), or the lysosomal membrane glycoprotein 1 (LAMP-1), a marker of lysosomes (Zhang *et al.*, 2004), were stained simultaneously with the BMPR-IB antibody using

rabbit polyclonal anti-EEA-1 (Abcam, Cambridge, UK) diluted 1:20 in 2% NGS in wash buffer, and rabbit polyclonal anti-LAMP-1 (Insight Biotechnology, Middlesex, UK) diluted 1:10 in 2% NGS in wash buffer, following the routine procedure described above. Non-specific rabbit IgG was used instead of the primary antibody for EEA-1 and LAMP-1, as the negative controls. Secondary goat anti-rabbit IgG antibody conjugated with 15 nm gold (Agar Scientific) was used to visualise the EEA-1 and LAMP-1 proteins, specifically.

2.5. Cell-enzyme-linked immunosorbent assay (C-ELISA)

2.5.1. Principle of C-ELISA

The C-ELISA is based on the well-established ELISA principle for detection of cell surface and/or cytoplasmic proteins on or in intact cells (Stocker and Heusser, 1979). In a C-ELISA, monolayers of cells adhering to the bottom of wells of a 96-well polystyrene plate comprise the solid phase of a conventional ELISA, in which an protein is absorbed to the bottom of each well. The cell monolayers are then reacted with a primary antibody and an enzyme-conjugated secondary antibody reacted. An enzyme substrate is then added, which gives rise to a soluble coloured product, indicating the presence of the protein of interest. The absorbance (A_{nm}) of the coloured product is measured using a spectrophotometer, and corresponds to the relative level of the protein.

2.5.2. C-ELISA protocol for BMPR proteins

The AB cells were seeded into flat-bottom 96-well plates at a density of $1x10^4$ cells/cm^2 and cultured for 48 h, after which they were subjected to the treatment described in each experiment. After washing with 20 mM Tris buffer with 0.1% bovine serum albumin (BSA), pH 7.6 (wash buffer), the cells were then fixed with 4% paraformaldehyde for 30 min at RT, with subsequently permeabilising by 0.1% saponin for 30 min at RT. The cells were treated with 10% normal donkey serum when using a secondary donkey anti-goat IgG antibody, or with NGS when using a secondary goat anti-mouse IgG antibody, in PBS with 0.1% saponin for 30 min, to block non-specific binding of antibody. The wells were washed and then incubated with primary goat anti-human BMPR-IA antibody or mouse mAbs against BMPR-IB and -II diluted 1:100 in FCS-PBS

for 3 h at RT. NGS or non-specific mouse IgG isotypes IgG2a and IgG2b were used instead of the primary antibodies for BMPR-IA, -IB and -II, respectively, as the negative controls. After washing with wash buffer, the wells were then incubated with the appropriate HRP-conjugated secondary antibodies diluted 1:500 in FCS-PBS for 1 h at RT, extensively washed and then the substrate 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) (100 µl) added for 30 min at RT. The reaction was stopped by the addition of 0.5 M H_2SO_4 (50 µl) and the A_{450nm} measured using an ELISA plate reader (Titertek Multiskan® Plus MKII; Labsystems, Helsinki, Finland). To determine the relative level of surface-associated protein, saponin was omitted from all the reagents used. The values obtained using the permeabilised and non-permeabilised samples are thus equivalent to the total cellular and surface-associated protein, respectively, and the differences between these two values were used to calculate the level of intracellular protein.

2.6. Protein quantification

2.6.1. Principle of Bio-Rad *DC* protein assay

The Bio-Rad *DC* protein assay is a colorimetric assay, based on the binding of copper ions to proteins under alkaline conditions. The reaction is similar to the well-documented Lowry assay, but reaches 90% of its maximum colour development within 15 min thereby saving time, and the colour intensity is more than 95% stable for 1 h and 90% for 2 h after the addition of reagents. In this assay, the protein reacts with an alkaline copper tartrate solution to form a copper-protein complex, which then reduces the Folin reagent to generate a characteristic blue coloured product with a maximum absorbance at 750 nm.

2.6.2. Protocol of Bio-Rad *DC* protein assay

The assay was performed using the Bio-Rad *DC* protein assay kit (Bio-Rad, Hemel Hempstead, UK), in accordance with the manufacturer's instructions. Briefly, working reagent A was first prepared by adding 20 µl of reagent S to each 1 ml of reagent A that will be needed for all the samples. BSA was used as a protein standard and was prepared at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml in the same buffer as the sample to obtain a standard curve, which was prepared each time the assay was performed. After adding 5 µl of the standards and of the samples to a 96-well plate, 25 µl of working reagent A was added

into each well. Two hundred microlitres of reagent B (Folin reagent) was then added into each well followed by gently agitating the plate to mix the reagents. The samples were incubated for 15 min at RT and the absorbance at 750 nm measured using an ELISA plate reader (Titertek Multiskan® Plus MKII). The standard and sample protein solutions were assayed in triplicate.

2.7. Western blotting (WB)

2.7.1. Principle of WB

WB is an analytical method to determine the presence of a specific protein in a sample containing many other proteins. It is based on the separation of individual proteins on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and then detection of the specific protein using a specific antibody. In WB, proteins are first transferred electrophoretically from the SDS-PAGE gel to a membrane, *e.g.*, nitrocellulose or polyvinylidene difluoride (PVDF) membrane, which binds proteins very strongly. The membrane is then saturated (blocked) with non-antigenic (non-reacting) protein to prevent non-specific immunoglobulin binding to the membrane, after which it is treated with the primary antibody or an enzyme-conjugated primary antibody raised against the target protein. When a non-conjugated primary antibody is used, an enzyme-conjugated secondary antibody that binds to the primary antibody is then added, followed by a substrate which forms an insoluble product and produces a visible band on the membrane. A diagram summarising the principle of WB is shown in Figure 2.2.

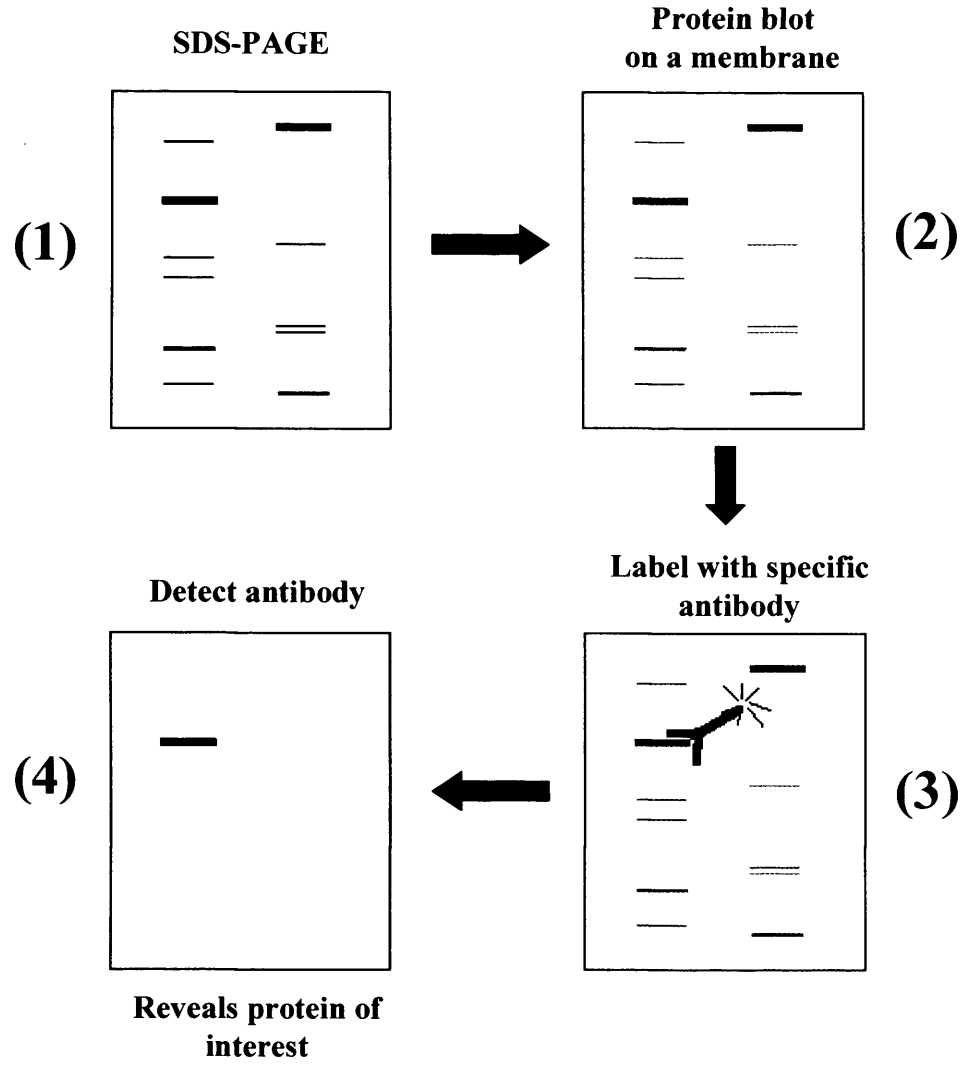


Figure 2.2. A diagram depicting the principle of WB. Proteins are separated by gel electrophoresis, usually SDS-PAGE (1). The proteins are transferred from the SDS-PAGE gel to a membrane (2). Non-specific binding of antibodies is blocked with non-antigenic proteins, such as BSA or non-fat (skimmed) milk. An antibody is then added which is able to bind to its specific protein (3). The antibody is conjugated to an enzyme (*e.g.*, HRP) which can be revealed by incubating it with a colourless substrate, *e.g.*, DAB or Lumigen PS-3 Acridan, that forms an insoluble coloured or x-ray-emitting product, respectively (4).

(Adapted from http://www.bio.davidson.edu/courses/genomics/method/westernblot.html)

2.7.2. Preparation of protein sample and WB protocol

2.7.2.1. Protein extraction

Exponentially growing cultures were washed with PBS and lysed for 20 min in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and freshly prepared protease inhibitors phenyl-methyl-sulfonyl fluoride (PMSF) 1 mM, aprotinin, leupeptin and pepstatin (1 μg/ml each) (all from Sigma). Lysates were cleared by centrifugation at 6000 rpm at 4°C for 10 min. Protein concentration was measured using the Bio-Rad *DC* protein assay kit and 150 μg of protein used for SDS-PAGE (except for the measurement of α-tubulin in which 50 μg of protein was used), as described below.

2.7.2.2. SDS-PAGE

Twenty microlitres of the cell lysates (50-150 μg of protein) for each sample was re-suspended in 5 μl of 5x reducing WB sample buffer (0.3 M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol and 100 mM dithiothreitol (DTT)), incubated at 90°C for 5 min, subjected to SDS-PAGE on 12% acrylamide gels (except for the detection of soluble forms of BMPR in which 20% acrylamide gels were used) at 100 V and 30 mA for 2 h in running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine and 0.1% SDS) and then transferred to PVDF membranes (Immobilon-P Transfermembrane, Sigma) at 100 V and 200 mA for 45 min in blotting buffer (12 mM Tris, 192 mM glycine and 20% methanol).

2.7.2.3. Immunoblotting

After transfer of the proteins to the PVDF membranes, they were placed in 100% methanol for 10 s to remove water and then blocked with 5% BSA in PBS (blocking buffer) for 1 h at RT. Immunoblotting was carried out by incubating the membranes overnight at 4°C with the following primary antibodies diluted 1:1000 in blocking buffer: biotinylated BMPR-IA, -IB and -II (R&D Systems); Smad1/5/8 (Upstate Biotechnology, Milton Keynes, UK); phospho (p)-Smad1/5/8 (New England Biolabs, Hitchin, UK) and α-tubulin (Insight Biotechnology, Wembley, UK). The membranes were washed three times with 1% Tween-20 in PBS and strepavidin-HRP or the appropriate HRP-conjugated secondary antibodies, diluted 1:2500 in blocking buffer, were added for 1 h at RT. After three washes with 1% Tween-20 in PBS, the immunoreactive bands were visualised using

the enhanced chemiluminescence ECL Plus system (Amersham Biosciences, Little Chalfont, UK), with Lumigen PS-3 Acridan as substrate, in accordance with the manufacturer's instructions.

The ECL Plus treated-membrane was exposed to an X-ray film which was then developed and the immunoreactive bands photographed. The intensity of the band was measured using the Scion Image program (Scion Corporation, Frederick, MD, USA).

2.8. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

2.8.1. Principle of RT-PCR

There are a number of procedures for studying specific mRNA transcripts in a sample of total cellular RNA, including Northern blot analysis, RT-PCR and *in situ* hybridization. However, RT-PCR is a sensitive technique for mRNA detection and measurement in small samples, being able to determine mRNA levels semi-quantitatively in samples as little as a single cell (Liu *et al.*, 2004).

The initial step in RT-PCR is the production of one single-stranded complementary DNA (cDNA) copy of each mRNA present in the total RNA extracted from the sample of interest, using a retroviral reverse transcriptase such as that from the Moloney murine leukemia virus (M-MuLV). This enzyme uses each single-stranded mRNA as a template to generate a cDNA strand, based on the pairing of RNA base pairs (A, U, G, C) to their DNA complements (dT, dA, dC, dG). The reverse transcription reaction uses oligo dT primers which hybridize with the poly-A tail of each mRNA, after which the enzyme, in concert with a mixture of the deoxyribonucleotide triphosphate (dNTP), extends each primer strand, resulting eventually in a cDNA strand. The number of each cDNA strand corresponds to the number of mRNA transcripts present in the total RNA sample. It is then followed by exponential amplification of a specific cDNA of interest by PCR, as follows.

The PCR reaction contains the necessary components for DNA amplification, *i.e.,* a sample containing the cDNA sequences, an excess of the four deoxynucleotides which are utilised to produce more cDNA, excess amounts of primers that recognise specific regions of the particular gene of interest, and a DNA polymerase. The polymerase widely used is the Taq polymerase, named after *Thermus aquaticus*, from which it was isolated and is invaluable because of its thermostable property at relatively high temperature (>90°C) (Saiki *et al.*, 1988).

The three stages of PCR are carried out at different temperatures. The first stage of the process involves heating at 90-95°C in order to ensure that all of the cDNA is in a single-stranded form (and not bound to the complementary 'parent' mRNA strand or to its complementary cDNA strand after the first cycle of the amplification). Secondly, the reaction is then cooled to 50-65°C at which the specific primers (*i.e.,* forward and reverse primers) bind or 'anneal' to each of the single cDNA strands. The final step of the reaction is to make a complete copy of each cDNA strand. Since the Taq polymerase works best at around 70-80°C (Chien *et al.*, 1976), the temperature of the reaction is raised to this high temperature, at which the complementary cDNA strands do not re-anneal, and the enzyme begins adding nucleotides to the primer, eventually making a complementary copy of the original cDNA strand. This completes one PCR cycle, resulting in the generation of a double-stranded DNA corresponding to the mRNA of interest.

Beginning again with heating at 90-95°C to separate the two cDNA strands, the protocol is subsequently repeated, with each newly synthesised cDNA sequence acting as a new template at each cycle. Theoretically, the amplification for a given number of cycles can be calculated using the formula: $Y = A \times 2^n$, where Y = total number of copies after n cycles; A = initial number of copies; n = number of cycles. The amount of cDNA formed after the reverse transcription and PCR is thus proportional to the initial number of mRNA transcripts present in the total RNA extracted from the sample of interest.

The initial cycles of PCR are characterised by an exponential increase in target amplification in the presence of an excess of reactants and when the enzyme is fully active. However, when these conditions no longer exist, the product stops accumulating

exponentially until a plateau is reached and there is little or no net increase in the PCR product. Thus, for semi-quantitation, the PCR reaction must be terminated when the product from a particular gene is both detectable and also being amplified within an exponential/linear range of PCR amplification. Since it is possible to ensure that these conditions are met, RT-PCR has become the standard, rapid, inexpensive, simple and sensitive method of choice for detecting and measuring relative gene expression. In this study, RT-PCR was therefore used to assess the activity of the target genes. However, as with using different antibodies in FCM, while it is possible to make semi-quantitative comparisons of the same gene between different samples, it is less accurate to compare different gene sequences.

2.8.2. RT-PCR protocol

2.8.2.1. RNA extraction

Total RNA was extracted from the cells using RNeasy® Mini Kit (Qiagen, West Sussex, UK), in accordance with the manufacturer's instructions. Briefly, cells were trypsinised, centrifuged and the pellet disrupted by addition of 350 µl of Buffer RLT in a 15 ml Falcon tube. The sample was then homogenised by pipetting and vortexing for 1 min. Then, 350 µl of 70% ethanol was added to the homogenised lysate, after which 700 µl of the sample, including any precipitate that may have formed, were transferred to an RNeasy mini-column placed in a 2 ml collection tube and centrifuged for 15 s at 10000 rpm. The sample was then washed with 700 µl of Buffer RW1 and the column was centrifuged for 15 s at 10000 rpm. The sample was washed twice with 500 µl of Buffer RPE and the column centrifuged for 2 min at 10000 rpm. Following centrifugation, the column was transferred to a new 2 ml collection tube and centrifuged for another 1 min. To elute the RNA sample, the column was then transferred to a new 1.5 ml collection tube, 25 µl of RNase-free water was added directly and the column centrifuged for 1 min at 10000 rpm. The elution step was performed twice and the total volume of 50 µl of the RNA sample stored at -70°C until further analyses.

2.8.2.2. Quantification of RNA

After thawing the RNA sample, 5 µl of the RNA sample was diluted with 95 µl of RNase-free water (1/20 dilution) in a cuvette and the concentration of RNA determined

by measuring the A_{260} using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK). RNase-free water was used as the blank. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per 1 ml and the concentration of RNA sample was therefore calculated (in µg/ml) from the following formula:

$$\text{Concentration of RNA sample} = 40 \times A_{260} \times \text{dilution factor}$$

2.8.2.3. Reverse transcription

The reverse transcription reaction was carried out in a thermocycler (GeneAmp PCR System 2400, Applied Biosystems, Cheshire, UK), and 1 µg of total RNA was used with 5 ng of oligo-dT (Promega, Madison, WI) in 40 µl of RNase-free water. After 5 min at 65°C, the first strand of cDNA was synthesised in a total volume of 50 µl, containing 50 U of M-MuLV reverse transcriptase, 1x M-MuLV buffer, 40 µM of each dNTP and 40 U of RNase block (all Stratagene, La Jolla, CA). After incubation at 37°C for 60 min, the mRNA/cDNA strands were separated, detached single mRNA strands were degraded and the enzyme was inactivated by incubation for 5 min at 90°C. The cDNA were stored at -20 °C until required.

2.8.2.4. PCR

The PCR primer sequences, the amount of cDNA and the number of cycles used for each gene examined in this study are shown in Table 3. A required volume of each cDNA sample was added to a 25 µl reaction mixture containing 2 U REDTaq DNA polymerase, 1x REDTaq PCR buffer, 50 µM of each dNTP (all from Sigma) and 2 µM of the respective forward and reverse primer pair sequences (Sigma-Genosys, Pampisford, UK).

The PCR reaction was carried out in a thermocycler (GeneAmp PCR System 2400). An initial denaturation step of 2 min at 94°C was followed by the amplification cycles (as indicated in Table 3) and final extension for 7 min at 72°C to ensure the complete generation of a double-stranded DNA. The optimal number of PCR cycles within the linear range of PCR amplification was determined for each gene. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified to assess the quality of the extracted RNA and as a baseline for calculating the relative level of each gene. The PCR products were separated by agarose gel electrophoresis on a 2% agarose gel, consisting of 2% agarose in 1x TAE buffer (40 mM Tris base, 40 mM acetic acid and

1 mM EDTA, pH 8.0), with 0.5 μg/ml of ethidium bromide (all from Sigma), which is a fluorescent dye that intercalates between bases of nucleic acids and allows visualisation of DNA fragments in the gel under UV light. The eletrophoresis was carried out using 1x TAE buffer with the voltage applied at 5 volts per one centimetre of the distance between the two electrodes of an electrophoresis tank.

To obtain a semi-quantitative estimate of the levels of mRNA of interest, the intensities of the bands corresponding to the genes of interest and to the GAPDH gene product were measured using the Scion Image program and the ratio of the band intensity of each target gene to that of GAPDH gene calculated.

Table 3. Detailed primers and conditions used for PCR

Genes	Sequence (5'-3'): Forward (F); Reverse (R); [amplification cycle condition]	cDNA (μl)	Cycles
BMPR-IA (474 bp)	F: GGGTGGGCACCAAACGCTAC; R: CCACTCTAATTCCACCCATGCC; [94°C 30 s, 61°C 30 s, 72°C 30 s]	5	36
BMPR-IA (510 bp)*	F: ACATCAGATTATTGGGAGCC; R: TGTAACAAAAGCAGCTGGAG; [94°C 1 min, 55°C 1 min, 72°C 1 min]	5	34
BMPR-IB (512 bp)	F: ACTTGCTGTATTGCTGACCTGG; R: GGCTTTCTGCAGAGATGCTTAC; [94°C 30 s, 61°C 30 s, 72°C 30 s]	10	40
BMPR-IB (456 bp)*	F: ACTCCCATTCCTCATCAAAG; R: GGTGAAGAACACTTTCACAG; [94°C 1 min, 55°C 1 min, 72°C 1 min]	5	36
BMPR-II (572 bp)	F: TTTACTGAGACGAGAGCAACAAG; R: ACATCTTCTGCATGTTTAAATGATG; [94°C 30 s, 61°C 30 s, 72°C 30 s]	10	36
BMPR-II (471 bp)*	F: ACCGTTTCTGCTGTTGTAGCAC; R: CTGCAGTGACTCTCTCATCTCC; [94°C 30 s, 61°C 30 s, 72°C 30 s]	5	34
GAPDH (600 bp)	F: CCACCCATGGCAAATTCCCATGGCA; R: CTGGACGGCAGGTCAGGTCCACC; [94°C 30 s, 55°C 2 min and 72°C 2 min]	5	22
ALP (357 bp)	F: CCCAAAGGCTTCTTCTTG; R: CTGGTAGTTGTTGTGAGC; [94°C 1 min, 58°C 1 min, 72°C 1 min]	10	38
Col I (461 bp)	F: GGACACAATGGATTGCAAGG; R: TAACCACTGCTCCACTCTGG; [94°C 1 min, 58°C 1 min, 72°C 1 min]	2	20
OP (532 bp)	F: CACCTGTGCCATACCAGTTAAAC; R: GGTGATGTCCTCGTCTGTAGCATC; [94°C 30 s, 55°C 1 min, 72°C 1 min]	5	30
BSP (627 bp)	F: TGCTCAGCATTTTGGGAAT; R: TGCATTGGCTCCAGTGACACT; [94°C 1 min, 58°C 1 min, 72°C 1 min]	5	30
OC (297 bp)	F: ATGAGAGCCCTCACACTCCTC; R: GCCGTAGAAGCGCCGATAGGC; [94°C 1 min, 58°C 1 min, 72°C 1 min]	5	30
BMP-2 (171bp)	F: GGAATGACTGGATTGTGGCT; R: TGAGTTCTGTCGGGACACAG; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	26

Table 3. Detailed primers and conditions used for PCR (continued)

Genes	Sequence (5'-3'): Forward (F); Reverse (R); [amplification cycle condition]	cDNA (µl)	Cycles
BMP-4 (106 bp)	F: TCAGGCAGTCCTTGAGGATA; R: AAGCAGTCTGTGTAGTGTGTGG; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	26
BMP-7 (160 bp)	F: GTGGCAGCATCCAATGAAC; R: CTGGTAGGCGCTCATAATTACC; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	26
OPG (135 bp)	F: CTGCTTATAACTGGAAATGGCC; R: CTGTGGCAAAATTAGTCACTGG; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	20
RANKL (288 bp)	F: AGTGTCTAGAGAGGAGGGCTTTGA; R: CCGCACTGTGACTAGAACTTCAGA; [94°C 1 min, 57°C 1 min, 72°C 1 min]	5	38
Cbfa1 (443 bp)	F: CAGTTCCCAAGCATTTCATCC; R: TCAATATGGTCGCCAAACAG; [94°C 1 min, 58°C 1 min, 72°C 1 min]	2	28
Dlx5 (162 bp)	F: ACATTCCGCTTTTCATGG; R: CGCAACTGTGGACACTTTC; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	30
Msx2 (185 bp)	F: ATGGATGCTTGTTTCAAAGGG; R: TACACCAAGAAAAGCAGGGC; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	30
Smad6 (204 bp)	F: AAAGACGCACTTTGGCTTA; R: CGAATACTTTATTATCGAGTGACTG; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	26
Smurf1 (203 bp)	F: TCGTGAGTTTATTGCATATGTAACA; R: CTTCCCACTGTTTTTATCACTGA; [94°C 1 min, 57°C 1 min, 72°C 1 min]	2	22
Noggin (259 bp)	F: GGAGGAAGTTACAGATGTGGCTGT; R: CACTCGGAAATGATGGGGTACTG; [94°C 1 min, 60°C 1 min, 72°C 1 min]	5	30

* primers containing sequences that amplify the mRNA sequences corresponding to the transmembrane domain and a part of the extracellular domain of the target protein.

Primer sources: BMPR-IA, -IB, -II and -II* (Gobbi *et al.*, 2002); BMPR-IA* and -IB* (Takeda *et al.*, 2003); GAPDH (Das and Olsen, 2001); ALP, Col I and OC (Lomri *et al.*, 1999); OP (Mouri *et al.*, 2003); BSP and Cbfa1 (isoforms I, II and III) (Nakayama *et al.*, 2004); noggin (Diefenderfer *et al.*, 2003); the other genes (UniSTS, National Center for Biotechnology Information)

2.9. Quantitative real-time PCR (Q-PCR)

2.9.1 Principle of Q-PCR

PCR amplification comprises three phases, *i.e.,* exponential, linear and plateau phases, as shown in Figure 2.3. In early cycles of the PCR reaction with all of the reagents being freshly active and available, the kinetics of the reaction enable the amplification to occur 'exponentially', where doubling of the PCR product occurs very precisely every cycle. However, as the reaction progresses and some of the PCR reagents are being used, the reaction starts to slow down and the PCR product is no longer doubled at each cycle, resulting in a 'linear' amplification, which is variable. Eventually the reaction reaches the 'plateau' of amplification, where there are no more new PCR products synthesised.

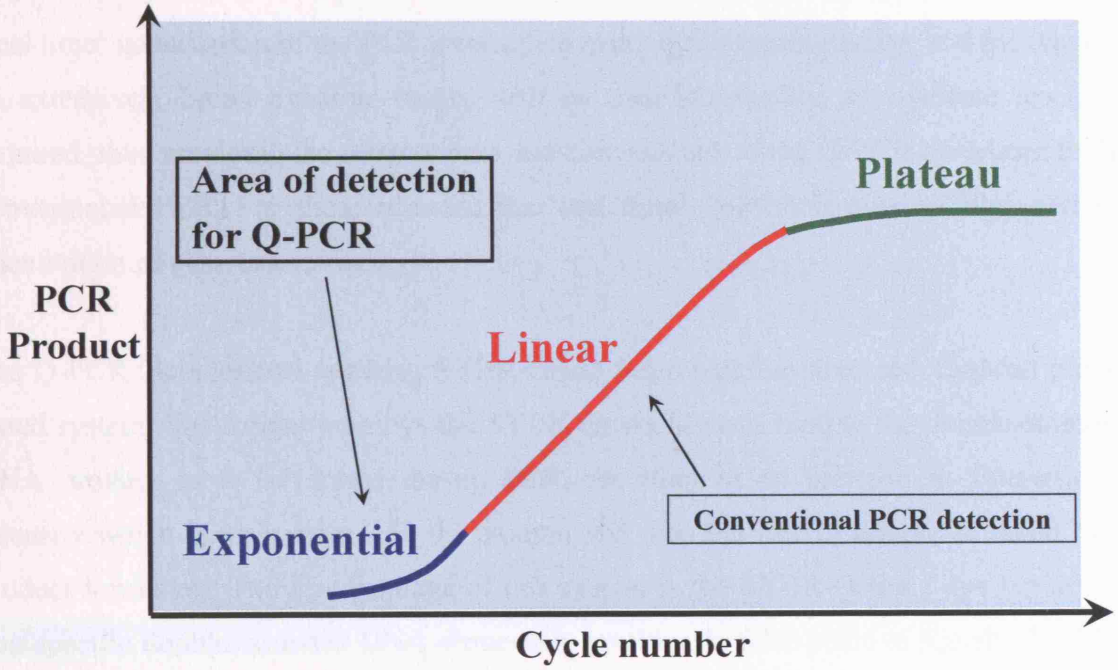


Figure 2.3. Basic phases of PCR amplification in a linear scale view. Three basic phases comprise the exponential, linear and plateau phase. Note that the exponential amplification occurs at the very beginning of the upturn of the curve, where the signal is detected in Q-PCR, while the area of detection for conventional PCR is in the linear range of the amplification.

In conventional PCR, detection of the PCR product by gel electrophoresis after a specific number of cycles is generally carried out when the amplification is in the linear phase, in which there is usually a sufficient amount of PCR product for detection by relatively insensitive ethidium bromide staining. In contrast, Q-PCR employs a much more sensitive detection system, allowing the low level of PCR product present in the exponential amplification to be detected. Detection of the PCR product in the exponential amplification phase is highly specific and precise, and provides a 'quantitative' measurement for Q-PCR compared to the 'semi-quantitative' conventional PCR. Most importantly, for the purpose of accurate and precise PCR-based results, it is necessary to determine the data at a point in which every sample is in the exponential phase of amplification. Practically, a dynamic range of conventional PCR can be analysed during end-point relative PCR. However, in order to extend this range, replicate reactions must be performed for a greater or lesser number of cycles, so that all of the samples can be analysed in the exponential phase. In contrast, Q-PCR performs this laborious process by 'real time' quantitation of the PCR products in every cycle automatically, and the result is an extensively broad dynamic range, with no user intervention or replicate reactions required, thus providing the relative ease and convenience of the Q-PCR compared to the conventional PCR. For these reasons, the 'real time' Q-PCR is now widely used for quantitation of gene expression.

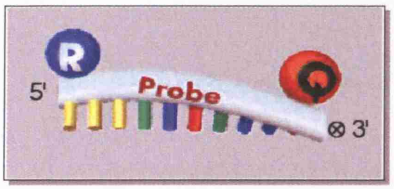
The Q-PCR includes two systems: SYBR Green I dye-based system and TaqMan probe-based system. The former employs the SYBR Green I dye to bind to the double-stranded DNA product as it is formed during PCR, resulting in an increase in fluorescence intensity which is proportional to the amount and thus the rate of double-stranded PCR product formation. The disadvantage of this system is the SYBR Green I dye binding to non-specific double-stranded DNA sequences, resulting in false positive signals. TaqMan probe-based system uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during the PCR cycles. This probe-based system combines the specificity of the primers and the probe, generating highly specific signals which are directly proportional to the initial level of the target cDNA. Therefore, the TaqMan probe-based Q-PCR system was used in the present study.

In this study, the TaqMan probe-based chemistry for each reaction comprises three major elements: primers specific for the target gene sequences; a TaqMan probe which contains a fluorescent reporter dye at the 5′ end and a non-fluorescent quencher at the 3′ end (Figure 2.4A); and a DNA polymerase with 5′ nuclease activity.

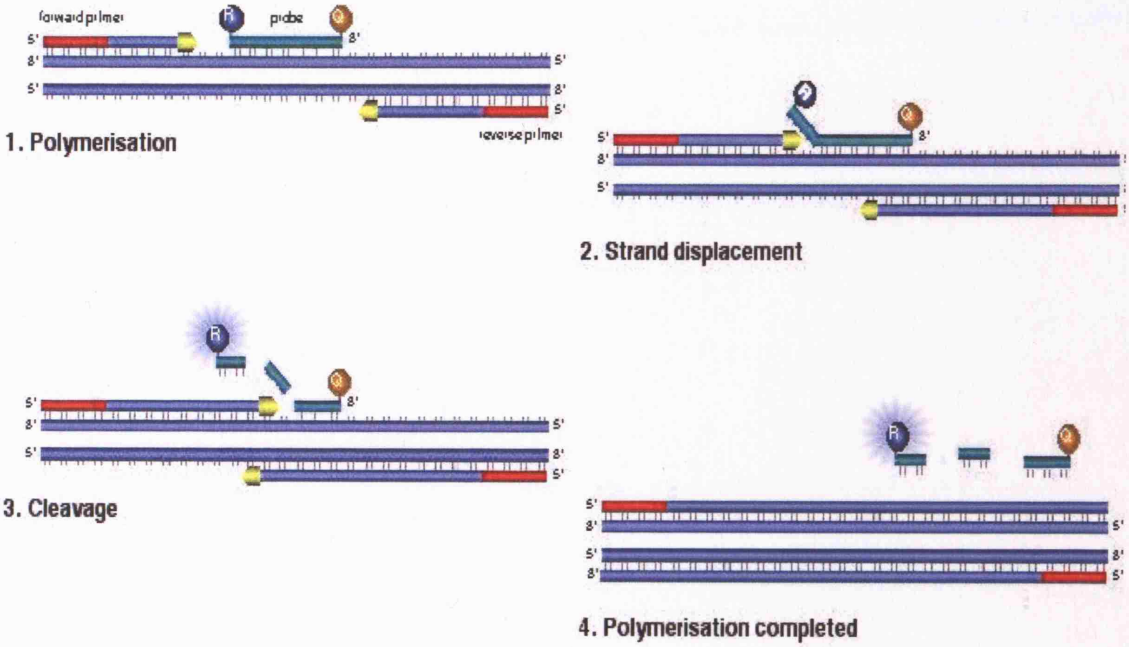
As outlined in Figure 2.4, during each PCR cycle, the probe specifically anneals between the forward and reverse primer binding sites in the presence of the target sequence. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During each extension cycle of PCR, the 5′ nucleolytic activity of the DNA polymerase cleaves the probe between the reporter and the quencher. The probe fragments are then displaced from the target, and polymerisation of the strand continues. Once separated from the quencher, the reporter dye emits its characteristic fluorescent signal (Figure 2.4B). Accumulation of PCR products is detected directly by monitoring the increase in fluorescence due to the released reporter dye. This is equivalent to the amount of PCR product at each time, enabling the rate of product formation and thereby the initial level of target cDNA to be accurately calculated.

Q-PCR allows reaction to be measured by the point in time during cycling when amplification of a PCR product achieves a fixed level of fluorescence, rather than the amount of PCR product generated after a fixed number of cycles as in the conventional semi-quantitative PCR. As shown in Figure 2.4C, in the initial cycles of PCR, there is no significant change in fluorescence signal. This range of PCR cycles is called the "baseline". The software first generates an amplification plot by calculating a mathematical trend using "normalised reported fluorescence" (R_n) values corresponding to the baseline cycles. Then, an algorithm searches for the point on the amplification plot at which the R_n value crosses the threshold (*i.e.,* the fluorescence level which is set, automatically by the software or manually, to be above the baseline and sufficiently low to be within the exponential amplification of PCR). The fractional PCR cycle at which this occurs is defined as the "threshold cycle" (C_t).

(A)


R
5'
Probe
Q
3'

(B)


forward primer
R
probe
Q
1. Polymerisation
reverse primer
2. Strand displacement
3. Cleavage
4. Polymerisation completed

(C)

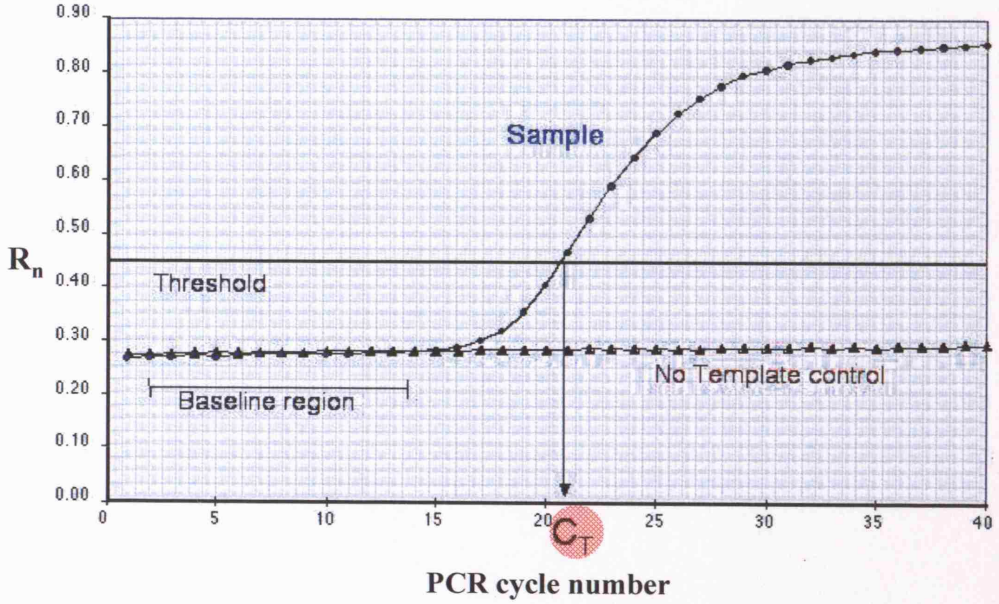

Sample
Threshold
No Template control
Baseline region
R_n
C_T
PCR cycle number

Figure 2.4. Schematic illustration showing the principle of TaqMan probe-based Q-PCR. In (A), a TaqMan probe contains a fluorescent reporter dye (R) linked to the 5′ end of the probe and a non-fluorescent quencher (Q) at the 3′ end. (B) depicts the principle of TaqMan-based chemistry, which shows the forklike-structure-dependent, polymerization-associated 5′ nuclease activity of the DNA polymerase during PCR. The PCR reaction exploits the 5′ nuclease activity of the DNA polymerase to cleave a TaqMan probe during PCR. During the reaction, cleavage of the probe separates the fluorescent reporter dye and the non-fluorescent quencher dye, consequently resulting in an increase in fluorescence of the unbound-reporter which lacks the fluorescence suppressive effect from the quencher. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any non-specific amplification is not detected. (C) shows an example of the amplification plot features, depicting threshold cycle (C_T) which is the calculated fractional cycle number at which the PCR product crosses a threshold of detection.
(Adapted from http://cgr.otago.ac.nz/slides/taqman/sld001.htm)

For relative quantitation of the target gene of interest, the comparative C_T method was used (Livak and Schmittgen, 2001). The software first calculates a ΔC_t value, *i.e.*, the C_t of a target gene normalised to that of an endogenous control (*e.g.*, GAPDH). This ΔC_t is then compared with that of a calibrator (*e.g.*, an untreated control sample), giving a $\Delta\Delta C_t$ value. Calculations of the relative quantitation values "$2^{-\Delta\Delta Ct}$" (*i.e.*, changes of the relative fluorescent signal of the tested sample compared with that of the calibrator, defined as 1.0) are displayed automatically.

2.9.2 Q-PCR protocol for BMPR-IB and OC genes

Total RNA was isolated and a first strand cDNA synthesised from 1 μg RNA as described in Section 2.8.2.1 and 2.8.2.3. The first strand cDNA products were used for amplifications performed with specific primers for BMPR-IB and OC gene. Primer sequences were designed with the Primer Express® program from Applied Biosystems; the Assay ID for BMPR-IB and OC genes used in this study were Hs00176144_m1 and Hs01587813_g1, respectively. For Q-PCR analysis in an ABI Prism® 7300 Sequence Detector (Applied Biosystems), the TaqMan® Gene Expression Assays consisting of unlabelled specific PCR primers and TaqMan® MGB probes with FAM™ dye-labelling were used. TaqMan PCR reactions were set up in a 96-well plate format as recommended by the manufacturer. Briefly, the cDNA products were first diluted (1:2) in nuclease-free water, and a 5 μl aliquot of the diluted cDNA was used in a final volume of 25 μl reaction mixture (for each well on a 96-well plate) containing 12.5 μl of 2X TaqMan® Universal PCR Master Mix (P/N 4304437), 1.25 μl of 20X Assays-on-Demand™ Gene Expression Assay mix and 6.25 μl of nuclease-free water (all from Applied Biosystems). Thermal cycler conditions were used as recommended by the manufacturer which consisted of AmpErase® UNG activation at 50°C for 2 min, AmpliTaq Gold® DNA polymerase activation at 95°C for 10 min and 40 PCR cycles, each of which was 95°C for 15 s and 60°C for 60 s. The data were automatically collected and analysed by the SDS software (Applied Biosystems).

For quantitation of the BMPR-IB and OC transcripts by a comparative C_T method, a validation experiment for each gene was first performed, in accordance with the manufacturer's instructions, and demonstrated that efficiencies of each of the two target genes and GAPDH amplifications were approximately equal (Appendix I-Figure 1). All

PCR reactions were performed in triplicate and each of the BMPR-IB and OC signals was normalised to the GAPDH signal simultaneously detected in the same plate. The data are presented as the mean fold induction (± SE) compared with that of the untreated control sample, defined as 1.0.

2.10. ALP activity assay

2.10.1. Principle of the ALP activity assay

ALP activity can be determined by measuring the rate of hydrolysis of various phosphate esters, with *p*-nitrophenyl phosphate one of the most widely used substrates (Chentoufi *et al.*, 1993). The enzymatic sequence of the ALP assay is as follows:

$$p\text{-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} p\text{-Nitrophenol} + H_3PO_4$$

Although *p*-nitrophenyl phosphate is colourless, the *p*-nitrophenol product has strong absorbance at 405 nm, and the A_{405} is thus proportional to the enzyme activity.

2.10.2. Protocol for ALP activity assay

The cells were seeded into flat-bottom 96-well plates at a density of $1x10^4$ cells/well and cultured for 48 h, after which they were subjected to the treatment described for each experiment. The activity of ALP, a membrane-bound enzyme (Chentoufi *et al.*, 1993), was measured in triplicate wells after washing the cells twice with Tris buffered saline (TBS) containing 50 mM Tris, pH 7.4, and 0.15 M NaCl and then incubating with 200 µl of 5 mM *p*-nitrophenyl phosphate in 50 mM glycine, 1 mM $MgCl_2$ and 150 mM 2-amino-2-methlyl-1-propanol buffer (all from Sigma), pH 10.5, at 37°C for 1 h. Then, 50 µl of 3 M NaOH was added to stop the enzyme reaction and the A_{405} measured spectrophotometrically. A standard curve of known concentrations of *p*-nitrophenol (Sigma) was concurrently obtained and used to determine the enzyme activity of the test samples, defined as nmol of *p*-nitrophenol produced per min per well at 37°C.

2.11. Small interfering RNA (siRNA)-mediated RNA interference (RNAi)

2.11.1. Principle of siRNA-mediated RNAi

RNAi has recently been employed to investigate gene function *in vitro* and *in vivo* by causing degradation of specific mRNA. This relatively new procedure is based on the observation that exogenous double-stranded RNA (dsRNA) can induce potent silencing of endogenous gene expression by sequence-specific mRNA cleavage in *Caenorhabditis elegans* (Fire *et al.*, 1998). It was also reported that the long dsRNA-mediated RNAi caused 'non-specific' inhibition of gene expression in mammalian cells (Kumar and Carmichael, 1998). However, this could be circumvented by using synthetic siRNA duplexes of approximately 21-25 nucleotides, that are too short to cause non-specific gene silencing (Caplen *et al.*, 2001). The siRNA duplexes can be delivered into a diverse range of cell types either by electroporation or transfection with chemical reagents. The schematic diagram illustrating the principle of siRNA-mediated RNAi is shown in Figure 2.5. Once siRNA is introduced into cells, an RNA-induced silencing complex (RISC) unwinds the siRNA and the antisense siRNA strand is incorporated into the RISC. This antisense siRNA strand then guides the RISC to the complementary sense mRNA molecule which is then cleaved by the RISC, leading to specific gene silencing. The RISC-antisense siRNA strand complex can subsequently be recycled and further bind to next target complementary mRNA molecules (Mello and Conte, Jr., 2004; Kim, 2005; Mattick and Makunin, 2005; Rao and Sockanathan, 2005).

In the present study, the siRNA was introduced into the AB cell cultures using lipofectamine-mediated reverse transfection (*i.e.*, transfection in suspension) (Amarzguioui, 2004; Ovcharenko *et al.*, 2005), the transfection efficiency of which was found to be approximately 98% (Appendix I-Figure 2).

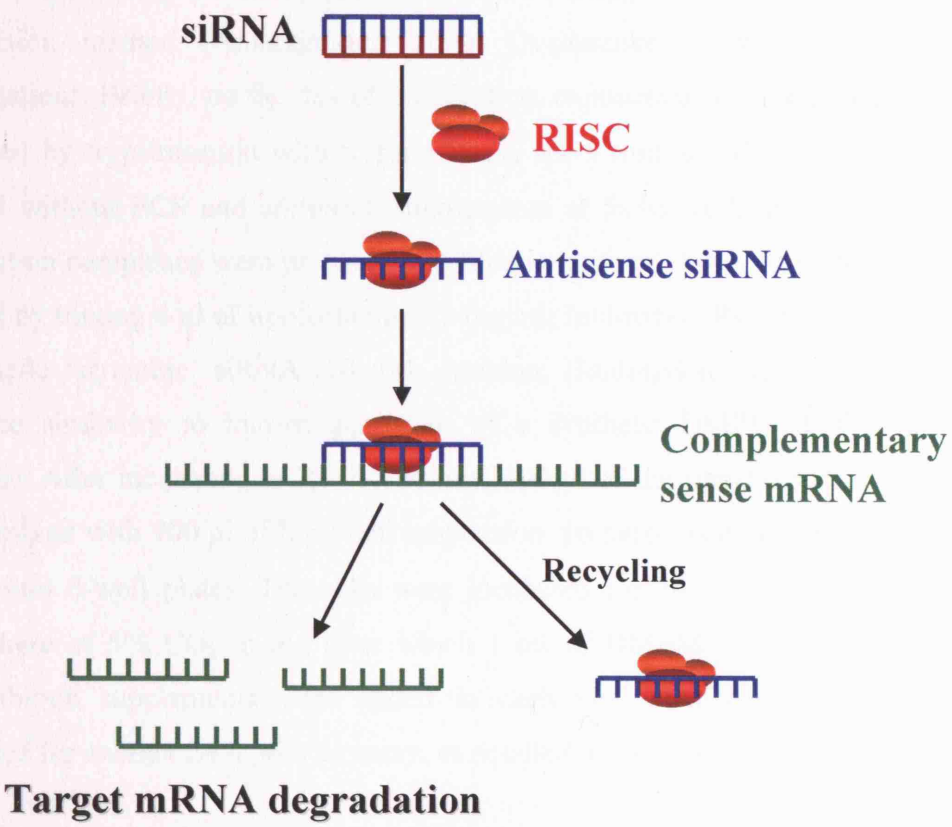


Figure 2.5. Schematic diagram showing the principle of siRNA-mediated RNAi. Antisense siRNA strand is incorporated into RISC, which contains proteins that exhibit structural homology to RNase H and acts as the 'slicer' enzyme that cleaves target mRNA (Kim, 2005), leading to sequence-specific target mRNA degradation and the RISC-antisense siRNA complex recycled.

2.11.2. Preparation of cells and reverse transfection with siRNA targeting BMPR-IB

The cell cultures were transfected with siRNA using a previously reported reverse transfection method (Amarzguioui, 2004; Ovcharenko *et al.*, 2005), with some modifications. Briefly, on the day of transfection, exponentially growing bone cells were harvested by trypsinisation with trypsin-EDTA for 5 min at 37°C and re-suspended in DMEM without FCS and antibiotic supplements at $5x10^5$ cells/ml. For each reaction, transfection complexes were prepared in a final volume of 100 µl of FCS/antibiotics-free DMEM by mixing 4 µl of lipofectamine (2 mg/ml; Invitrogen, Paisley, UK) with 10 µl of a synthetic 'scramble' siRNA (10 µM; Ambion, Huntingdon, UK), which has limited sequence similarity to known genes, or of a synthetic BMPR-IB siRNA (10 µM; Ambion). After incubating at RT for 30 min, 100 µl of the transfection complexes were gently mixed with 900 µl of bone cell suspension, prepared as described above, and then placed into 6-well plates. The cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air, after which 1 ml of DMEM containing 20% FCS and 2x antibiotic supplements were added to each well. The cells were subsequently incubated for another 24 h prior to assay, as detailed in each experiment.

In preparation of transfection for subsequently performing of the ALP activity assay of the transfected cells, a 96-well plate was used, and the number of cells and the amount of reagents adjusted accordingly, in accordance with the manufacturer's recommendation.

2.12. Statistical Analysis

The data are presented as the mean ± SE of measurements from three independent experiments performed in triplicate in all experiments throughout the thesis, unless otherwise noted. Statistical differences between the means were analysed by one-way ANOVA, followed by the *post-hoc* Bonferroni test for multiple comparisons, with $p<0.05$ considered significant. In some experiments where the data were obtained as values relative to that of the control group, defined as 1.0 or 100%, statistical differences between the mean of the test groups and a specified value (*i.e.,* 1.0 or 100) were analysed by single sample *t*-test, with $p<0.05$ considered significant. The ANOVA, Bonferroni and *t*-test programs in the SPSS 11.0 software (SPSS, Chicago, IL) were used for the analyses.

CHAPTER 3
ISOLATION AND CHARACTERISATION OF PRIMARY HUMAN AB CELLS

3.1. Introduction

A number of tissues are often used to obtain cells of osteoblastic lineage, including cancellous bone specimens derived from long bones (femora and tibiae), from intra-oral AB and from calvarial bones from fetal or neonatal animals. In addition, several different methods have been described for isolating osteoblastic cells from such bone tissues, including enzymatic digestion or explant culture of bone fragments *ex vivo*. Proteolytic enzymes, *e.g.*, collagenase and trypsin, have been successfully used for obtaining osteoblast-enriched cultures from the calvarial bones of fetal and neonatal rodents and fetal chicks, while for heavily mineralised bones of the adult the use of explant cultures is generally the procedure of choice (Aubin and Herbertson, 1998), including from human adult AB (Salih *et al.*, 2001; Pradel *et al.*, 2005).

Although definitive and specific markers of the osteoblast lineage have not yet been identified unequivocally, such cells are generally considered to be defined by the expression of a number of phenotypic and functional features, including terminal differentiation to form mineralised ECM in osteogenic culture (Salih *et al.*, 2001). A number of osteoblast-associated markers have also been reported, including ALP, a marker of early osteoblast differentiation; Col I, a major protein component in bone matrix; BSP, a marker of intermediate to late osteoblast differentiation; OC, a late marker of osteoblast differentiation; and Cbfa1, an osteoblastic-associated transcription factor (Doglioli and Scortecci, 1991; Ducy *et al.*, 1997; Aubin, 2001; Salih *et al.*, 2001; Pradel *et al.*, 2005). Moreover, cells of osteoblastic lineage are also known to be responsive to Dex induction of ALP activity, which is thus considered to be characteristic of osteoblasts (Rickard *et al.*, 1994).

In the present study, AB cells were isolated from human AB fragments by explant culture and were characterised by examining the expression of ALP, Col I, BSP, OC and Cbfa1

mRNA transcripts as well as the ability of cells to respond to Dex induction of ALP activity and to form mineralised ECM in osteogenic medium (OM).

3.2. Materials and Methods

Cultures of AB cells from three different donors were prepared and maintained as described in Section 2.1 and cells between passages 3 and 5 (P3-P5) used in the experiments. RT-PCR were used to examine the expression of ALP, Col I, BSP, OC and Cbfa1 transcripts, as described in Section 2.8. Additional methods used in the present chapter are described below.

3.2.1. Treatment of cells

For Dex-induced ALP activity assay, exponentially growing AB cells were treated with 10 nM Dex (Sigma) for 72 h (Kuru *et al.*, 1999) and were then subjected to histochemical staining of ALP-positive cells as described below. For assay of mineralisation ability, confluent AB cells were incubated for 28 days in OM, which consisted of standard culture medium supplemented with 0.1 mM L-ascorbic acid 2-phosphate and 10 mM β-glycerophosphate (all from Sigma) (Takamizawa *et al.*, 2004), with the culture medium being changed every three days and the cultures subsequently subjected to Alizarin Red S staining as described below.

3.2.2. Histochemical staining of ALP-positive cells

Cells were cultured at a density of $1x10^4$ cells/cm^2 on a 6-well plate for 48 h, then treated as described in Section 3.2.1. After rinsing the cell monolayers with cold (4°C) TBS, the cells were fixed with cold 4% paraformaldehyde in PBS for 10 min, washed with cold distilled water and allowed to dry for 30 min, then incubated for 30 min with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (Sigma) at 37°C. The reaction was stopped by removing the substrate solution and washing with distilled water. The cell monolayers were photographed, the ALP-positive cells staining a dark blue colour.

3.2.3. Alizarin Red S staining

Mineralisation of the ECM was examined histochemically by staining of calcium with Alizarin Red S, which reveals the presence of calcium deposits (Stanford *et al.*, 1995). Briefly, confluent AB cells were obtained after culturing cells at a density of $1x10^5$ cells/cm^2 on a 12-well plate for 48 h in standard culture medium. OM was then added, as described in Section 3.2.1, and after 28 days the cell monolayers were washed with cold PBS and the cells fixed with 4% paraformaldehyde in PBS for 10 min at RT, then washed with distilled water. The samples were incubated for 30 min at RT with Alizarin Red S (Sigma) (2% w/v) in distilled water, adjusted to pH 4.2 with 10% ammonium hydroxide (Sigma), then rinsed twice with methanol, air dried and photographed. Mineralisation of the ECM was visualised as bright red deposits.

3.3. Results

3.3.1. Outgrowth of the AB cells

Explant cultures of the AB cells were established as described in Section 2.1.2 and the outgrowth of cells observed microscopically. The results in Figure 3.1 show that after approximately 10 days of explant culture, an outgrowth of AB cells with a spindle morphology was evident (black arrows). The cells subsequently proliferated, and by day 16 there were more cells and they showed a heterogeneous morphology, *i.e.,* comprising both spindle and polygonal morphology (black and white arrows, respectively). The cultures reached confluence in regions adjacent to the explants after approximately 21 days, with the heterogeneous population of spindle and polygonal cells (as shown by black and white arrows) still apparent.

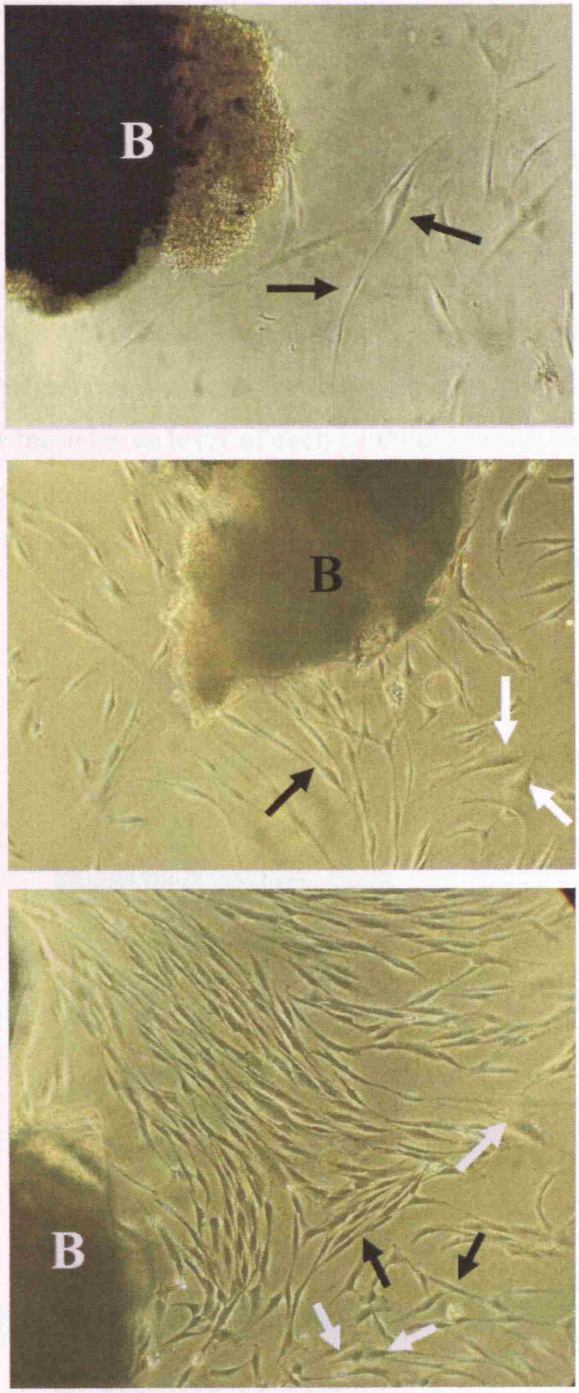


Figure 3.1. Explant cultures of human AB fragments. Microscopic photographs show an outgrowth of cells from a fragment of explanted bone tissue after 10, 16 and 21 days of culture. Note the spindle-shaped cells (black arrows) at day 10 and both spindle- and polygonal-shaped cells (white arrows) observed at days 16 and 21. B: AB fragments; original magnification x10.

3.3.2. Expression of osteoblast-related genes by the AB cells

The AB cells obtained from three different donors at P3 were subjected to RNA extraction and the expression of osteoblast-related genes examined by RT-PCR, as described in Section 2.8. The results in Figure 3.2 show that PCR products of 357, 461, 627, 297 and 443 base pairs (bp), corresponding to ALP, Col I, BSP, OC and Cbfa1 transcripts, respectively, were readily detected in all three cell samples. Thus, the AB cells from all three donors expressed mRNA corresponding to these genes, although the relative transcript levels of each gene appeared to be different in each of the cell samples. Despite these differences between the cell samples, the results in Figure 3.3 further demonstrate that the relative level of each of these mRNA was also maintained when the cells were further cultured until P4 and P5. Therefore, in the present study AB cells between P3 and P5 were used.

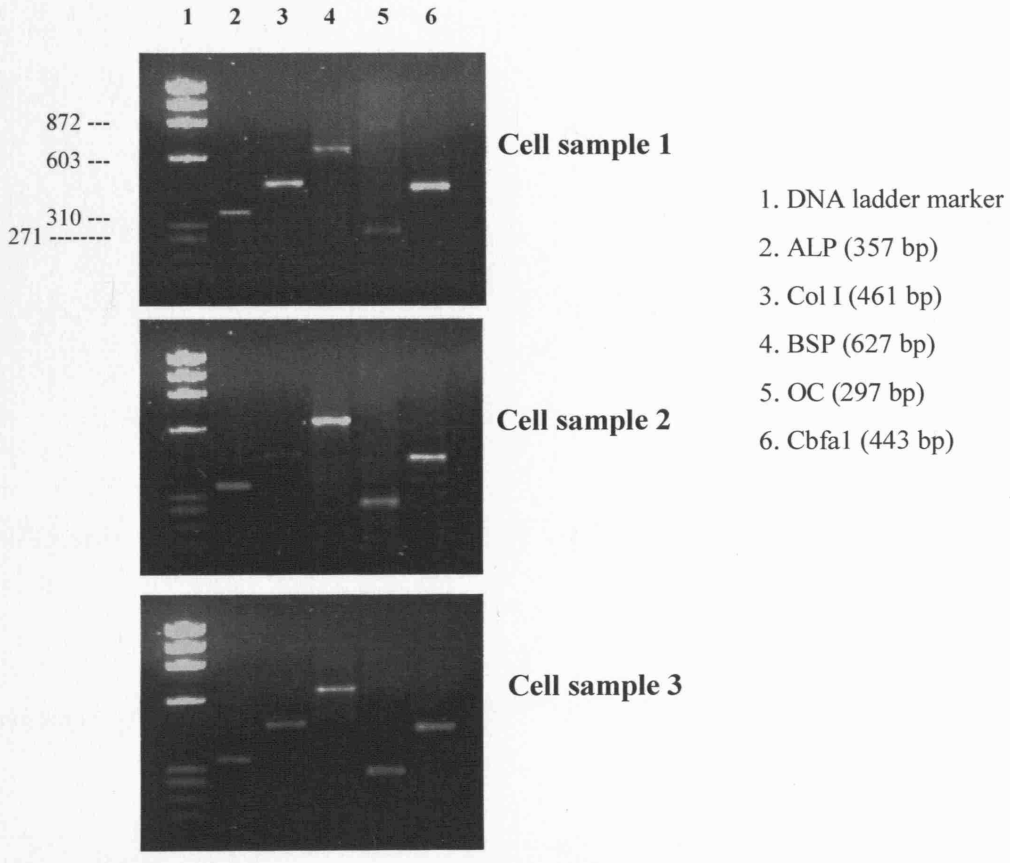
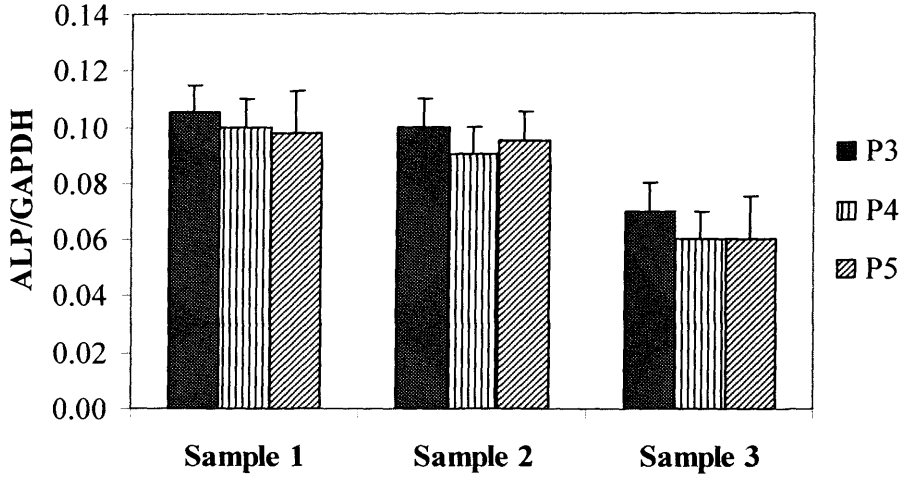
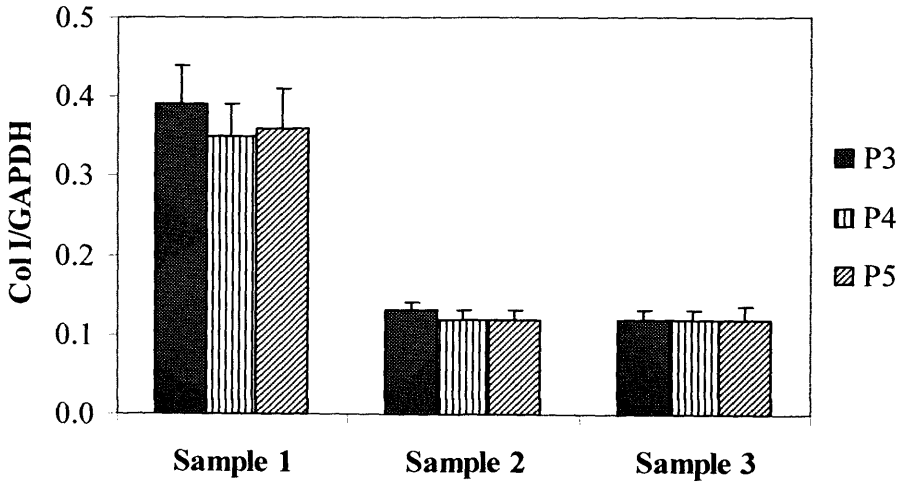
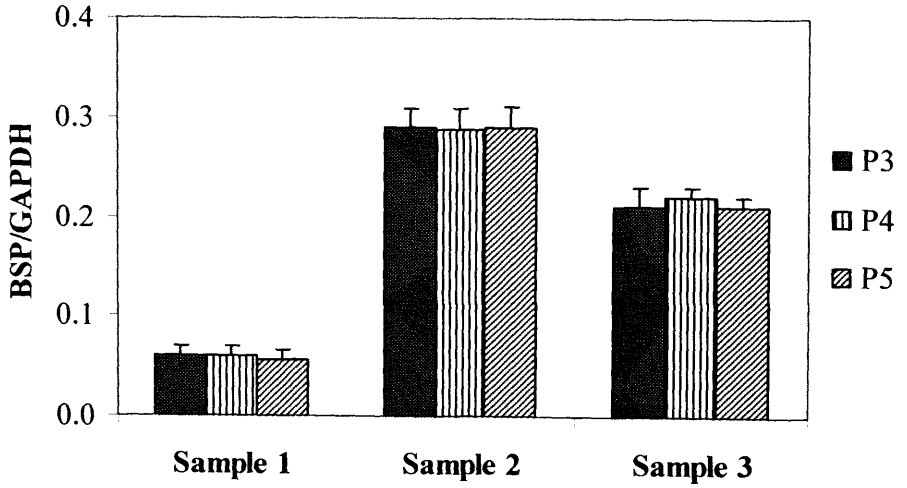


Figure 3.2. Representative RT-PCR gels showing RT-PCR products of the expected molecular sizes of 357, 461, 627, 297 and 443 bp, corresponding to ALP, Col I, BSP, OC and Cbfa1 transcripts, respectively, examined in three different AB cell samples at P3.


0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ALP/GAPDH
P3
P4
P5
Sample 1
Sample 2
Sample 3


0.5
0.4
0.3
0.2
0.1
0.0
Col I/GAPDH
P3
P4
P5
Sample 1
Sample 2
Sample 3


0.4
0.3
0.2
0.1
0.0
BSP/GAPDH
P3
P4
P5
Sample 1
Sample 2
Sample 3

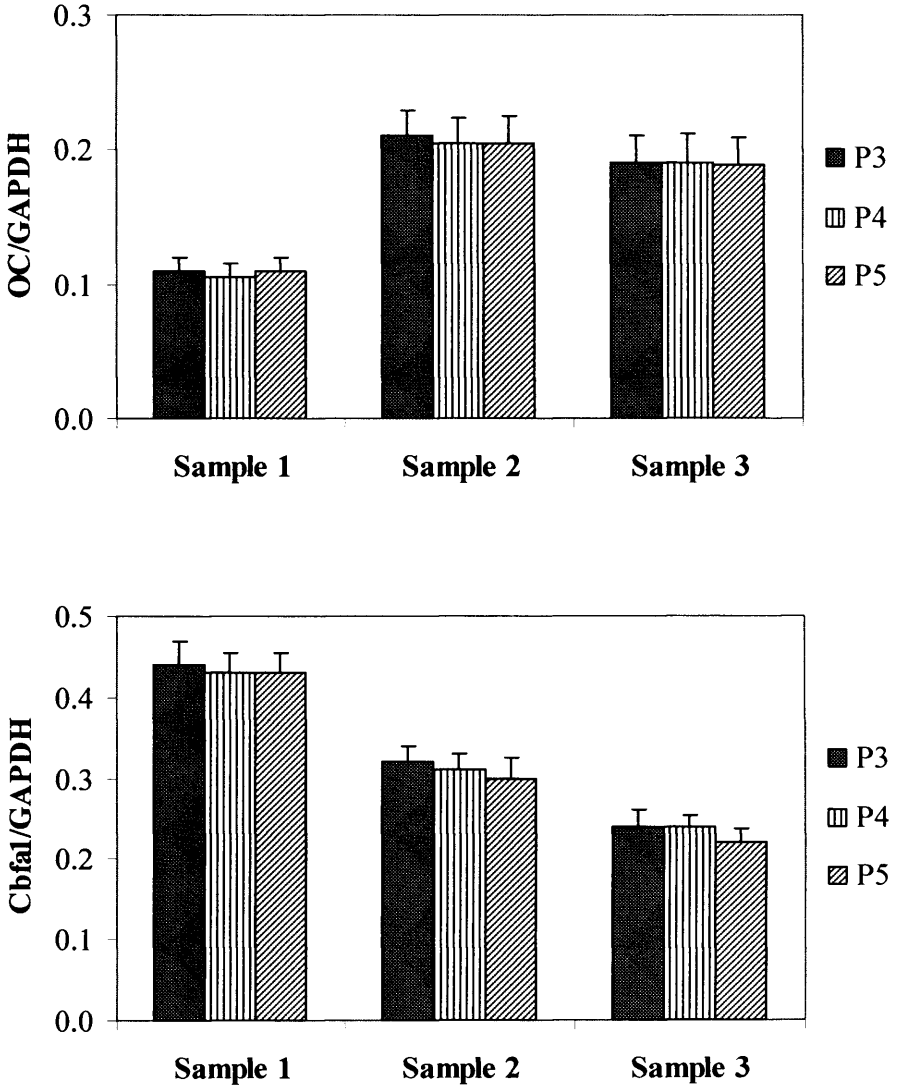


Figure 3.3. Relative levels of osteoblast-related gene expression in AB cells at P3, P4 and P5. RT-PCR reactions were performed using specific primers, as described in Section 2.8. The results are presented as the mean ± SE of the level of each of mRNA transcript relative to that of GAPDH, obtained from triplicate experiments. Note that the relative level of each of these mRNA appeared to be similar in AB cell at P3, P4 and P5.

3.3.3. Dex-induced ALP activity in the AB cells

The results in Figure 3.4 show that although untreated AB cells expressed a relatively low level of ALP activity, following treatment with 10 nM Dex for 72 h all three AB cell samples had a markedly higher level of ALP activity, as shown by the intense dark blue staining (Figure 3.4).

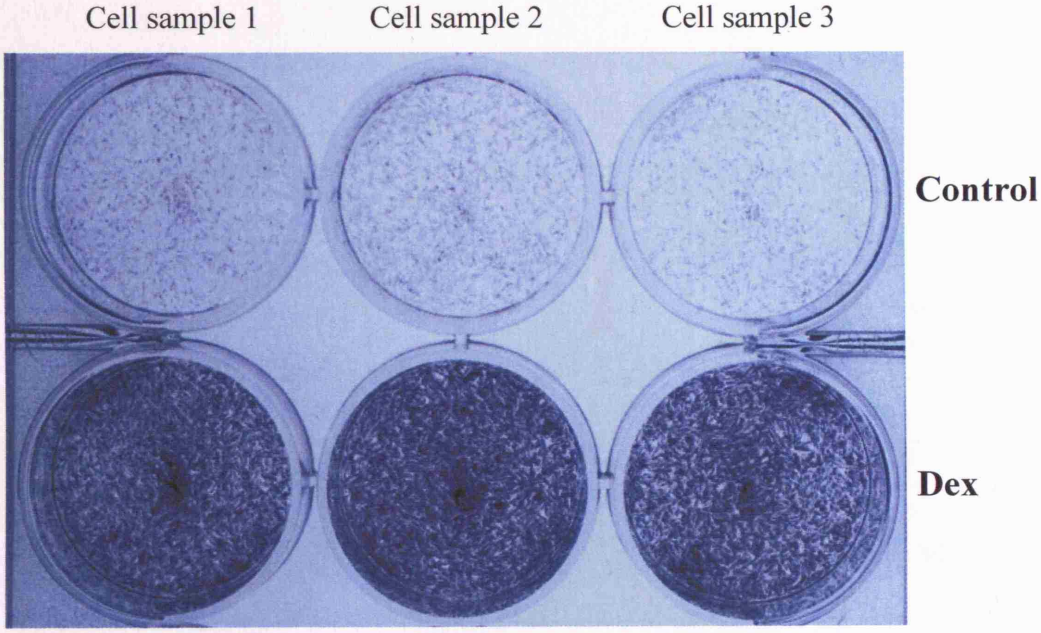
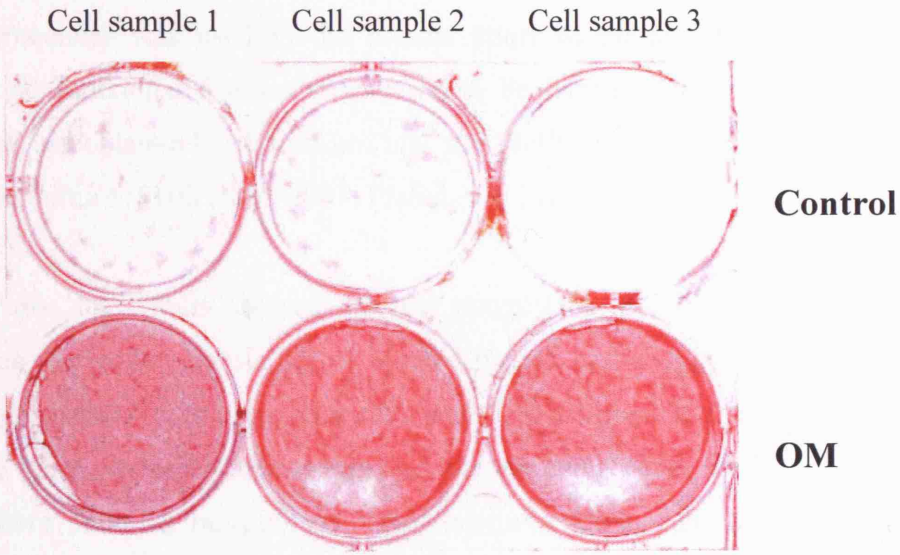


Figure 3.4. A 6-well plate showing the histochemical staining of ALP activity of the three AB cell samples (at P3) in response to Dex treatment for 72 h.

3.3.4. Mineralisation of the ECM by the AB cells

Mineralisation of the ECM was determined histochemically by staining of calcium deposits with Alizarin Red S, as described in the Materials and Methods. The results showed that AB cells cultured for 28 days in standard culture medium (control) showed no clear evidence of positive Alizarin Red S staining whereas cells cultured in OM exhibited a bright red stained, sheet-like structure (arrows) (Figure 3.5). Thus, all three AB cell cultures showed evidence of calcium deposits, indicative of the formation of a mineralised matrix, when cultured in OM.

(A)



(B)

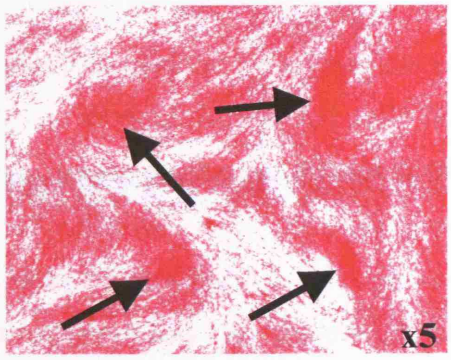


Figure 3.5. Alizarin Red S staining of the three AB cell samples. In (A), a 6-well plate shows Alizarin Red S staining of the three cell samples (at P3) cultured in standard medium (Control) and in OM for 28 days. In (B), bright red stained, sheet-like structures are indicated by arrows in higher magnification (x5).

3.4. Discussion

The use of cell cultures enables the function and regulation of cellular activity to be studied at the molecular level *in vitro*. While established long-known cell lines are often used for this purpose, the use of primary cells is advantageous because they retain genotypic characteristics of cells in normal tissue *in vivo* (Di-Silvio and Gurav, 2001). A number of methods have been discussed for obtaining such primary cells, including tissue fragment explants and enzymatic digestion. However, while the use of proteolytic enzymes (collagenase and trypsin) was previously shown to be suitable with calvarial bones of fetal or neonatal rodents and fetal chicks, primary explant has been suggested as the method of choice for highly mineralised adult bone (Aubin and Herbertson, 1998). The latter procedure was used in the present study to obtain cells which exhibited phenotypic and functional features considered to be characteristic of osteoblasts, *e.g.*, expression of osteoblast-related markers and the ability to form mineralised ECM in differentiation culture (Salih *et al.*, 2001; Pradel *et al.*, 2005).

Morphologically, the results showed that the outgrowths of AB explants contained a heterogeneous population consisting of spindle-shaped and polygonal cells. This may indicate the presence of both progenitor cells and more differentiated cells of osteoblastic lineage since it has previously been suggested that the morphology of cells changes from spindle to more cuboidal morphology when precursor bone cells undergo osteogenic differentiation (Aubin, 2001).

Phenotypic characterisation also indicated the presence of osteoblastic cells in the AB cell cultures. Thus, RT-PCR analysis showed that the AB cells expressed readily detectable levels of osteoblast-associated transcripts, *i.e.*, ALP, Col I, BSP, OC and Cbfa1, again suggesting that the cultures contained cells of osteoblastic lineage. In addition, histochemical analysis demonstrated that the AB cell cultures were responsive to Dex induction of ALP activity, as has previously been reported for cells of osteoblastic lineage (Rickard *et al.*, 1994), further supporting the presence of such cells in these AB cell cultures.

The present results have also shown that when cultured in OM, the AB cells were capable of forming mineralised ECM, one of the most important characteristic functions of

osteoblasts *in vitro*. Mineralised nodular structures have previously been observed to be formed in osteogenic cultures of mesenchymal stromal cells and less differentiated osteoprogenitor cells (Chen *et al.*, 1998; Gilbert *et al.*, 2000; Jorgensen *et al.*, 2004), although more mature cells of osteoblastic lineage do not appear to produce identifiable nodular structures *in vitro* but rather more uniform mineralised sheet-like structures (Aubin, 2001). In the present study, cultures of the AB cells in OM produced a mineralised ECM with a sheet-like structure, rather than a nodular morphology, as demonstrated by Alizarin Red S staining, suggesting that the AB cell cultures used here are likely to have comprised more mature cells of osteoblastic linage. This is also supported by the finding that expression of OC transcripts, a marker of late osteoblast differentiation, is readily detected in non-stimulated AB cell cultures.

Although contamination of the cultures with a small proportion of osteoclasts is possible, in prolonged culture as used here, the presence of such cells is usually maintained only by exogenous supplementation with Dex and a mixture of macrophage-colony stimulating factor (MCSF), receptor activator of nuclear factor-kappa B ligand (RANKL) and TGF-β1 (Susa *et al.*, 2004). It is thus likely that a substantial proportion of the AB cells isolated here are of osteoblastic lineage.

In conclusion, the AB cells obtained from explant cultures of cortico-lamellar bone fragments of AB appear to comprise a substantial proportion of cells with certain phenotypic and functional features of osteoblasts, possibly ranging from immature osteoblastic cells to fully differentiated osteoblasts within the same cultures.

CHAPTER 4
EXPRESSION, LOCALISATION AND SURFACE TRANSLOCATION OF BMPR

4.1. Introduction

It has previously been suggested that BMP/BMPR signalling is species- and cell type-specific and that the responsiveness of cells to the BMP depends at least in part on the expression of the BMPR (Cheifetz, 1999). The expression of the BMP-specific receptors in AB cells is thus of major importance in BMP-mediated AB growth and regeneration and ultimately the success of regenerative therapies. Although BMP-2 has been reported to stimulate osteoblast differentiation of human bone cells derived from mandibulae (Takiguchi *et al.*, 1998), thus far there has been no direct evidence of the expression of BMPR-IA, -IB and -II in these cells.

Moreover, while the BMPR, like other growth factor receptors, are generally considered to be transmembrane proteins, a number of these have also been reported to have an intracellular localisation at which they may have biological functions other than initiating and facilitating signal transduction processes (Zwaagstra *et al.*, 2000; Wells and Marti, 2002). For example, it has been reported that the EGFR accumulate in the nucleus and are functionally involved in modulating gene transcription, acting directly as transcription factors and enhancing mitogenesis (Wells and Marti, 2002). Such data about the cellular localisation of growth factor receptors, including the BMPR, are therefore essential for understanding the mechanisms and diversity of activity of growth factors, including the BMPs.

The cellular localisation of a number of cell surface receptors, including for growth factors, has previously been shown to be modulated by various factors *in vitro* (Zwaagstra *et al.*, 2000; Sobue *et al.*, 2002; Nohe *et al.*, 2003). For example, treatment of mink lung epithelial Mv1Lu cells and human lung carcinoma A549 cells with TGF-β1 promoted a redistribution of cytoplasmic TGFβR-I, resulting in their accumulation in the peri-nuclear and nuclear regions (Zwaagstra *et al.*, 2000). TGF-β1 has also been shown to decrease nuclear translocation of the cytoplasmic FGFR-I but enhance nuclear translocation of

cytoplasmic FGFR-II in human osteosarcoma MG63 cells (Sobue *et al.*, 2002). Recently, BMP-2 was reported to induce intracellular and peri-nuclear translocation of TGFβR-II from the cell surface in osteoblasts (Chang *et al.*, 2002). In addition, BMP-2 has been shown to directly affect the clustering of both BMPR-IA and -II on the plasma membrane (Nohe *et al.*, 2003), suggesting the possibility of BMP-2-mediated BMPR redistribution. The absence of serum factors in culture media is also known to cause translocation of surface and cytoplasmic TGFβR-I into the nucleus in Mv1Lu cells (Zwaagstra *et al.*, 2000). These studies thus clearly indicate that TGF-β1, BMP-2 and serum mediators all affect receptor redistribution.

While it remains unclear to what extent changes in BMPR localisation might affect the biological activity of the BMP, the expression and possibly re-organisation of BMPR at the cell surface undoubtedly play an important part in BMP signalling (Gilboa *et al.*, 2000; Nohe *et al.*, 2003). It is possible that certain factors such as the ligand BMP-2, TGF-β1 and serum factors could modulate localisation and intracellular transport of BMPR, thus changing the response of the cells to the BMP. The present study was therefore carried out to examine the expression and localisation of BMPR-IA, -IB and -II, and to determine the effect of BMP-2, TGF-β1 and FCS on the surface expression and translocation of these receptors in AB cells *in vitro*.

4.2. Materials and Methods

Cell culture, RT-PCR, FCM, WB, ICC, EM and C-ELISA were performed as described in Chapter 2 and some additional methods used in this chapter are described below. The osteoblast-like cell line HOS (TE85), which is rountinely used in the Division of Biomaterials and Tissue Engineering, Eastman Dental Institute, was cultured in the same way and used, in some FCM experiments, as a baseline for comparison of protein levels with the primary-derived cells. In the FCM experiments to examine the expression of BMPR in osseous and non-osseous intra-oral cells, primary human periodontal ligament (PDL) cells and gingival fibroblasts (GF) were also included as non-bone-derived cells which are functionally associated with the teeth. PDL and GF cells were obtained from the PDL tissue at the middle third portion of the root of tooth and from the gingival tissue, respectively. Primary explantation and maintenance of these cells were carried out in the same manner as described for AB cells in Section 2.1.

4.2.1. Treatment of cells

Exponentially growing cells were incubated for 24 h in DMEM containing 1% FCS (1% FCS-DMEM) and then BMP-2 (at 25, 50 and 100 ng/ml), TGF-β1 (at 0.1, 1 and 10 ng/ml) or FCS (at 10, 25 and 50%) added for 30-120 min. In all experiments, cycloheximide (10 μM; Sigma) was added for 1 h prior to the addition of BMP-2, TGF-β1 or FCS and present throughout the time of the factor treatment, to inhibit new protein synthesis. In some experiments, to block intracellular protein transport or internalisation of surface protein, the cells were also incubated with 10 μM of monensin (Sigma) for 1 h before or after TGF-β1 treatment, respectively. In experiments using chemical inhibitors of the TGF-β1 signalling pathway, cells were pre-treated for 2 h, with SB203580 (10 μM), U0126 (1 μM), SP600125 (10 μM), bisindolylmaleimide IX (Bis IX) (2 μM) and wortmannin (0.1 μM) (all from Sigma), which are inhibitors of p38 MAPK, ERK1/2, JNK1/2/3, PKC and PI3K, respectively (Heldin *et al.*, 1998; Massague and Chen, 2000; Miyazono *et al.*, 2005; Webber *et al.*, 2005), followed by the addition of 0.1 ng/ml of TGF-β1 in the continuing presence of the inhibitors. The concentrations of each of the inhibitors were selected on the basis of previous studies (Vermes *et al.*, 2002; Sobue *et al.*, 2002; Luo *et al.*, 2005). At the concentrations and conditions used here, all the inhibitors used were found to cause less than 5% cell death of the AB cells, as determined by trypan blue exclusion tests performed in duplicate. C-ELISA, FCM, ICC

and WB analysis were carried out on untreated (*i.e.,* only cycloheximide treated) and treated cells.

To examine BMP-2-induced formation of phosphorylated Smad1/5/8 (p-Smad1/5/8), cells were incubated with 100 ng/ml BMP-2 for 30 min. The optimal dose and exposure time for the ligand BMP-2 to activate Smad1/5/8 phosphorylation were initially determined (Appendix II-Figure 1) and used in all subsequent experiments.

4.2.2. Measurement of BMP-2 binding by C-ELISA

The relative level of specific BMP-2 binding to the AB cells was determined using C-ELISA, as described in Section 2.5.2, with some modifications. Briefly, cells were first pre-treated with cycloheximide for 1 h, then with 0.1 ng/ml of TGF-β1 for the indicated times at 37°C in the continuing presence of cycloheximide. Subsequently, 1 μg/ml of BMP-2 was added to the cultures for another 2 h at 4°C. The cell monolayers were then fixed with 4% paraformaldehyde for 30 min and subjected to C-ELISA at RT using mouse anti-human BMP-2/4 primary antibody (R&D Systems) (1:500 for 1 h) and HRP-conjugated goat anti-mouse IgG secondary antibody (1:500 for 1 h).

To determine whether BMP-2 was also able to bind to other cell surface molecules in addition to the BMPR-IA, -IB and -II, the cells were pre-treated for 1 h at 4°C with a mixture of 2 μg/ml of each of polyclonal antibodies against BMPR-IA, -IB and -II (before incubating with BMP-2) to block specific binding of the BMP-2 to these three BMPR. The samples were then incubated with BMP-2 and subjected to the C-ELISA assay described above.

4.3. Results

4.3.1. Expression of BMPR transcripts in the AB cells

Using primer sequences specific for the human BMPR-IA, -IB and -II genes, RT-PCR products corresponding to the expected molecular sizes of 510, 456 and 471 bp, respectively, were visualised under UV light, as shown in Figure 4.1A. This representative gel shows the expression of BMPR-IA, -IB and -II transcripts in the AB cells. Cells obtained from the samples 1, 2 and 3 also expressed all three BMPR transcripts, as shown in Figure 4.1B.

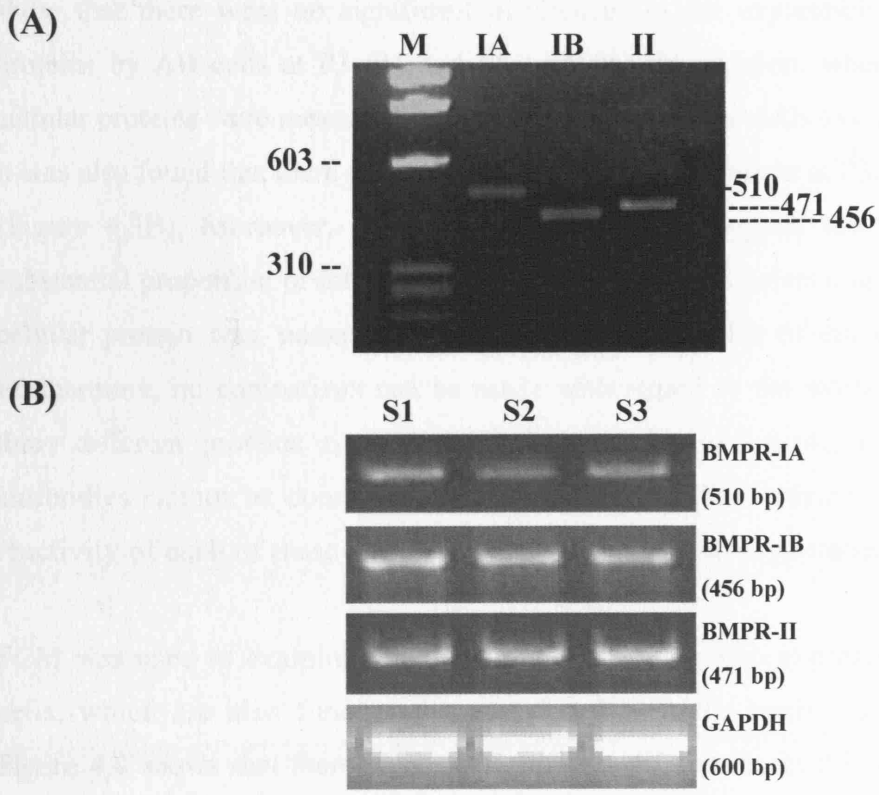


Figure 4.1. Expression of BMPR transcripts in the AB cells analysed by RT-PCR. In (A), a representative RT-PCR gel shows RT-PCR products of the expected molecular sizes of 510, 456 and 471 bp, corresponding to BMPR-IA, -IB and -II, respectively. The data were obtained from cell sample 1 (S1). M: DNA ladder marker. In (B), expression pattern of each of the BMPR and the housekeeping gene, GAPDH in the three different AB cell samples (S1, S2 and S3) is shown.

4.3.2. Expression of the BMPR proteins in the AB cells

In addition to the BMPR transcripts, the AB cells also expressed the BMPR-IA, -IB and -II proteins, which were detected by WB as the approximately 55, 55 and 75 kDa polypeptides, respectively (Figure 4.2A). Moreover, the cell surface expression of the corresponding proteins was evident by FCM analysis after immunostaining of the cells with the respective specific antibodies, as shown by the fluorescence profiles in Figure 4.2B.

To determine whether any changes in the expression of BMPR-IA, -IB and -II in the AB cells occurred between P3 and P5, which were used throughout the experiments, the cell surface levels of these three BMPR were measured by FCM. The results in Figure 4.3A show that there were no significant differences in the expression of the cell surface proteins by AB cells at P3, P4 and P5 ($p>0.05$). In addition, when the total levels of cellular proteins were measured by first permeabilising the cells to enable antibody entry, it was also found that there were also no significant differences at P3, P4 and P5 ($p>0.05$) (Figure 4.3B). Moreover, the results in Figure 4.3 suggest that these cells have a substantial proportion of intracellular-localised BMPR-IB protein, since the level of total cellular protein was, unexpectedly, much higher than that of the cell surface protein. Furthermore, no comparison can be made with regard to the relative expression of the three different proteins since the AFI values obtained using the different BMPR antibodies cannot be compared with each other as they indicate only differences in reactivity of each of these antibodies with their respective proteins.

FCM was used to examine whether the BMPR were also expressed by PDL and GF cells, which are also functionally associated with the teeth, but non-bone-derived. Figure 4.4 shows that there were no significant differences in the relative total cellular level of each individual receptor between any of these three different cell types studied ($p>0.05$), except for the significantly elevated level of BMPR-IB in the AB cells ($p<0.05$).

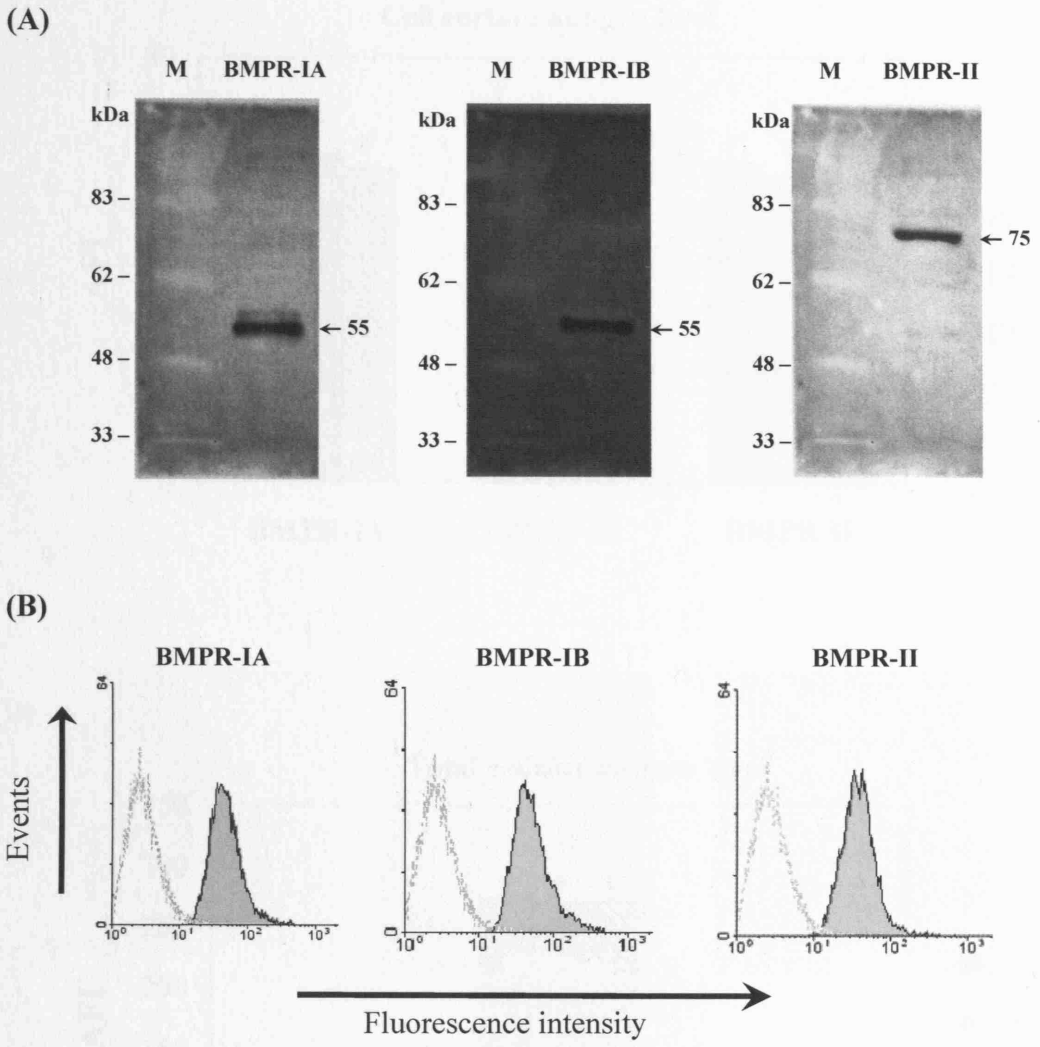
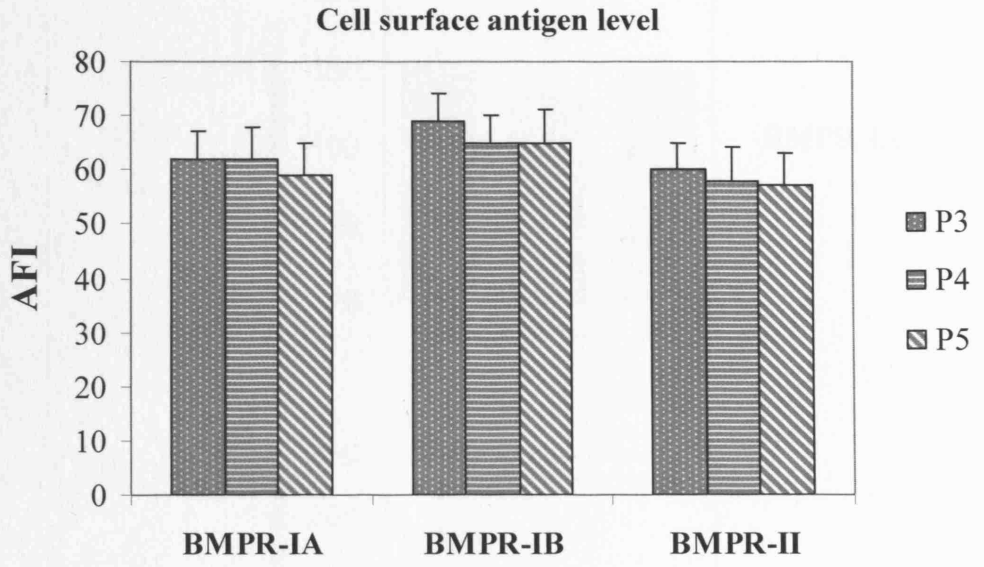


Figure 4.2. Expression of BMPR proteins in the AB cells (cell sample 1) analysed by WB and FCM. In the representative WB (A), the immunoreactive black bands show the electrophoretic migration of the approximately 55, 55 and 75 kDa polypeptides corresponding to BMPR-IA, -IB and -II proteins, respectively. In (B), representative histograms obtained from FCM analysis show the cell surface expression of the three BMPR proteins. The expression of cell surface BMPR is evident by the shift of the shaded histograms (the fluorescence of specific BMPR staining) to the right compared with the fluorescence of the isotype-matched antibodies used as controls (open histograms).

(A)



(B)

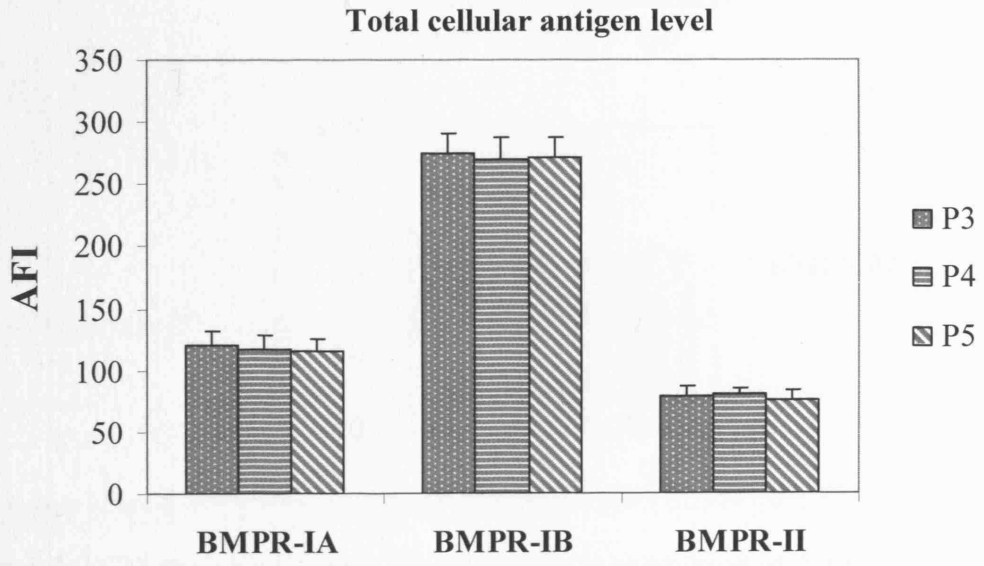


Figure 4.3. Expression profiles of cell surface (A) and total cellular (B) BMPR proteins in the AB cells at P3, P4 and P5, analysed by FCM. Data are presented as the mean AFI ± SE obtained from triplicate experiments.

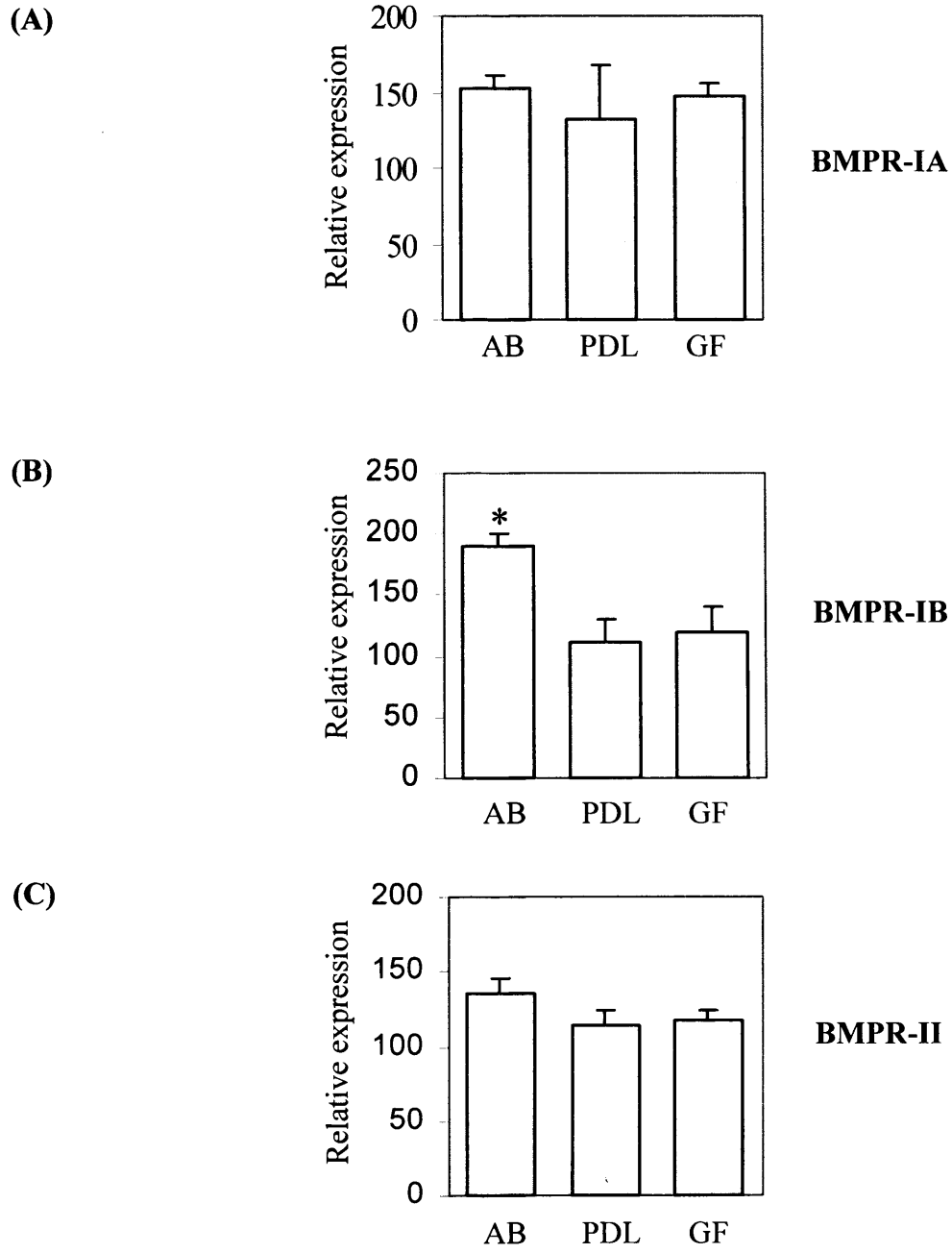


Figure 4.4. FCM analysis showing the total cellular expression of the BMPR-IA (A), -IB (B) and -II (C) proteins in the AB, PDL and GF cells. The relative levels of BMPR are presented as the mean AFI ± SE of three different cell samples for each cell type relative to that expressed by HOS cells, which is defined as 100. * Note the significantly elevated BMPR-IB level in the AB cells compared with that expressed by the PDL and GF cells ($p<0.05$).

4.3.3. FCM analysis of cellular distribution of BMPR in the AB cells

A summary of the FCM results obtained from three different AB cells (from three different donors) in Figure 4.5 shows that the distribution of the three BMPR was found to be markedly different. Thus, the average surface-associated levels of BMPR-IA, -IB and -II were 47 ± 0.9 %, 26 ± 1.4 % and 76 ± 5.6 % of the total cellular protein, respectively. This demonstrates that whereas BMPR-IA was relatively equally distributed between the cell surface and the intracellular region, BMPR-IB had a far more pronounced intracellular distribution and BMPR-II was present primarily at the plasma membrane.

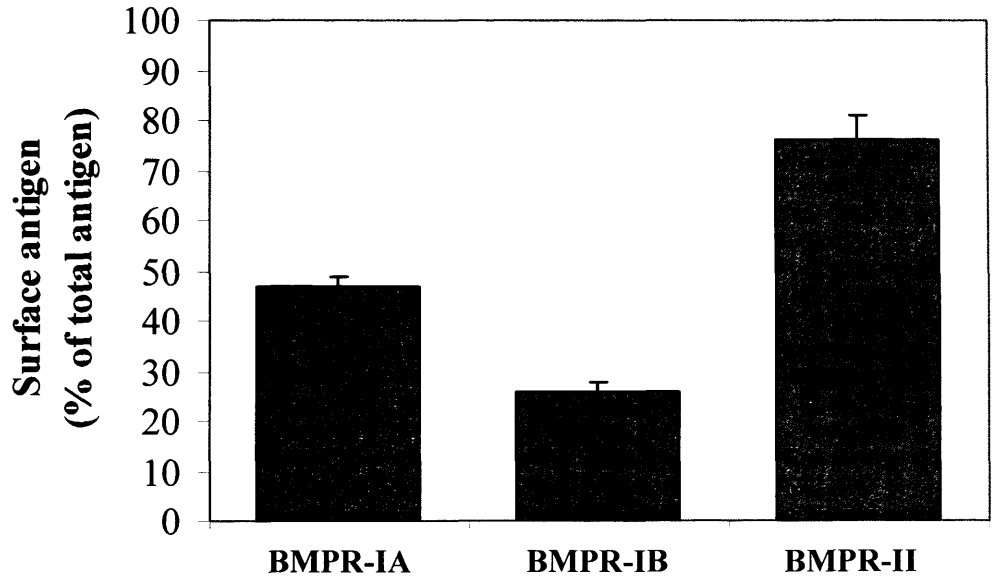
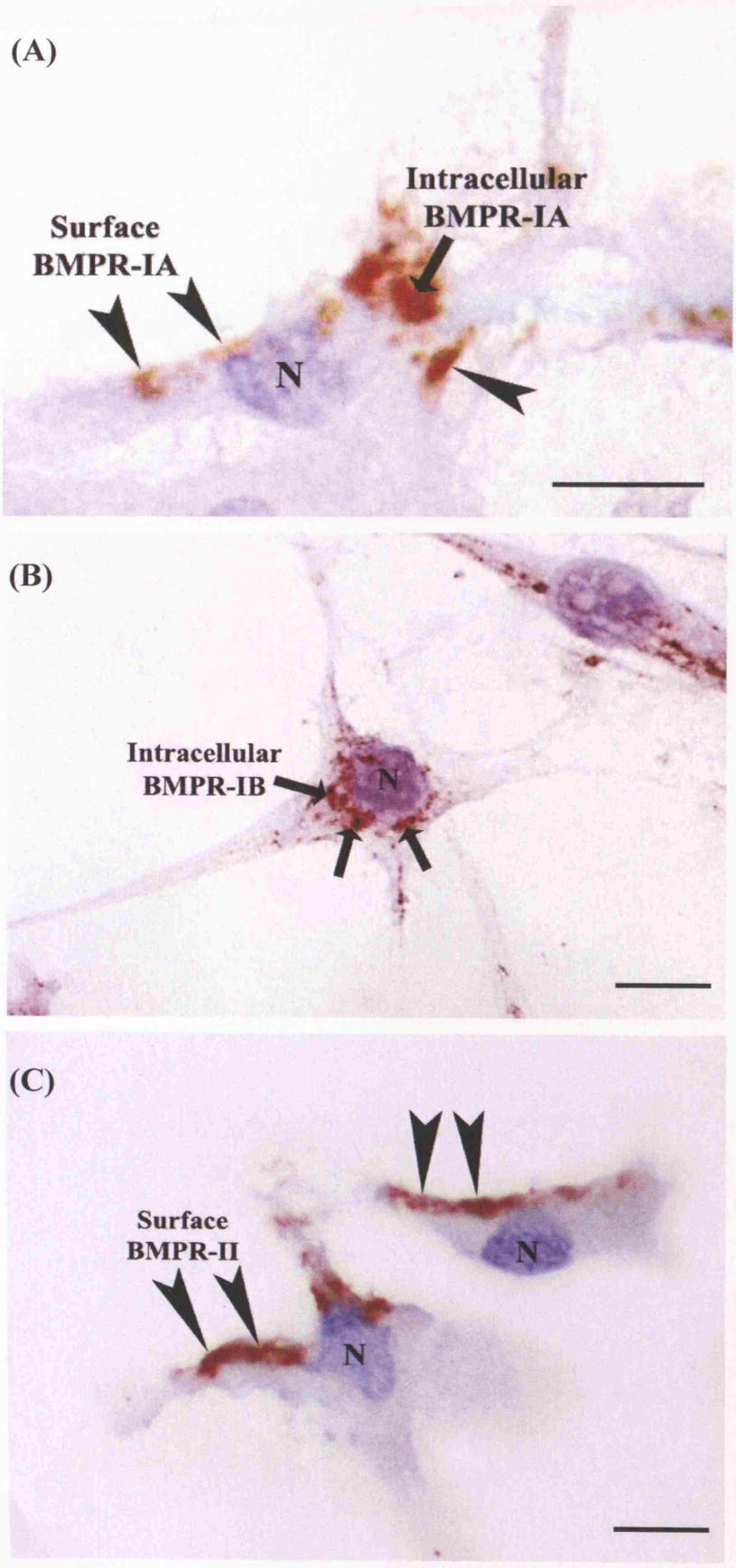


Figure 4.5. FCM analysis showing the average proportion of cell surface BMPR-IA, -IB and -II proteins in three different AB cell samples. The relative levels of cell surface and total cellular BMPR expression were measured by FCM on non-permeabilised and permeabilised cells, respectively, and the average proportion of the cell surface protein calculated. The data are presented as the mean ± SE of three different AB cell samples.

4.3.4. ICC analysis of surface and intracellular BMPR proteins

All three BMPR were also readily detected by ICC of the AB cells, as shown in Figure 4.6, although the localisation pattern of each protein again differed markedly, as suggested by the FCM results. Thus, BMPR-IA was expressed both at the cell surface and in the cytoplasm (Figure 4.6A), whereas staining for BMPR-IB was found to be predominantly intracellular, with the protein apparently accumulated mainly in the peri-nuclear region (Figure 4.6B). In contrast, while only little staining for BMPR-II was detected in the cytoplasm, a substantial amount of this protein was observed at the cell membrane (Figure 4.6C). No staining was detected in the negative control (Figure 4.6D).


(A)
Surface
BMPR-IA
Intracellular
BMPR-IA
N
(B)
Intracellular
BMPR-IB
N
(C)
Surface
BMPR-II
N
N

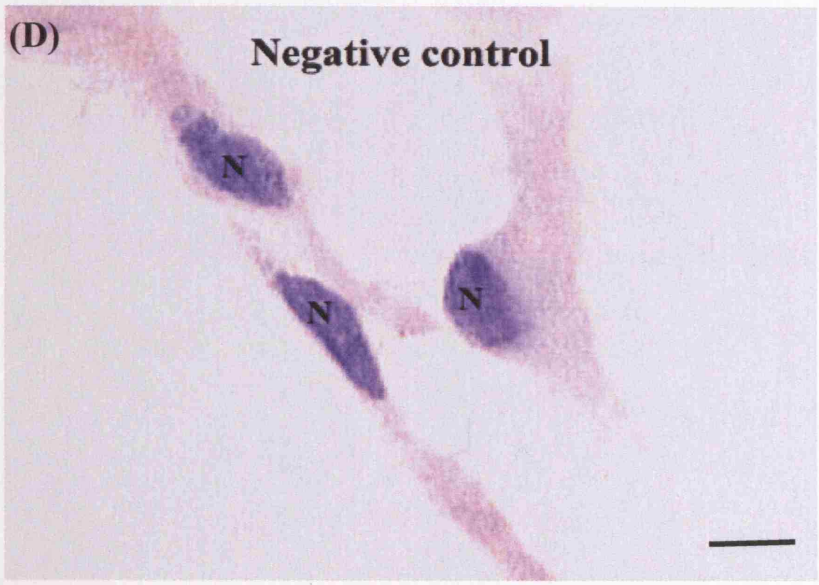


Figure 4.6. ICC analysis showing the differences in the subcellular distribution of the three BMPR in the AB cell sample 1. BMPR-IA (A) was detected both at the cell surface (arrowheads) as well as in the cytoplasm (arrow), while BMPR-IB (B) was present mainly intracellularly (arrows). The majority of BMPR-II (C) was found predominantly at the cell membrane (arrowheads). There was no evidence of BMPR expression in the negative control, which was immunostained with non-specific IgG isotypes as described in Section 2.2.2 (D). The specific brown-coloured staining indicates the presence of the BMPR and the nucleus counter-stained with Mayer's hematoxylin is observed in blue. N, nucleus; Bar = 10 μm.

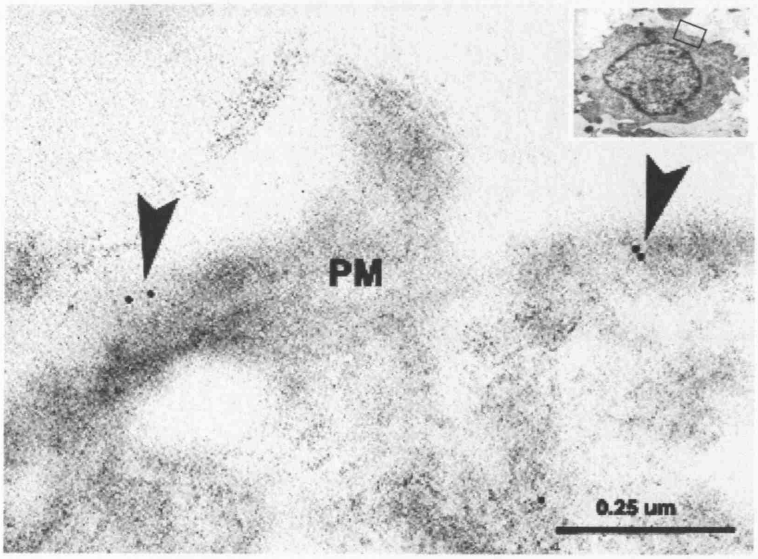
4.3.5. Ultrastructural localisation of the BMPR-IB protein

FCM and ICC indicated that the BMPR-IB protein in particular is localised predominantly in the cytoplasm of the AB cells, despite being widely recognised as a transmembrane protein. Immunogold EM was therefore carried out to determine the precise subcellular localisation of this protein specifically.

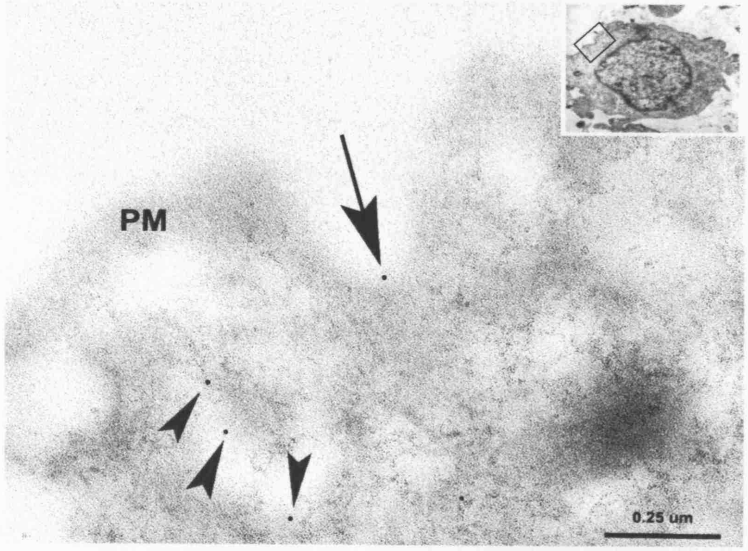
The results in Figure 4.7A show that 10 nm gold particles corresponding to BMPR-IB were evident at the plasma membrane, with no apparent clustering in any particular site. This protein was also detected in a region of invaginated plasma membrane, as indicated by the arrow in Figure 4.7B, while other gold particles (indicated by arrowheads) were found in vesicle-like structures near the plasma membrane, presumably endosomes. However, Figure 4.7C shows that BMPR-IB was prominently associated with membranous cytoplasmic structures, possibly corresponding to the ER and Golgi/*trans*-Golgi organelles. Notably, this receptor was also detected at the nuclear membrane (Figure 4.7D) and even within the nucleus (Figure 4.7E). No gold particle was detected in the negative control (Figure 4.9A).

Double-labelling immunogold EM analysis established that the 10 nm gold particles corresponding to the BMPR-IB protein co-localised with the 15 nm gold particles corresponding to the EEA-1 antibody, a marker of early endosomes (Zhang *et al.*, 2004) (Figure 4.8A), and with the LAMP-1 antibody, a marker of lysosomes (Zhang *et al.*, 2004), showing that BMPR-IB was also present in organelles in the peri-nuclear region, presumably lysosomes (Figure 4.8B). No gold particle was detected in the negative control (Figure 4.9B), and no cross-reactivities of 15 nm gold-conjugated goat anti-rabbit IgG secondary antibody to mouse anti-BMPR-IB antibody and of 10 nm gold-conjugated anti-mouse IgG secondary antibody to rabbit anti-EEA-1/LAMP-1 antibodies were observed (Figure 4.9C and 4.9D, respectively).

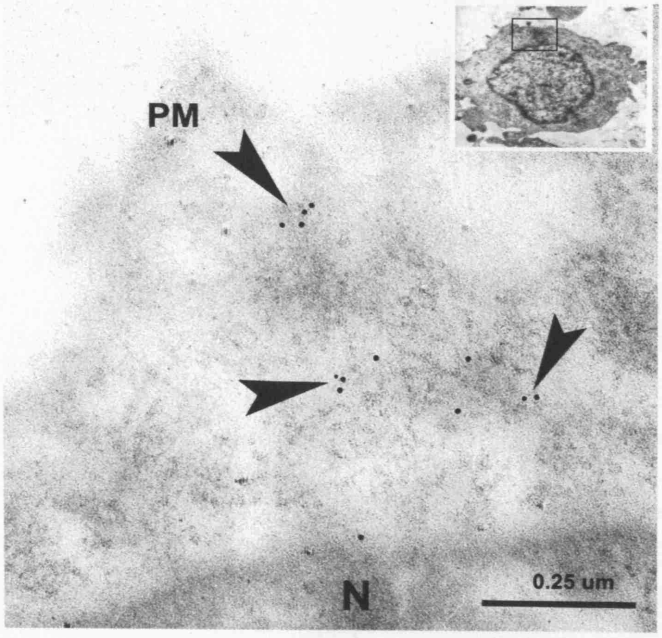
(A)



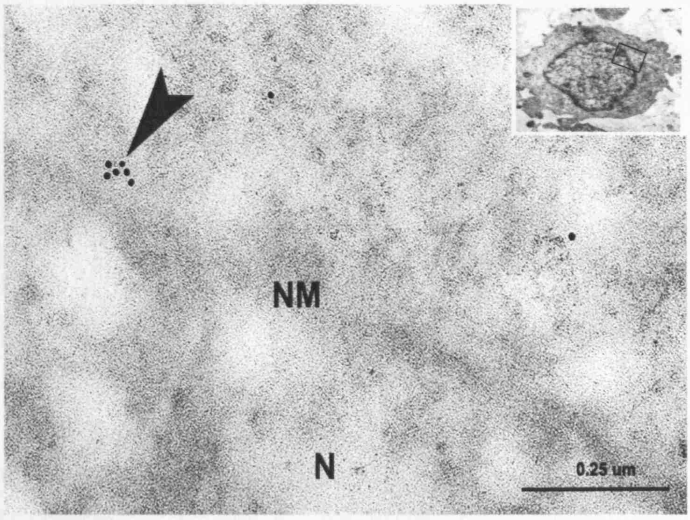
(B)



(C)



(D)



(E)

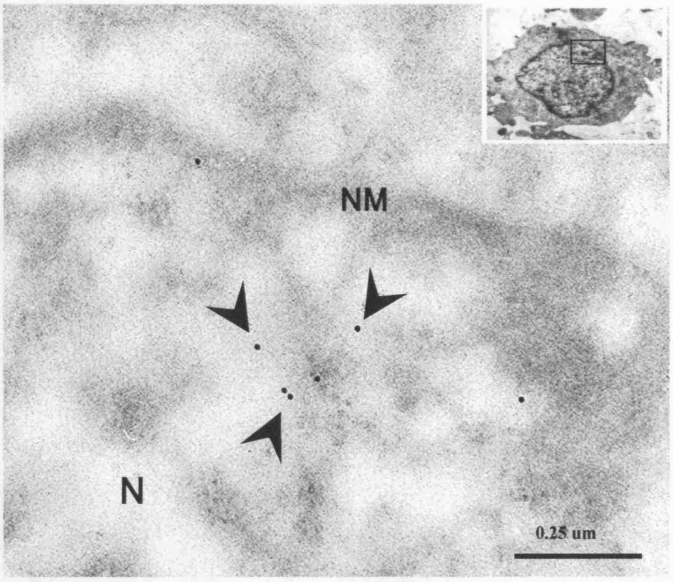
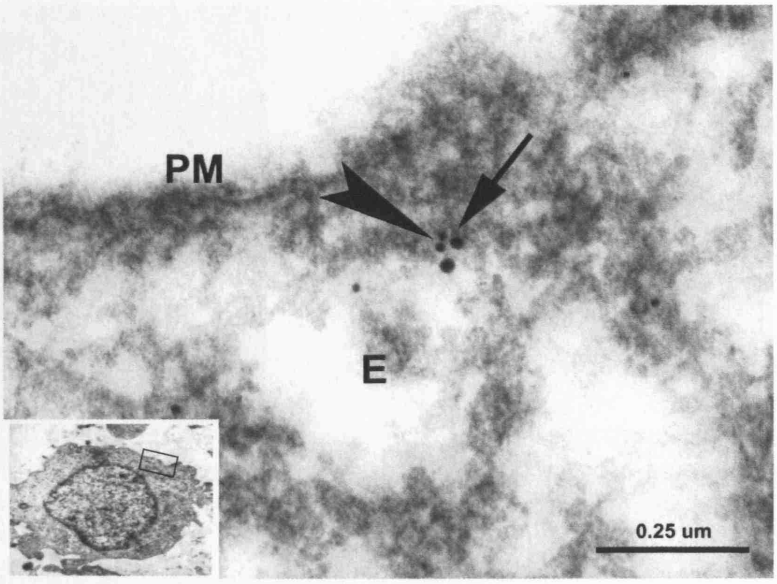


Figure 4.7. Immunogold EM showing the ultrastructural localisation of BMPR-IB in the AB cells. The small round black spheres (arrowheads) are the 10 nm gold particles corresponding to the presence of BMPR-IB. Note the localisation of BMPR-IB at the plasma membrane (A), in an invaginated plasma membrane (B; arrow) and vesicle-like structures near the plasma membrane (B; arrowheads), in membranous cytoplasmic structures (C), at the nuclear membrane (D) and within the nucleus (E). Inset: a sample cell at a low magnification indicating the location from which the immunogold EM were taken. PM, plasma membrane; N, nucleus; NM, nuclear membrane.

(A)



(B)

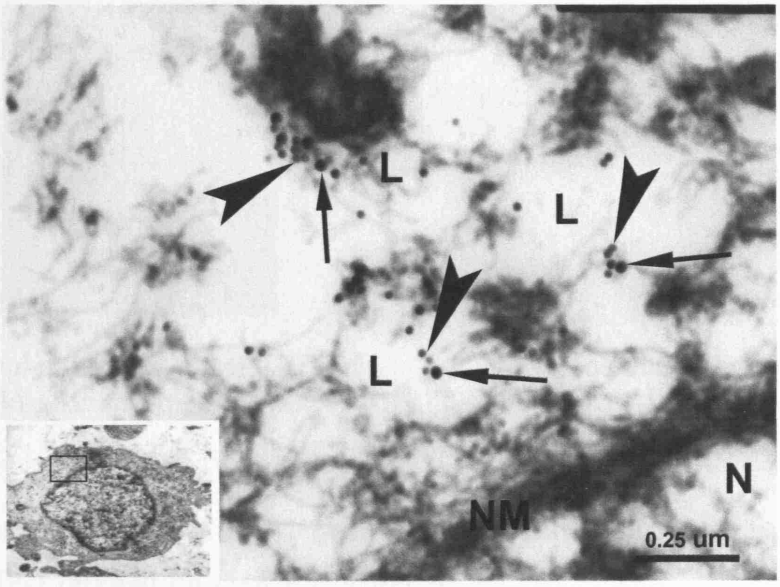
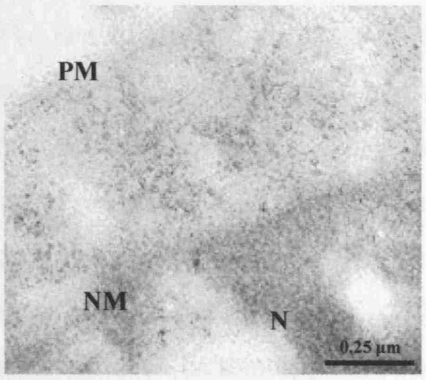


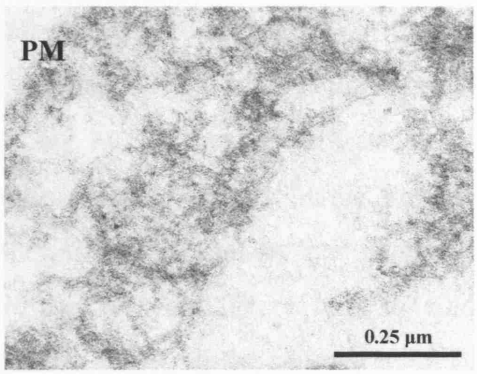
Figure 4.8. Double-labelling immunogold EM demonstrating the co-localisation of BMPR-IB with EEA-1 (A) and LAMP-1 (B) in the AB cells. The small round black spheres (arrowheads) are the 10 nm gold probes corresponding to the presence of BMPR-IB, and the large round black spheres (arrows) are the 15 nm gold probes corresponding to the EEA-1 or LAMP-1. Note the presence of BMPR-IB co-localised with EEA-1 (A) and with LAMP-1 (B). Inset: a sample cell at a low magnification indicating the area from which the immunogold EM were taken. PM, plasma membrane; E, early endosome; L, lysosome; N, nucleus; NM, nuclear membrane.

(A)



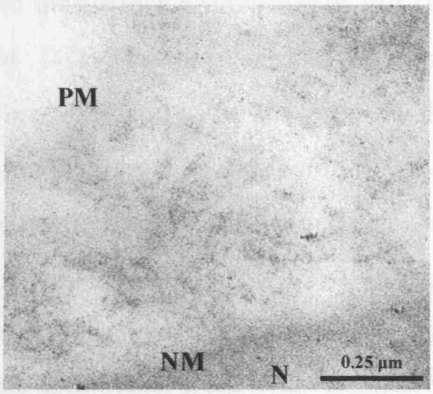
1°: non-specific mouse IgG2a
2°: 10 nm gold-goat anti-mouse IgG

(B)



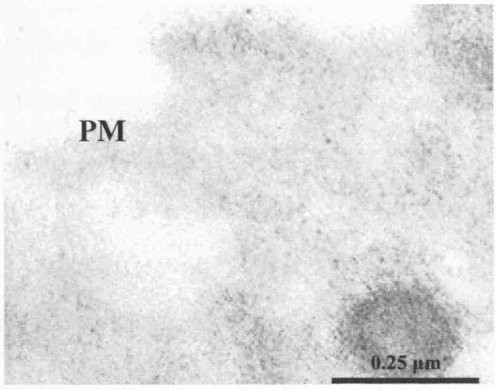
1°: non-specific rabbit IgG
2°: 15 nm gold-goat anti-rabbit IgG

(C)



1°: monoclonal mouse anti-BMPR-IB IgG
2°: 15 nm gold-goat anti-rabbit IgG

(D)



1°: polyclonal rabbit anti-EEA-1 and anti-LAMP-1 IgG
2°: 10 nm gold-goat anti-mouse IgG

Figure 4.9. Immunogold EM showing the negative controls performed for BMPR-IB (A), EEA-1/LAMP-1 (B) and for double labelling of BMPR-IB with EEA-1/LAMP-1 (C and D). The sample sections were immunogold-stained with primary (1°) and secondary (2°) antibodies, as noted below each figure. No gold particle was detected in all the negative control sections. Panel A and B show the negative control sections for BMPR-IB and EEA-1/LAMP-1 primary antibodies, respectively. Note that no cross-reactivities of 15 nm gold-conjugated goat anti-rabbit IgG antibody to mouse anti-BMPR-IB antibody (C) and of 10 nm gold-conjugated anti-mouse IgG antibody to rabbit anti-EEA-1/LAMP-1 antibodies (D) were observed.

4.3.6. C-ELISA analysis of the effects of BMP-2, TGF-β1 and FCS on surface expression of the BMPR

The sensitivity and response of cells to the BMP depend at least partly on the surface expression of BMPR, which could possibly be regulated by growth factors and serum factors. To determine whether BMP-2, TGF-β1 and FCS affect the expression of cell surface BMPR, the AB cells were incubated with these mediators in the presence of cycloheximide (to prevent any new protein synthesis) and the cell surface expression of BMPR measured by C-ELISA after 0, 30, 60, 90 and 120 min. The results showed that none of these factors appeared to have any detectable effect on the relative level of cell surface BMPR-IA compared with the cells at 0 min (control cultures) (Figure 4.10). In contrast, the relative level of surface BMPR-IB protein was found to be up-regulated by TGF-β1 treatment (Figure 4.11B), whereas BMP-2 and FCS had very little, if any, effect on the surface expression of this receptor (Figure 4.11A and 4.11C). Moreover, the effect of TGF-β1 was found to be time-dependent, with the maximal induction at 60 min (approximately 200% of the level found in untreated control cells at 0 min). However, this decreased to the level at approximately 150% of that in untreated control cells after 90 min, and by 120 min decreased further to the same low level present in untreated control cells (Figure 4.11B). In addition, TGF-β1 concentrations of 0.1, 1.0 and 10 ng/ml had the same transient effect on BMPR-IB surface expression, as shown in Figure 4.11B. As with BMPR-IA, the relative level of cell surface BMPR-II was not significantly affected by any of the factors tested ($p>0.05$) (Figure 4.12).

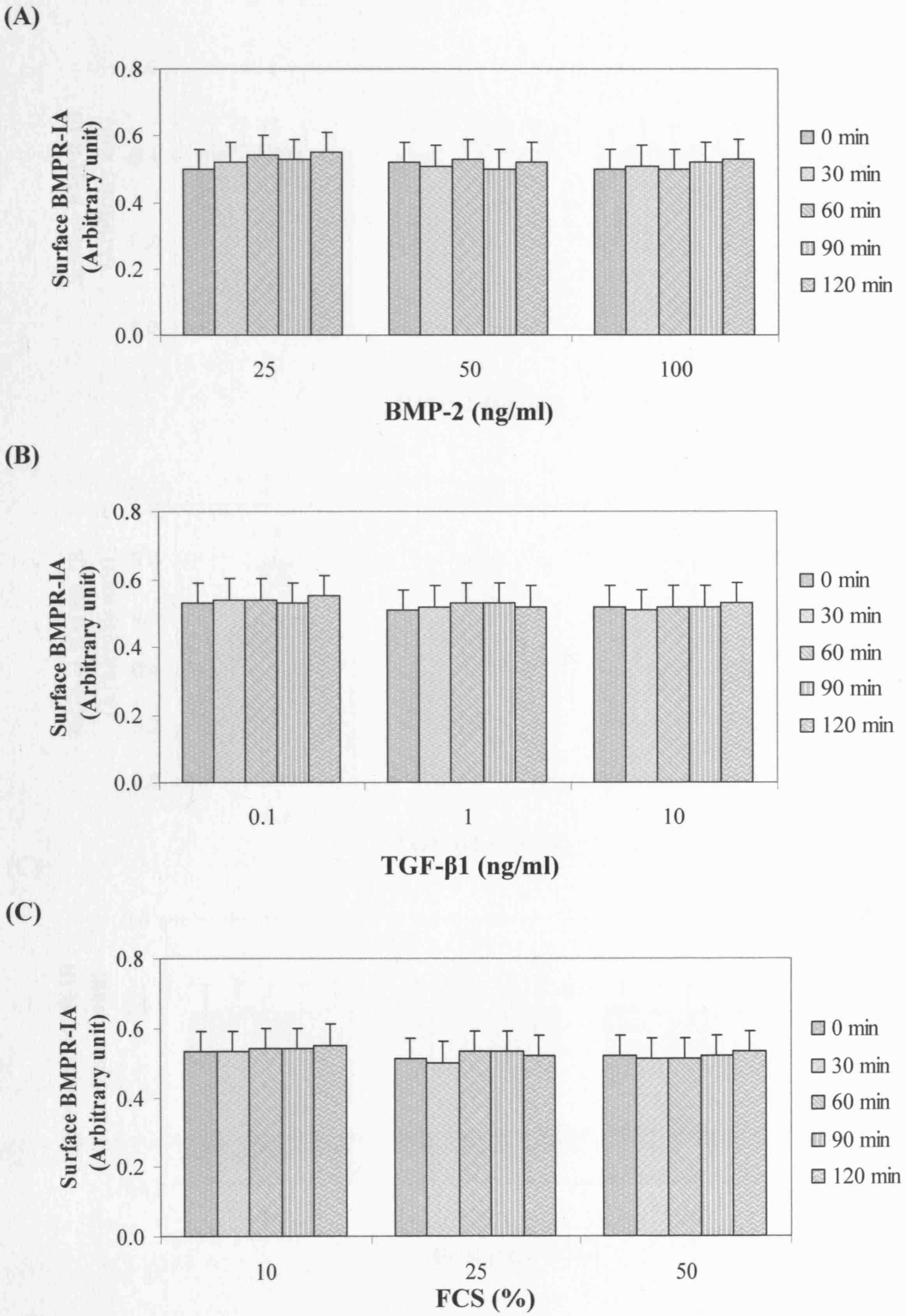


Figure 4.10. Effect of BMP-2 (A), TGF-β1 (B) and FCS (C) on the expression of surface BMPR-IA measured by C-ELISA. The data are presented as the mean ± SE of triplicate experiments.

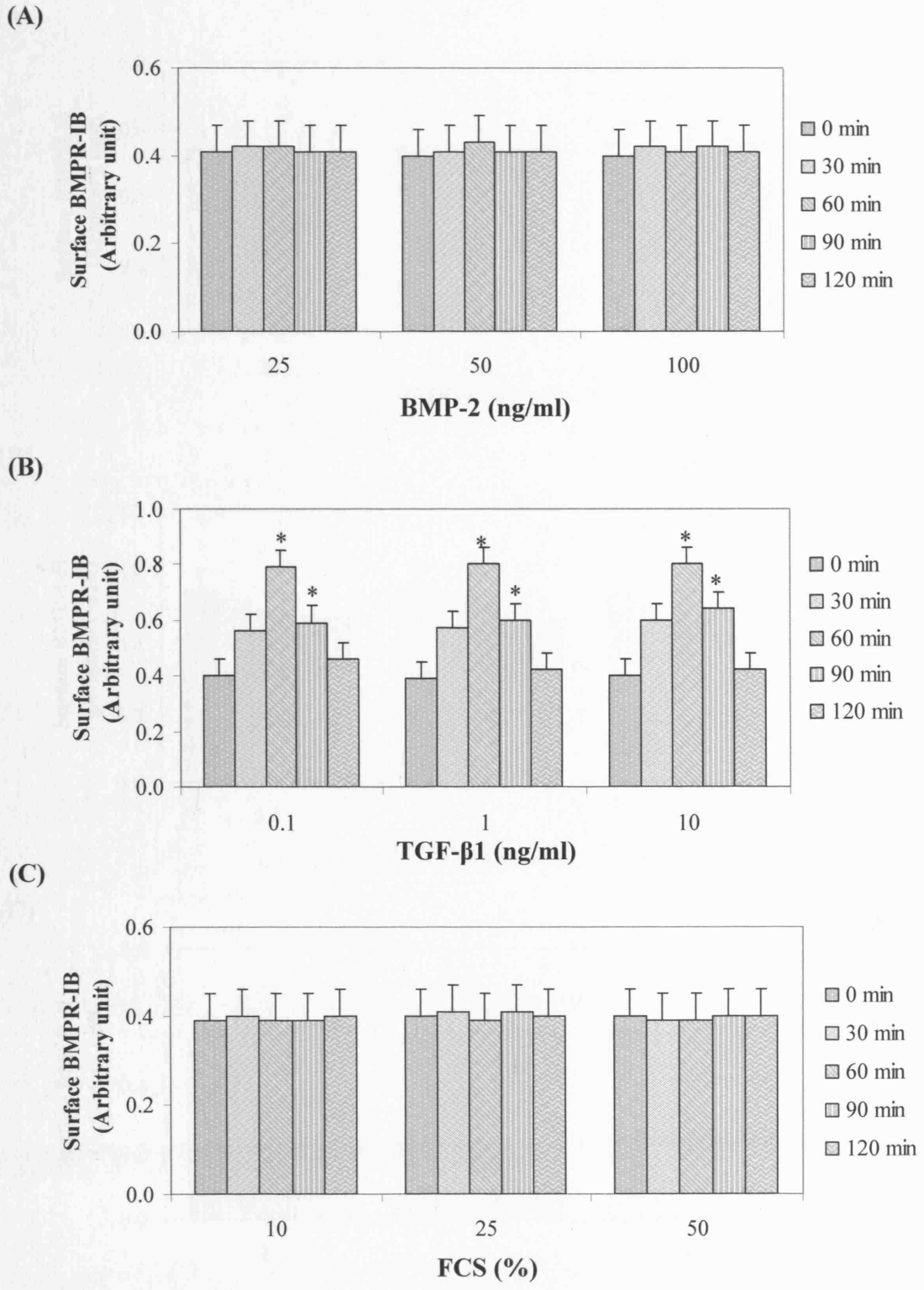


Figure 4.11. Effect of BMP-2 (A), TGF-β1 (B) and FCS (C) on the expression of surface BMPR-IB measured by C-ELISA. The data are presented as the mean ± SE of triplicate experiments.* $p<0.05$ vs untreated control cells (at 0min).

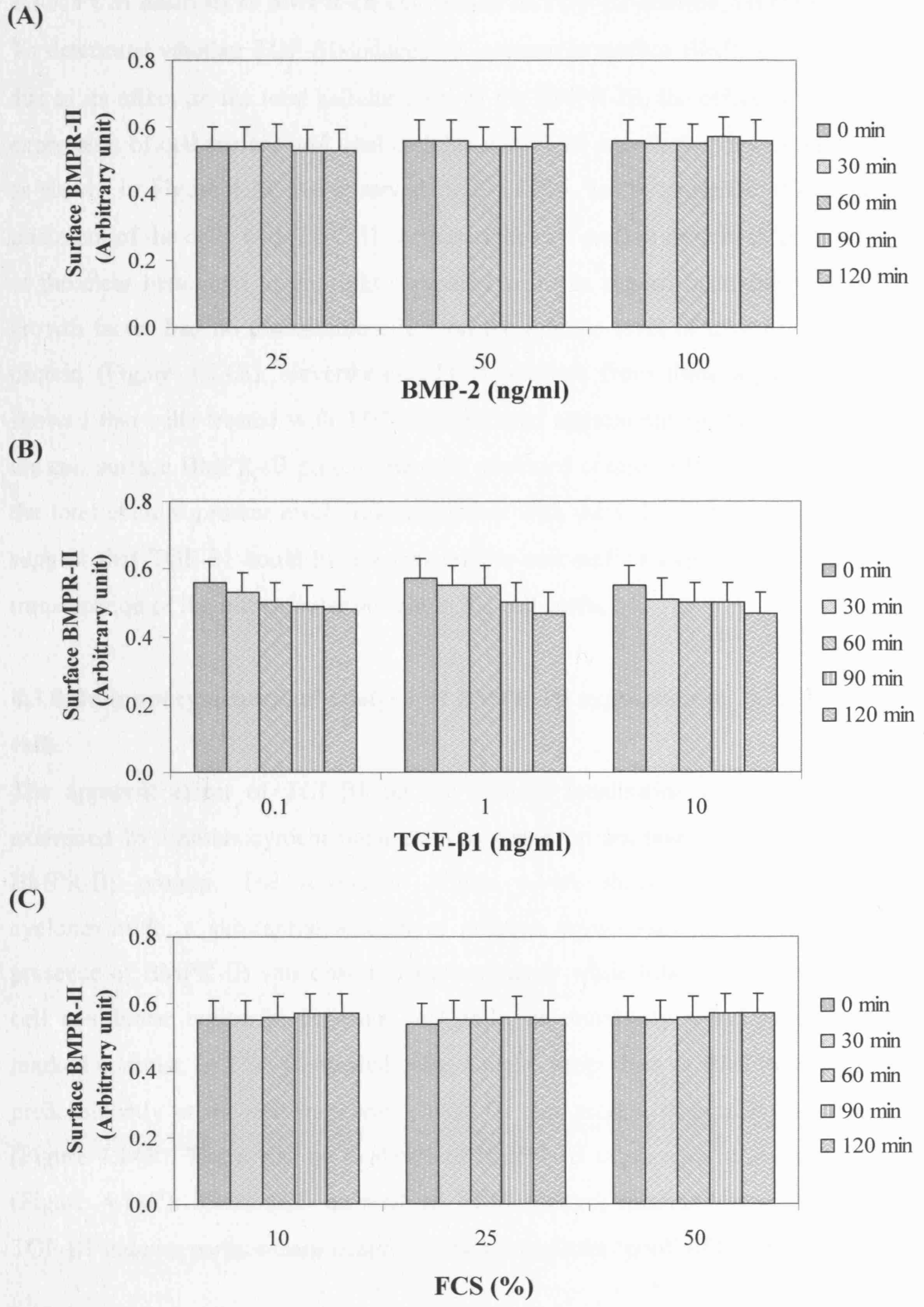


Figure 4.12. Effect of BMP-2 (A), TGF-β1 (B) and FCS (C) on the expression of surface BMPR-II measured by C-ELISA. The data are presented as the mean ± SE of triplicate experiments.

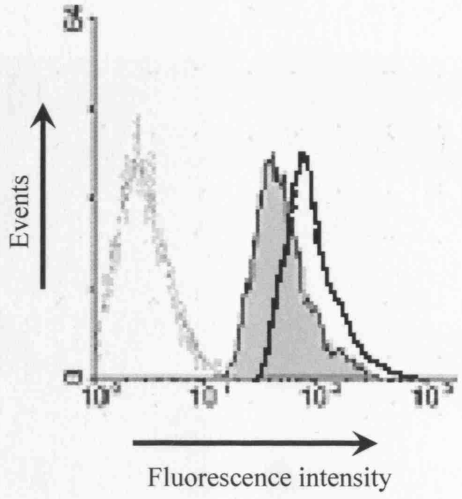
4.3.7. FCM analysis of BMPR-IB expression in TGF-β1-treated AB cells

To determine whether TGF-β1-induced an increase in surface BMPR-IB expression was due to its effect on the total cellular level of the BMPR-IB, the effect of TGF-β1 on the expression of cell surface and total cellular BMPR-IB was further investigated by FCM, as shown in Figure 4.13. As observed by C-ELISA, in the presence of cycloheximide, treatment of the cells with TGF-β1 increased the cell surface protein (Figure 4.13A; shift of the clear histogram to the right compared with the shaded histogram). However, the growth factor had no discernable effect on the relative level of total cellular BMPR-IB protein (Figure 4.13B). Nevertheless, FCM analysis from three separate experiments showed that cells treated with TGF-β1 expressed approximately 2-fold higher levels of the cell surface BMPR-IB protein than the untreated control cells, despite no increase in the total cellular protein level. Taken together with the C-ELISA results, these findings suggest that TGF-β1 could have enhanced the cell surface expression of BMPR-IB by translocation of the intracellular protein to the cell surface.

4.3.8. Immunocytochemical analysis of BMPR-IB expression in TGF-β1-treated AB cells

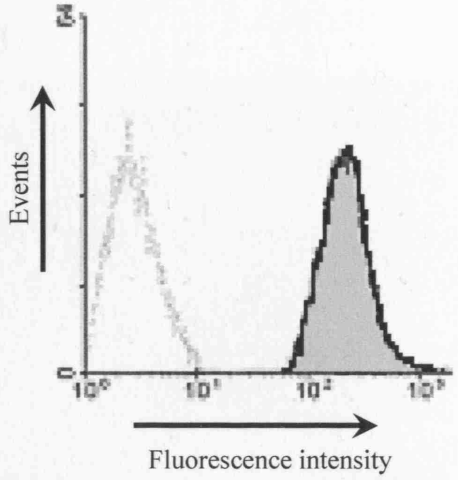
The apparent effect of TGF-β1 on the cellular localisation of BMPR-IB was also examined by immunocytochemical staining using an antibody specific for the human BMPR-IB protein. The results in Figure 4.14A show that in the presence of cycloheximide, a substantial amount of positive brown staining corresponding to the presence of BMPR-IB was observed intracellularly while little protein was found at the cell membrane region in untreated AB cells, as previously noted (Section 4.3.4). In marked contrast, in TGF-β1-treated cells, a major proportion of BMPR-IB was localised predominantly at the cell membrane, with far less protein detectable in the cytoplasm (Figure 4.14B). There was no evidence of BMPR-IB expression in the negative control (Figure 4.14C). Thus, ICC as well as FCM (above) analyses strongly indicate that TGF-β1 induces surface translocation of the intracellular 'pool' of BMPR-IB.

(A) Cell surface BMPR-IB



(195 ± 11%)

(B) Total cellular BMPR-IB



(105 ± 4%)

Figure 4.13. FCM analysis showing the effect of TGF-β1 (0.1 ng/ml for 60 min) on the expression of cell surface (A) and total cellular (B) BMPR-IB. In (A), the increase in the relative level of surface BMPR-IB is shown by the shift of the clear (solid line) histogram (TGF-β1-treated cells) to the right compared with the shaded histogram (untreated cells). In (B), no discernable effect of TGF-β1 on the relative level of total cellular BMPR-IB is detected. The numbers in brackets show the mean percentage increase (± SE) of three separate experiments, defined as 100% in the untreated cells. The dotted histogram represents the fluorescence of the isotype-matched sample used as a control.

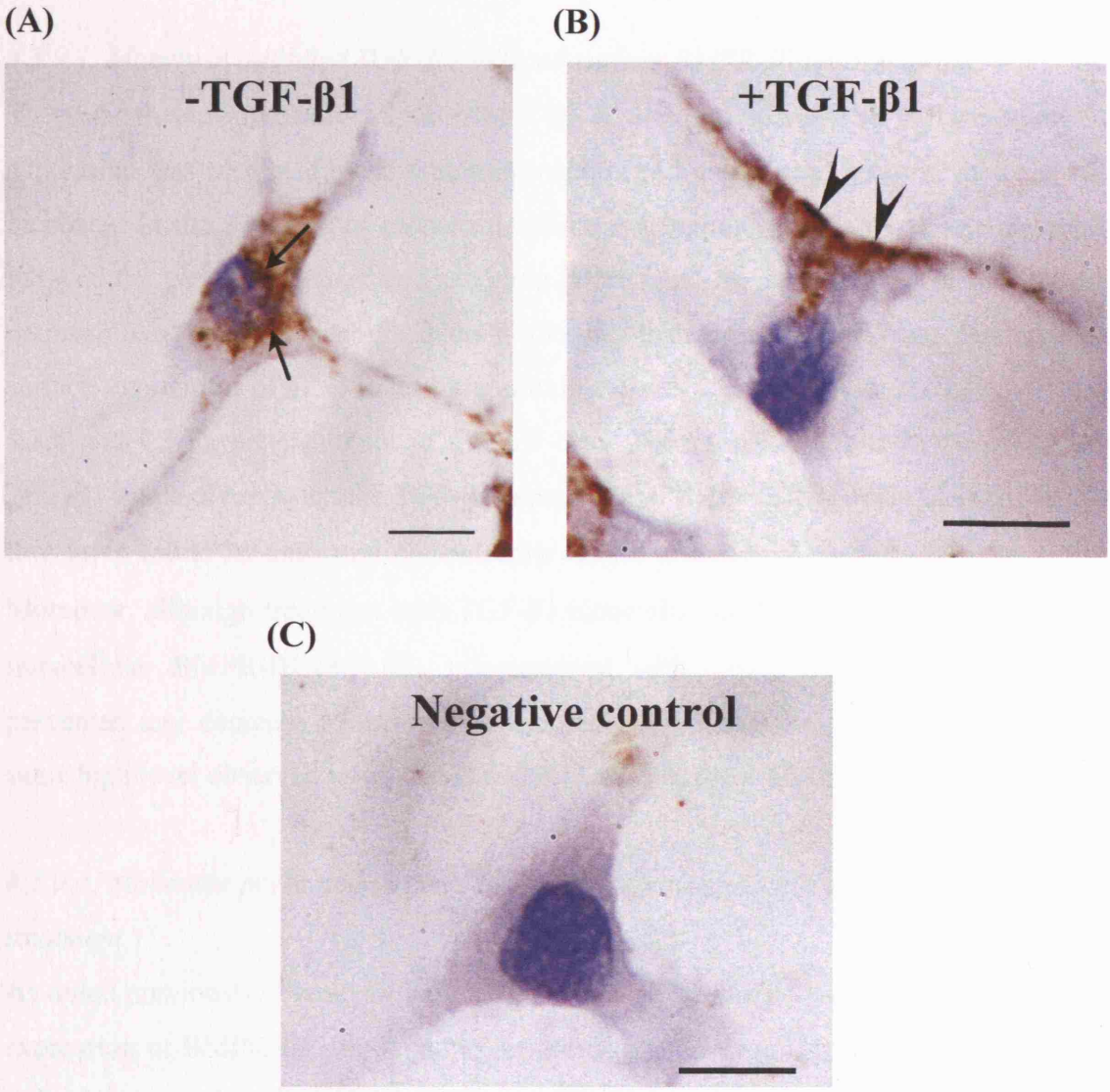


Figure 4.14. Immunocytochemical micrographs showing the expression of BMPR-IB protein in AB cells (A) and in AB cells after TGF-β1 treatment (0.1 ng/ml for 60 min) (B). Note that BMPR-IB in untreated cells (A) was observed mainly intracellularly (arrows), whereas a major proportion of the receptor in TGF-β1-treated cells was found at the cell membrane (arrowheads) (B). There was no evidence of BMPR-IB expression in the negative control (C). Bar = 10 μm.

4.3.9. Effect of monensin on the expression of the BMPR-IB protein in TGF-β1-treated AB cells

4.3.9.1. Monensin inhibited TGF-β1-induced surface BMPR-IB up-regulation

To establish unequivocally whether the TGF-β1-induced increase in surface BMPR-IB expression was mediated by surface translocation of intracellular receptor, the cells were incubated in the presence of monensin, which is generally known to effectively inhibit intracellular protein transport and secretion (Dinter and Berger, 1998). C-ELISA of non-permeabilised and permeabilised cells showed that while TGF-β1 up-regulated cell surface expression of BMPR-IB by approximately 2-fold, as previously noted (Section 4.3.6 and 4.3.7), pre-treatment of the AB cells with monensin prior to the addition of TGF-β1 markedly reduced the TGF-β1-induced BMPR-IB surface expression to the same low level found in untreated control cells in the absence of TGF-β1 (Figure 4.15A). Moreover, although treatment with TGF-β1 alone also significantly reduced the level of intracellular BMPR-IB ($p<0.05$), pre-treatment with monensin prior to TGF-β1 also prevented any decrease in the intracellular level of BMPR-IB, which remained at the same high level observed in untreated control cells (Figure 4.15A).

4.3.9.2. Monensin prevented surface BMPR-IB down-regulation after 90 min of TGF-β1 treatment

As noted previously (Section 4.3.6), after 90 min of incubation with TGF-β1, the surface expression of BMPR-IB was found to progressively decrease. To determine whether this reduction in surface BMPR-IB level was due to internalisation of the surface protein, monensin, which has also been shown to block internalisation (Mueller *et al.*, 2002), was added 60 min after TGF-β1 treatment (the time at maximal TGF-β1-induced surface BMPR-IB expression) to prevent any possible internalisation of the cell surface protein. Measurement of BMPR-IB expression by C-ELISA at 120 min after TGF-β1 stimulation (and 60 min after monensin addition) showed that monensin-treated cells still had a significantly higher level of TGF-β1-induced surface BMPR-IB compared with cells treated with TGF-β1 alone (without monensin) ($p<0.05$) (Figure 4.15B). Moreover, the addition of monensin immediately after the peak of TGF-β1-induced BMPR-IB surface expression (60 min after TGF-β1 addition) concomitantly resulted in a significantly lower

amount of intracellular BMPR-IB compared with that in cells treated with TGF-β1 alone (without monensin) ($p<0.05$) (Figure 4.15B).

Taken together, these findings strongly suggest that increased surface BMPR-IB expression observed within a short period following treatment with TGF-β1 is at least partly mediated by rapid translocation of the intracellular receptor to the cell surface. Moreover, these data also suggest that the subsequent loss of TGF-β1-induced surface BMPR-IB may be due to internalisation of this receptor from the cell surface.

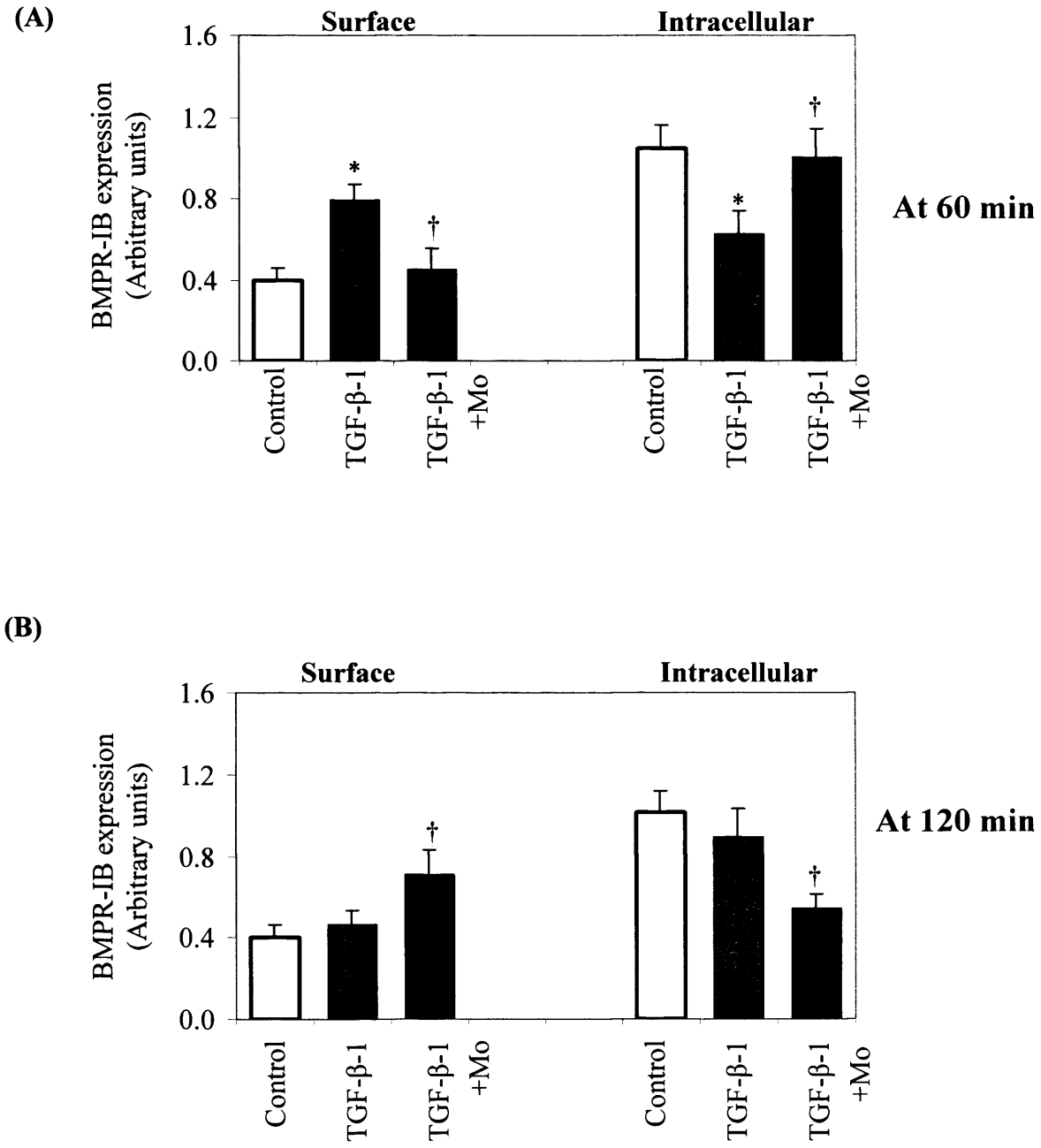


Figure 4.15. C-ELISA analysis of the effect of monensin (Mo) on the expression of cell surface and intracellular BMPR-IB protein at 60 min (A) and 120 min (B) after TGF-β1 stimulation (0.1 ng/ml). In (A), cells were treated with TGF-β1 for 60 min or pre-treated with Mo followed by TGF-β1 treatment for 60 min (in the continuing presence of Mo). In (B), cells were treated with TGF-β1 for 120 min with and without adding Mo at 60 min. The data are presented as the mean ± SE of triplicate experiments.

* $p<0.05$ vs untreated control cells; † $p<0.05$ vs cells treated with TGF-β1 alone.

4.3.10. The role of TGF-β1 signalling pathways in up-regulation of surface BMPR-IB expression

Three MAPK (p38, ERK1/2 and JNK1/2/3), the PKC and the PI3K proteins have been shown to have a major role in intracellular signalling initiated by TGF-β1 (Massague and Chen, 2000; Wilkes *et al.*, 2005). The chemical compounds SB203580, U0126, SP600125, Bis IX and wortmannin are specific inhibitors of these pathways, respectively, as noted in the legend to Figure 4.13, and were used to investigate the signalling pathways involved in the up-regulation of surface BMPR-IB expression observed in TGF-β1-treated cells. A summary of triplicate experiments shows that while the inhibitors alone had little if any effect on 'constitutive' surface BMPR-IB protein expression, two of the inhibitors (SB203580 and Bis IX) significantly ablated TGF-β1-induced up-regulation of BMPR-IB protein on the cell surface, by approximately 78% and 73%, respectively ($p<0.05$) (Figure 4.16). In contrast, U0126, SP600125 and wortmannin, inhibitors of ERK1/2, JNK1/2/3 and PI3K, respectively, had very little effect on BMPR-IB up-regulation by TGF-β1 in the AB cells (less than 5% inhibition). Thus, cell surface up-regulation of BMPR-IB protein induced by TGF-β1 appears to be mediated through two TGF-β-associated pathways, *i.e.,* p38 MAPK and PKC.

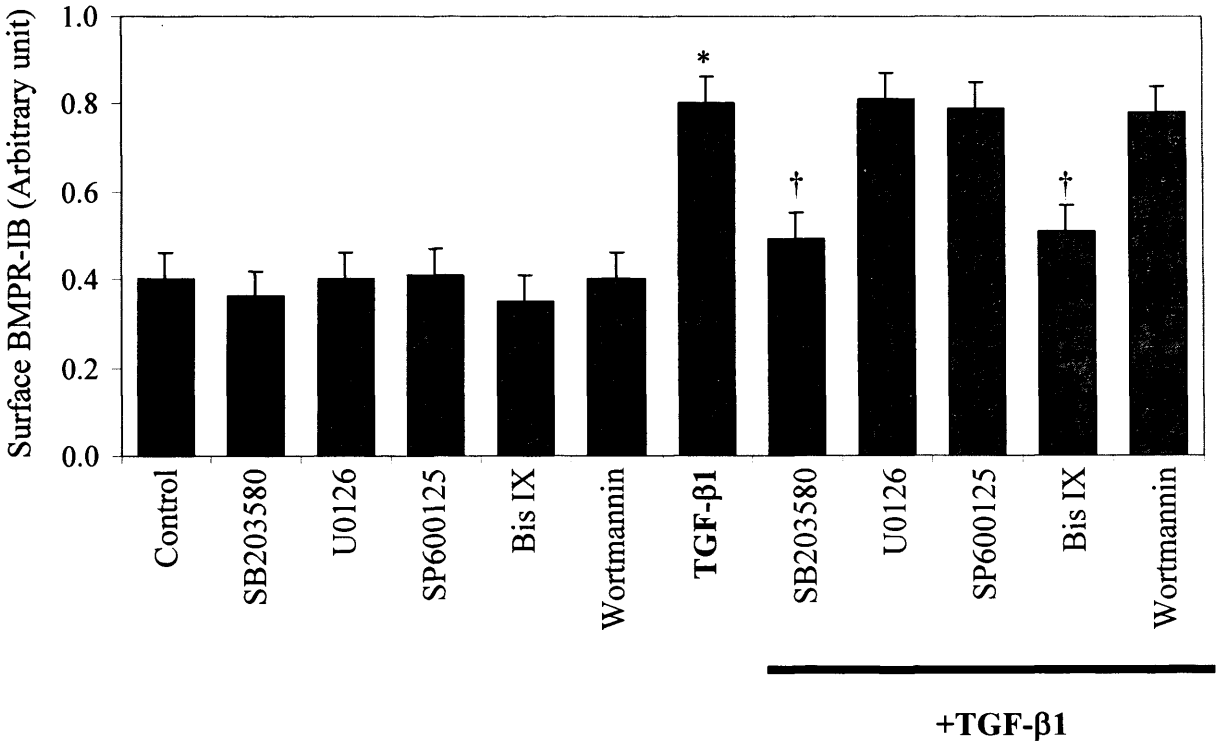


Figure 4.16. C-ELISA analysis of signalling pathways involved in TGF-β1-induced up-regulation of surface BMPR-IB expression. Cells were pre-treated with each of the inhibitors (for 2 h) and then treated with TGF-β1 (0.1 ng/ml for 1 h) in the continuing presence of the inhibitors. The proteins SB203580, U0126, SP600125, Bis IX and wortmannin are specific inhibitors of the p38, ERK1/2, JNK1/2/3, PKC and PI3K, respectively. The samples were then subjected to C-ELISA of non-permeabilised cells. Data are presented as mean ± SE from triplicate experiments. * $p<0.05$ vs control cultures, † $p<0.05$ vs TGF-β1-treated cultures.

4.3.11. Effect of TGF-β1 on BMP-2 binding to AB cells

To determine whether the TGF-β1-induced increase in surface BMPR-IB expression was of functional importance, the binding of BMP-2 to TGF-β1-treated AB cells was measured by C-ELISA, as described in the Materials and Methods. The results in Table 4 show that the relative level of BMP-2 binding to the AB cells markedly and progressively increased during incubation of the cells with TGF-β1, from an initial value of 0.491 units to a maximum of 0.891 units at 60 min (approximately 67% increase in BMP-2 binding compared with the base line value at 0 min). Thereafter, BMP-2 binding, like surface BMPR-IB expression, declined and at 120 min was similar to the 0 min sample. In control experiments to determine the 'basal' level of BMP-2 binding to non-BMPR sites (apart from BMPR-IA, -IB and -II), the AB cells were blocked with three blocking antibodies against BMPR-IA, -IB and -II and then incubated with BMP-2, a relatively low signal of BMP-2 binding of approximately 0.058 unit was detected by C-ELISA using a specific antibody against BMP-2, suggesting the presence of only a very low level of non-BMPR sites on the surface of these cells (Table 4). These data suggest that the increase in surface BMPR-IB expression induced by TGF-β1 results in a significant, if transient, increase in the binding of BMP-2 to the AB cells ($p<0.05$), which is thus likely to enhance signal transduction via the BMPR pathway, as described below (Section 4.3.12).

Table 4. Effect of TGF-β1 on BMP-2 binding to AB cells

Conditions		**BMP-2 binding (Arbitrary units)**	**% Binding increase [(a)]**
Anti-BMPR (2 μg/ml each)	**TGF-β1 (0.1 ng/ml)**		
+	-	0.058 ± 0.009	-
-	0 min	0.491 ± 0.010	-
-	30 min	0.692 ± 0.011	41
-	60 min	0.819 ± 0.009	67*
-	90 min	0.745 ± 0.010	52*
-	120 min	0.511 ± 0.009	4

The data are present as the mean ± SE obtained from triplicate experiments.

* $p<0.05$ vs the level measured at 0 min. [(a)] compared with the level at 0 min.

4.3.12. Effect of TGF-β1 on BMP-2-induced Smad1/5/8 phosphorylation

To determine whether the TGF-β1-induced increase in BMPR-IB surface expression and BMP-2 binding was functionally relevant, the effect of TGF-β1 on the BMP-2-induced phosphorylation of Smad1/5/8 to form p-Smad1/5/8 was measured since these have been considered to be immediate downstream effectors in the BMPR signalling pathway (ten Dijke *et al.*, 2003a). The optimal dose and exposure time (100 ng/ml for 30 min) for the ligand BMP-2 to induce p-Smad1/5/8 formation in the AB cells were previously determined (Appendix II-Figure 1) and used in the experiments below. As shown in Figure 4.17, incubation of the cells with BMP-2 alone resulted in readily detectable levels of p-Smad1/5/8, as previously reported (Yamamoto *et al.*, 1997; Lai and Cheng, 2002; Noth *et al.*, 2003), whereas incubation with TGF-β1 alone either for 60 or 120 min (in the absence of the ligand BMP-2) did not activate Smad1/5/8 phosphorylation. However, pre-incubation of the AB cells with TGF-β1 for 60 min enhanced the level of BMP-2-induced p-Smad1/5/8 by approximately 2.4-fold while pre-treatment with this growth factor for 120 min had little effect on BMP-2-induced p-Smad1/5/8 (Figure 4.17). Thus, the increase in surface BMPR-IB expression induced by TGF-β1 significantly enhanced BMP-2-induced p-Smad1/5/8 in AB cells ($p<0.05$).

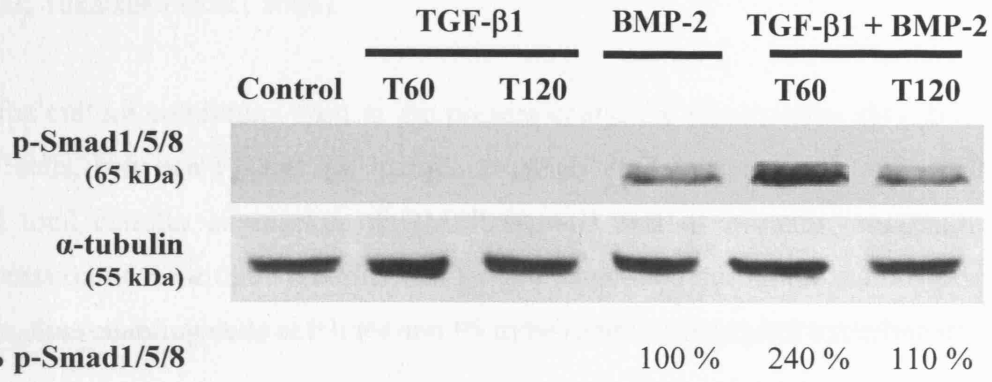


Figure 4.17. WB analysis showing the effect of TGF-β1 on BMP-2-induced Smad1/5/8 phosphorylation. Cells were incubated with TGF-β1 (0.1 ng/ml) for 0, 60 and 120 min (control, T60 and T120, respectively) followed by the addition of BMP-2 (100 ng/ml) for 30 min. The samples were then subjected to WB using antibodies specific for p-Smad1/5/8 and α-tubulin as the internal control. The numbers under the immunoreactive bands show the percentage of Smad1/5/8 phosphorylation, defined as 100% in cells treated with BMP-2 alone.

4.4. Discussion

It has previously been shown that the BMPs exert their functional activities through signal transduction via BMPR (Nohe *et al.*, 2002). The present study has shown, for the first time, that AB cells obtained from three different donors express all three BMPR-IA, -IB and -II transcripts and proteins, demonstrating that these cells are potentially BMP-responsive and that BMPR-IA, -IB and -II might be closely coordinated in the AB cells.

Examination of oral-derived PDL and GF cells as well as the AB cells showed that the expression of the three BMPR proteins was not specific for cells of only the osteoblastic lineage, as has previously been suggested (ten Dijke *et al.*, 1994b; Rosenzweig *et al.*, 1995; Nishitoh *et al.*, 1996), but was also present in non-osseous tissue-derived cells such as the PDL and GF cells. Each of the BMPR proteins was found to be expressed at a generally similar level by all of the three cell types, except for a significantly higher level of BMPR-IB expression by the AB cell. This may suggest a possibly unique functional role of this particular receptor in bone, and it is therefore notable that BMPR-IB has previously been shown to be highly active in transducing intracellular signals for osteoblast differentiation specifically, whereas BMPR-IA may be more important in adipocyte differentiation signalling (Chen *et al.*, 1998) and BMPR-II in BMP-mediated growth control of renal carcinoma and pulmonary artery smooth muscle cells (Kim *et al.*, 2003; Takahashi *et al.*, 2006).

In the culture conditions used in the present study, the results have also shown that the AB cells, between P3 and P5, maintained relatively constant levels of both the surface and total cellular expression of BMPR-IA, -IB and -II proteins, suggesting that the expression of these three proteins was largely unaffected during the culture passages used here, thus enabling cells at P3, P4 and P5 to be used in subsequent experiments.

Although the BMPR have been shown to be expressed in a number of different tissues and cell types (Mehler *et al.*, 1997; Reddi, 1997; Reddi, 2000; Nakashima and Reddi, 2003), the subcellular localisation of these proteins has previously not been investigated, including in bone cells. The results in this study have demonstrated, for the first time, that while a substantial proportion of the BMPR, particularly BMPR-II, is localised at the cell surface, as expected of transmembrane receptors that transduce intracellular signals for

cell growth and differentiation, under the normal cell culture conditions used here only approximately half of BMPR-IA and an even smaller proportion of BMPR-IB (approximately 26%) was detected at the plasma membrane. Since the BMPR are generally considered to be transmembrane proteins (Wu *et al.*, 1996), the present finding that BMPR-IB had a primarily intracellular localisation in the AB cells was thus somewhat unexpected. However, TGFβR-I has also been shown to have a predominantly intracellular localisation in unstimulated mink lung epithelial Mv1Lu and human lung carcinoma A549 cells (Zwaagstra *et al.*, 2000), and steroid hormone receptors are normally localised in the cytoplasm and nucleus, exerting their biological function directly as transcription factors after ligand binding in the cytoplasm (Lee and Chang, 2003). While the present results provide the first evidence of a predominantly intracellular localisation of BMPR-IB, further studies are required to determine whether the relatively high level of intracellular BMPR-IB has any functional role in the AB cells *in vitro* and whether this receptor also has a similar subcellular localisation *in vivo*.

Ultrastructural analysis using immunogold EM also revealed the presence of BMPR-IB protein in an "invaginated plasma membrane", a structure known to be characteristic of internalisation via endocytosis, which has previously been reported to occur for BMP/BMPR complexes *in vitro* (Jortikka *et al.*, 1997; Nohe *et al.*, 2005; Hartung *et al.*, 2006). In addition, the presence of the intracellular BMPR-IB within the endosomal/lysosomal compartment, as demonstrated by double labelling immunogold EM, further reflects the internalisation of this protein via endocytosis and subsequent intracellular transport processes. These are well-known to regulate the normal turnover of many cell surface receptors (Anders *et al.*, 1997; Jortikka *et al.*, 1997; Hayes *et al.*, 2002; Wang *et al.*, 2002; Di Guglielmo *et al.*, 2003; Wang *et al.*, 2003), and it is therefore possible that endocytic internalisation may also play an important part in the regulation of BMPR expression in AB cells. Recently, the BMPR localised in the endocytic pathway have been shown to have paramount biological function on their downstream signalling cascades, *e.g.*, Smad and p38 signalling pathways (Nohe *et al.*, 2005; Hartung *et al.*, 2006), as has previously been reported for EGFR- and PDGFR-mediated signal transduction from endosomes (Sorkin, 2001; Wang *et al.*, 2003).

Although the reason(s) for the presence of high levels of intracellular BMPR-IB are still unclear, it has previously been suggested that in the absence of the respective ligand, a number of other transmembrane growth factor receptors are capable of undergoing endocytosis via ligand-independent internalisation (Seto *et al.*, 2002). Thus, in the present study, it is possible that surface-associated BMPR could have been internalised by such a process. However, in the absence of cognate ligands, cell surface growth factor receptors are only very slowly internalised and are then rapidly recycled back to the plasma membrane, resulting in their accumulation at the cell surface, as is usually observed (Seto *et al.*, 2002). It is thus unlikely that the presence of intracellular BMPR-IB observed in unstimulated AB cells (*i.e.,* in the absence of the ligand) used in this study resulted from ligand-independent internalisation of cell surface BMPR, although it is possible that BMPs secreted from the AB cells themselves could act in an autocrine manner and prompt the internalisation of BMPR, as was evident by the co-localisation of BMPR-IB with the early endosomal/lysosomal compartments (Figure 4.8).

Alternatively, the presence of cytoplasmic BMPR-IB may represent newly-synthesised receptors. Although detailed information concerning the biosynthesis and processing of BMPR is thus far unavailable, analysis of TGFβR biosynthesis has indicated that this receptor is not wholly transported to the plasma membrane, with up to 50% of newly synthesised receptors remaining in the ER (Koli and Arteaga, 1997; Wells *et al.*, 1997). It is therefore possible that in the absence of a specific ligand or other stimulatory factor(s), as in the present experiments, the presence of intracellular BMPR-IB may also reflect the accumulation and retention of a 'pool' of newly-synthesised receptor, possibly in the ER/Golgi compartments. These would be available to be rapidly transported to the cell surface and exert their signal-transducing activity in the presence of the ligand.

Notably, under the EM, BMPR-IB staining was also observed at the nuclear membrane and within the nucleus, possibly as a result of a nuclear translocation process as has previously been reported for TGFβR-I (Zwaagstra *et al.*, 2000), FGFR (Sobue *et al.*, 2002) and EGFR (Wells and Marti, 2002). This observation suggests that BMPR-IB, and thus possibly the BMP/BMPR complex, might also directly induce BMP target gene transcription in the nucleus, for example, by up-regulating the osteoblast-specific Cbfa1 transcription factor (Ducy *et al.*, 1997). As noted previously, direct transcriptional activity

is the major mechanism for the action of steroid-steroid receptor complexes (Lee and Chang, 2003) and also the prostaglandin E_2-E prostanoid receptor complex (Schlotzer-Schrehardt *et al.*, 2002) on target cells.

The expression and re-organisation of BMPR on the cell surface are very likely to play an important part in BMP signalling and hence the biological activity of the BMP (Gilboa *et al.*, 2000; Nohe *et al.*, 2003). Although TGF-β1, BMP-2 and serum factors have previously been reported to affect the cellular redistribution of a number of receptors, such as TGFβR and FGFR as well as BMPR-IA and -II (Zwaagstra *et al.*, 2000; Sobue *et al.*, 2002; Nohe *et al.*, 2003), the results in the present study have shown that TGF-β1 was the only one of these factors capable of modulating the surface translocation of BMPR in the AB cells. Moreover, these results also showed, for the first time, that TGF-β1 markedly enhanced the level of cell surface BMPR-IB specifically by increasing the translocation of this receptor from an 'intracellular pool' to the plasma membrane. In contrast to BMPR-IB, the surface expression of BMPR-IA and -II was largely unaffected by any of the mediators, possibly because of the absence of a large cytoplasmic pool of these receptors, as previously shown in Section 4.3.3 and 4.3.4.

Under the experimental conditions used here, in which new protein synthesis was inhibited, it is unlikely that the increased cell surface expression of BMPR-IB induced by TGF-β1 treatment was due to an increase in new BMPR-IB protein synthesis. The results have also shown that monensin, an inhibitor of intracellular protein transport and secretion (Dinter and Berger, 1998), was able to ablate the TGF-β1-induced increase in BMPR-IB surface expression, suggesting that the stimulatory effect of this growth factor may be mediated through intracellular protein transport, *i.e.,* surface translocation of BMPR-IB. However, this cellular redistribution was only transient, and it is unclear from the present experiment why the TGF-β1-increased level of surface expression of BMPR-IB subsequently decreased to a lower level after 90 and 120 min of incubation with TGF-β1. A similar transient up-regulation has previously been reported for a chemokine receptor in human monocytes (Green *et al.*, 2006). The present results also showed that at later times monensin, which also inhibits internalisation of surface proteins (Mueller *et al.*, 2002), blocked the transport of the TGF-β1-induced surface BMPR-IB back to the cytoplasm (Figure 4.15B). It is thus likely that the more prolonged exposure to TGF-β1

(in the absence of monensin) resulted in the internalisation of this receptor, possibly reflecting cellular mechanisms of homeostatic control of intracellular and plasma membrane receptor levels. Such processes may involve a number of highly regulated, dynamic intracellular trafficking processes, including ER/Golgi-cell surface export, which is known to be under the control of intracellular processing, glycosylation and specific export signals of an individual protein (Duvernay *et al.*, 2005). While the precise mechanism by which TGF-β1 induces the surface translocation of BMPR-IB specifically is still unclear, by using inhibitors of key components of TGF-β1 signalling, the present study has revealed that the cell surface up-regulation of BMPR-IB protein induced by TGF-β1 is mediated via p38 MAPK and PKC. The former has recently been shown to play an important role in rapid up-regulation of the chemokine receptor CX3CR1 surface expression (Green *et al.*, 2006) while the role of the latter in the regulation of intracellular trafficking of other receptors has also been reported (Lu and Ziff, 2005; Mundell *et al.*, 2006).

As a result of TGF-β1-induced BMPR-IB surface translocation and surface expression, the results have also shown that AB cells treated with TGF-β1 possess a greater BMP-2 binding capacity. The biological importance of the increase in BMP-2 binding in AB cells induced by TGF-β1 was demonstrated by an increase in the level of Smad1/5/8 phosphorylation, immediate downstream effectors in the BMPR signalling pathway (ten Dijke *et al.*, 2003a). Moreover, since new protein synthesis was inhibited by cycloheximide, it is unlikely that the higher level of BMP-2-induced phosphorylated Smad1/5/8 elicited by incubation with the TGF-β1 was due to an increase in the level of Smad1/5/8 protein expression. These findings suggest that transient TGF-β1-induced surface translocation of BMPR-IB is functionally important in BMP/BMPR signalling in AB cells, similar to a previous report of osteogenesis induced by a short exposure (15 min) of adipose tissue-derived mesenchymal stem cells to BMP-2 (Knippenberg *et al.*, 2006).

In conclusion, the results show that AB cells express all three BMPR transcripts and proteins. Moreover, the BMPR were found to have unique profiles of subcellular distribution not only at the plasma membrane but also in a number of intracellular compartments and the nucleus, with possibly important functional consequences for the

response of target cells to the BMP. Moreover, the results in the present study have shown, for the first time, that TGF-β1 transiently up-regulated the expression of cell surface BMPR-IB through stimulation of BMPR-IB surface translocation, which subsequently resulted in enhanced responsiveness of the AB cells to the BMP-2, *i.e.*, increased BMP-2 binding and Smad1/5/8 phosphorylation.

CHAPTER 5
REGULATION OF CELL SURFACE BMPR BY SHEDDING

5.1. Introduction

Post-translational regulation of cell surface receptors has previously been shown to occur, at least partly, by internalisation via endocytosis and by ectodomain shedding from the cell surface (Anders *et al.*, 1997; Dello and Rovida, 2002; Hayes *et al.*, 2002; Di Guglielmo *et al.*, 2003), thereby reducing ligand binding and the initiation of downstream signal transduction. For example, types-I and -II TGFβR (Dore, Jr. *et al.*, 2001) and the BMPR (Jortikka *et al.*, 1997; Nohe *et al.*, 2005; Hartung *et al.*, 2006) have been shown to undergo endocytic internalisation. The process of proteolytic shedding has also been shown to down-regulate a wide range of surface proteins including adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cadherin and E-selectin (Garton *et al.*, 2003), and cell surface growth factor receptors, such as TGFβR, FGFR, PDGFR and EGFR (Lopez-Casillas *et al.*, 1991; Tiesman and Hart, 1993; Philip *et al.*, 1999; Hanneken, 2001; Dello and Rovida, 2002). The shedding of a transmembrane receptor can result in a 'soluble' form of the receptor (Dello and Rovida, 2002), although soluble forms of transmembrane proteins have also been shown to be generated by the formation of alternatively spliced mRNA transcripts (Rose-John and Heinrich, 1994; Muller-Newen *et al.*, 1996a), for example the sIL-6R isoform (Muller-Newen *et al.*, 1996b). The molecular mechanism and biological importance of shedding are discussed in Section 1.4.2.

While shedding could also regulate cell surface BMPR expression and thereby at least partly control cell responses to the BMP, this process has not previously been investigated. The present study therefore examined whether 'soluble' forms of the BMPR (sBMPR) could be shed from AB cells *in vitro* following treatment with a previously identified potent shedding inducer, *i.e.*, phorbol 12-myristate 13-acetate (PMA), which has generally been considered to be the most common inducer of ectodomain shedding, (Dello and Rovida, 2002; Vermes *et al.*, 2002). Stimulation of cells with PMA has been shown to result in PKC activation, a common signalling target of shedding of various cell surface proteins including growth factor receptors (Dello and Rovida, 2002). The

functional consequences of BMPR shedding were also examined, by measuring phosphorylation of Smad1/5/8, an early activation step in the BMPR pathway (ten Dijke *et al.*, 2003a).

5.2. Materials and Methods

Cell culture, RT-PCR, FCM and WB were performed as described in Chapter 2. To examine whether alternatively spliced forms of the BMPR were produced in response to PMA treatment which could have generated sBMPR, the primers that amplify the transcript sequences corresponding to the transmembrane domain and a part of the extracellular region of each of the three BMPR as indicated in Table 3 were used in order to detect possible alternatively spliced isoform(s) which lack exons coding for both the transmembrane and cytoplasmic domains, as has been reported for other 'soluble' receptors (Rose-John and Heinrich, 1994; Muller-Newen *et al.*, 1996a). Additional methods used in this chapter are described below.

5.2.1. Treatment of cells

Exponentially growing cultures of AB cells were subjected to serum starvation (1% FCS) for 24 h and then treated with 20 ng/ml of PMA (Sigma) in fresh medium containing 1% FCS for 4 h, the condition of which has previously been used for PMA-induced shedding of sIL-6R in primary human osteoblasts (Vermes *et al.*, 2002). In some experiments, 100 ng/ml of BMP-2 were also added to the cultures for 30 min (in the continuing presence of PMA) to initiate BMP/BMPR signalling. At the concentration used here, PMA was found to cause less than 5% cell death, as determined by trypan blue exclusion tests performed in duplicate. FCM analysis and RNA and protein extraction were carried out on untreated and PMA-treated cells as described below, and tissue culture media supernatants were collected and stored at -20°C until subsequent analysis.

5.2.2. Preparation of cell culture supernatants

Supernatants of treated and untreated cultures were decanted and centrifuged at 2000 rpm at 4°C for 10 min, then desalted by dialysis using Spectra/Por® 3500 MW cut-off dialysis tubing (Medicell International Ltd., London, UK). For each sample, 100 ml of supernatant collected from the cell cultures were dialysed in distilled water at 4°C for 2 h

and evaporated overnight at RT to a final volume of 200 µl. The concentrated supernatant samples were then incubated with 50 mg of protein G polystyrene beads (6.7 µm, 0.5% w/v) (Spherotech Inc., Libertyville, IL, USA) for 6 h at 4°C to remove non-specific protein G binding proteins. After centrifugation at 1000 rpm at 4°C for 5 min, the concentrated supernatant samples were collected and immunoprecipitated as described in Section 5.2.3.

5.2.3. Immunoprecipitation of concentrated supernatant samples

Four micrograms of each of the primary anti-BMPR antibodies, which recognises a specific epitope in the extracellular domain of each of the BMPR, was incubated with 50 mg of protein G polystyrene beads in 200 µl of PBS containing 1% BSA, for 6 h at 4°C. The beads were then washed twice in PBS containing 1% BSA. After centrifugation at 1000 rpm at 4°C for 5 min, the BMPR antibody-coated protein G polystyrene beads were incubated overnight at 4°C with 200 µl of the concentrated supernatant samples, obtained as described in Section 5.2.2, then washed twice with PBS containing 1% BSA. The samples were then centrifuged at 1000 rpm at 4°C for 5 min to obtain the bead pellet, after which sBMPR were then eluted from the beads by treatment with 18 µl of 100 mM glycine, pH 3.0, for 5 min at RT. After centrifugation at 1000 rpm at 4°C for 5 min, the eluted supernatants were neutralised with 2 µl of 1.5 M Tris, pH 8.8, and the final volume of 20 µl for each sample was re-suspended in 5 µl of 5x reducing WB sample buffer. The immunoprecipitated supernatants (25 µl for each sample) were subjected to SDS-PAGE and WB as described in Section 2.7.2.

5.2.4. BMP-2 binding assay

To determine whether sBMPR is functionally relevant, the ability of sBMPR to bind to the ligand BMP-2 was also examined. After the immunoprecipitated supernatants (from Section 5.2.3) were subjected to SDS-PAGE and the sBMPR transferred to a PVDF membrane, as described in Section 2.7.2.2, the membrane was incubated in 5% BSA in PBS (blocking buffer) for 1 h at RT. It was then treated with 1 µg/ml of BMP-2 (R&D Systems) in blocking buffer for 2 h at RT and subjected to immunoblotting, as described in Section 2.7.2.3, using biotinylated goat anti-human BMP-2/4 primary antibody (R&D systems) (1:1000 for 3 h) and streptavidin-HRP (1:2500 for 1 h). The immunoreactive

bands, representing the presence of BMP-2 binding, were visualised as described in Section 2.7.2.3.

5.3. Results

5.3.1. Effect of PMA on BMPR expression

In the present study, PMA was used to examine whether the BMPR could also be induced to undergo shedding by this phorbol ester, by examining its effect on the level of cell surface BMPR expression. The results of FCM analysis, carried out using antibodies specific for the extracellular domains of the three BMPR as described in Section 2.3.2, showed that incubation of the AB cells with PMA (20 ng/ml for 4 h) resulted in a decrease in cell surface expression of BMPR-IA, -IB and -II by approximately 52%, 54% and 43%, respectively, compared with that of untreated cells (Figure 5.1).

5.3.2. Detection of sBMPR in PMA-treated AB cell cultures

To determine whether the PMA-induced decrease in BMPR cell surface expression concomitantly generated a 'soluble' form of the BMPR, as has previously been reported for other 'soluble' receptors (Mullberg *et al.*, 1995; Vermes *et al.*, 2002), the culture supernatants of the PMA-treated cells were concentrated, immunoprecipitated and subjected to WB as described in the Materials and Methods. The results in Figure 5.2 show that 'soluble' forms of the BMPR-IA, -IB and -II were detected by SDS-PAGE, as approximately 18, 18 and 20 kDa polypeptides, respectively. Thus, simultaneously with the reduction of cell surface expression of the three BMPR examined, PMA treatment also resulted in the presence of the respective soluble proteins.

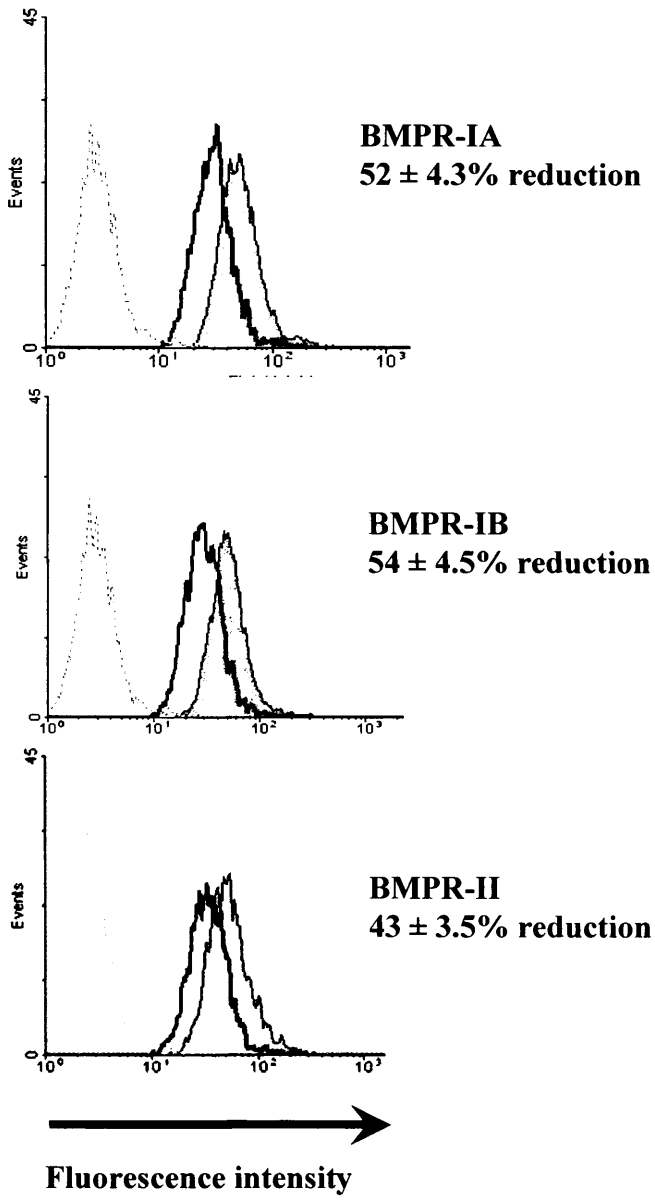


Figure 5.1. Effect of PMA on BMPR expression by AB cells. Cells were incubated with 20 ng/ml of PMA for 4 h and the surface expression of BMPR analysed by FCM. The reduction in the relative levels of the BMPR in PMA-treated cultures is shown by the shift of fluorescence (clear histograms, solid lines) to the left compared with the untreated control cultures (shaded histograms). The numbers show the mean percentage reduction (± SE) of the PMA-treated cultures of three separate experiments. The dotted histograms are the background fluorescence profiles of the isotype-matched control samples.

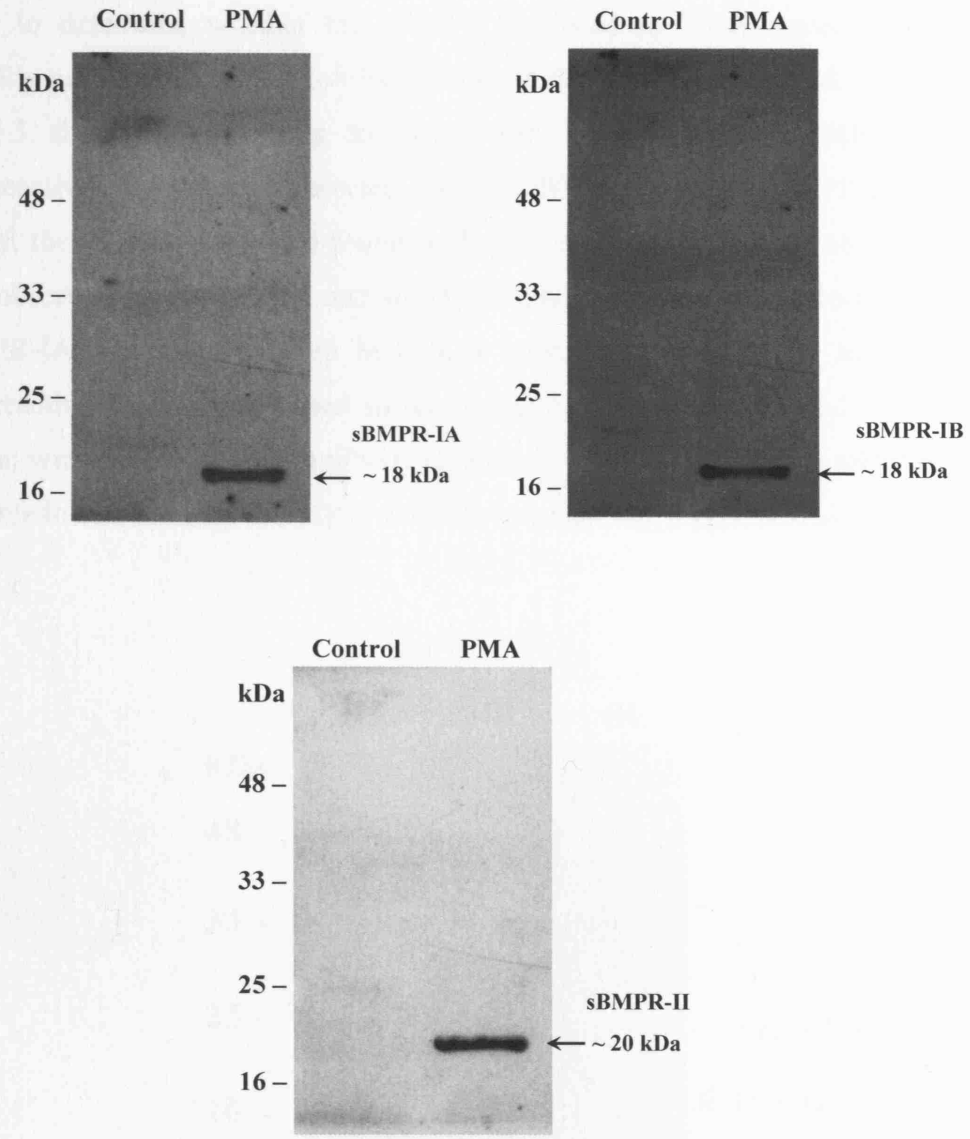


Figure 5.2. Effect of PMA on sBMPR expression. Cells were incubated with 20 ng/ml of PMA for 4 h and the presence of the three sBMPR in the cell culture supernatants determined by immunoprecipitation and WB. The immunoreactive (black) bands detected in the PMA-treated culture supernatant show the electrophoretic migration of the approximately 18, 18 and 20 kDa polypeptides corresponding to 'soluble' forms of BMPR-IA, -IB and -II, respectively. No immunoreactive bands were detected in the untreated (control) culture supernatant.

5.3.3. Binding of BMP-2 to sBMPR in PMA-treated cell culture supernatants

In order to determine whether the sBMPR produced in PMA-treated cultures was functionally active, their ability to bind the ligand BMP-2 was examined. The results in Figure 5.3 show that following treatment with BMP-2 and anti-BMP-2 antibody, immunoreactive bands were detected in the sBMPR-IA and sBMPR-IB samples. Moreover, these bands were also found in the same position (approximately 18 and 18 kDa) as observed for sBMPR-IA and sBMPR-IB, respectively, demonstrating the ability of sBMPR-IA and sBMPR-IB to bind their cognate ligand BMP-2. In contrast, no immunoreactive band was detected in the immunoprecipitated sBMPR-II sample after incubation with the anti-BMP-2 antibody (Figure 5.3), showing that only sBMPR-IA and -IB are able to bind the ligand BMP-2 at levels detectable by this procedure.

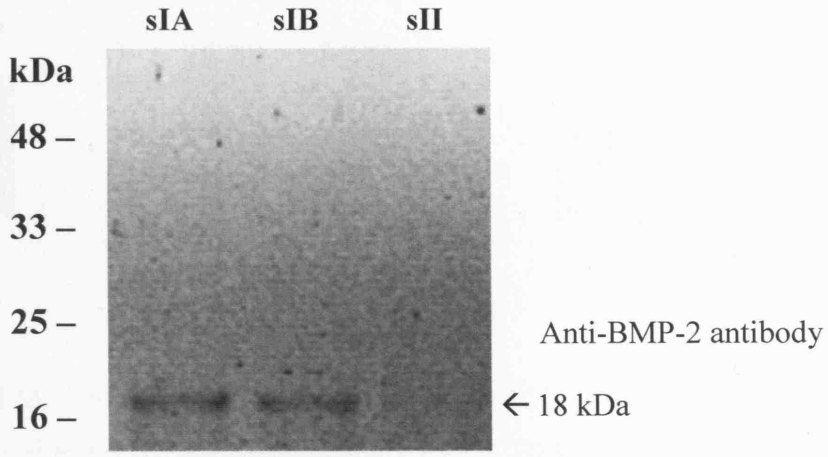


Figure 5.3. Binding of BMP-2 to the sBMPR obtained from PMA-treated AB cell culture supernatants. The cultures were incubated with 20 ng/ml of PMA for 4 h and the supernatants collected and concentrated. Immunoprecipitated samples obtained using each of the BMPR antibodies were prepared and transferred to a PVDF membrane and subjected to the BMP-2 binding assay as described in Section 5.2.4. The immunoreactive (black) bands detected in the immunoprecipitated sBMPR-IA (sIA) and sBMPR-IB (sIB) samples show the presence of BMP-2 binding to these respective sBMPR. No immunoreactive band was detected in the immunoprecipitated sBMPR-II (sII) sample.

5.3.4. Effect of PMA on the relative levels of intracellular BMPR

The relative intracellular level of each BMPR protein was calculated from the cell surface and total cellular protein level data obtained by FCM, as described in Section 2.3.2. The results in Figure 5.4 show that PMA stimulation (20 ng/ml for 4 h) had very little, if any, effect on the relative levels of intracellular BMPR-IA, -IB or -II proteins in the AB cells. Thus, while PMA treatment induced the shedding of soluble forms of all three BMPR concomitant with a decrease in the surface-associated level of these proteins, it had no effect on the intracellular 'BMPR pool'.

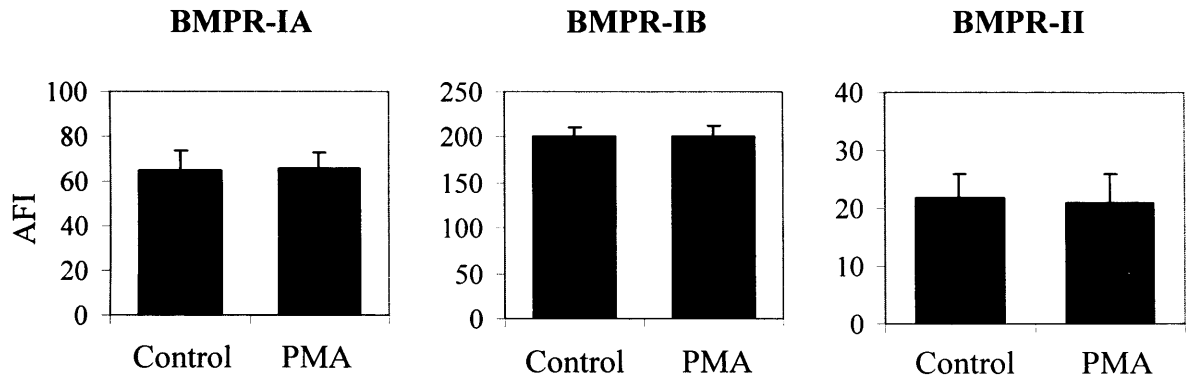


Figure 5.4. Effect of PMA on the relative levels of intracellular BMPR. Cells were incubated with 20 ng/ml of PMA for 4 h and the expression of BMPR analysed by FCM. The results represent the relative level of intracellular BMPR calculated from the relative levels of surface and total cellular BMPR as described in Section 2.3.2, and are presented as the mean AFI ± SE obtained from triplicate experiments.

5.3.5. Effect of PMA treatment on BMPR transcript expression

To examine the effect of PMA on the expression of BMPR transcripts and whether alternatively spliced forms of the BMPR were produced in response to PMA treatment, RT-PCR was carried out on untreated (control) and PMA-treated AB cell cultures. The results in Figure 5.5 show that for all the three BMPR genes examined, the band intensities of the PCR product of each gene in untreated control and PMA-treated cells appeared to be similar relative to the GAPDH gene, indicating that stimulation of the cells with PMA (20 ng/ml for 4 h) had very little apparent effect on the relative level of BMPR-IA, -IB and -II mRNA. Moreover, using specific primers that amplify sequences of the transmembrane domain and a part of the extracellular region of each of the three BMPR transcripts, only the same PCR products (of 510, 456 and 471 bp corresponding to the BMPR-IA, -IB and -II transcripts, respectively) were observed in both the untreated and PMA-treated cultures (Figure 5.5). These results indicated that the cells expressed only one form of each BMPR transcript and that no additional alternatively spliced transcripts which could have generated 'soluble' receptors were induced by PMA.

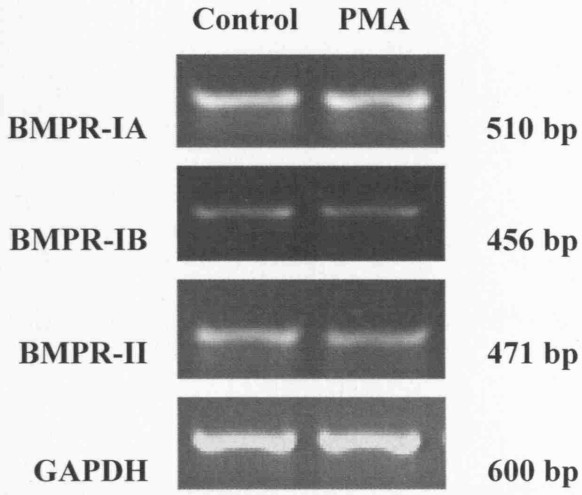


Figure 5.5. Effect of PMA treatment on BMPR transcript expression. Cells were subjected to PMA treatment (20 ng/ml) for 4 h, the RNA extracted and RT-PCR carried out on untreated (control) and PMA-treated cells. The figure shows representative electrophoresis gels of the RT-PCR products using primers containing sequences which amplify the mRNA sequences corresponding to the transmembrane domain and a part of the extracellular domain of BMPR-IA, -IB and -II.

5.3.6. Effect of PMA on BMP-2-induced Smad1/5/8 phosphorylation

To determine whether PMA-induced shedding of the BMPR resulted in the loss of BMP/BMPR-dependent signal transduction, the effect of PMA on Smad1/5/8 phosphorylation was measured after 30 min of exposure of the cells to BMP-2, as described in Section 5.2.1. The results in Figure 5.6 show that while no immunoreactive band corresponding to p-Smad1/5/8 could be detected in untreated control cells, treatment with BMP-2 alone resulted in the formation of p-Smad1/5/8 as shown by the presence of an approximately 65 kDa polypeptide band on SDS-PAGE (Figure 5.6). In addition, no apparent effect on Smad1/5/8 protein expression was evident in these cultures, based on the similar band intensities (Figure 5.6). However, exposure of the AB cells to PMA prior to BMP-2 treatment markedly reduced the level of BMP-2-induced Smad1/5/8 phosphorylation, by approximately 80% compared with the level in the BMP-2-treated cells (without PMA pre-treatment) ($p<0.05$) (Figure 5.6). Thus, PMA-induced shedding of the BMPR significantly diminished BMP/BMPR signalling in AB cells.

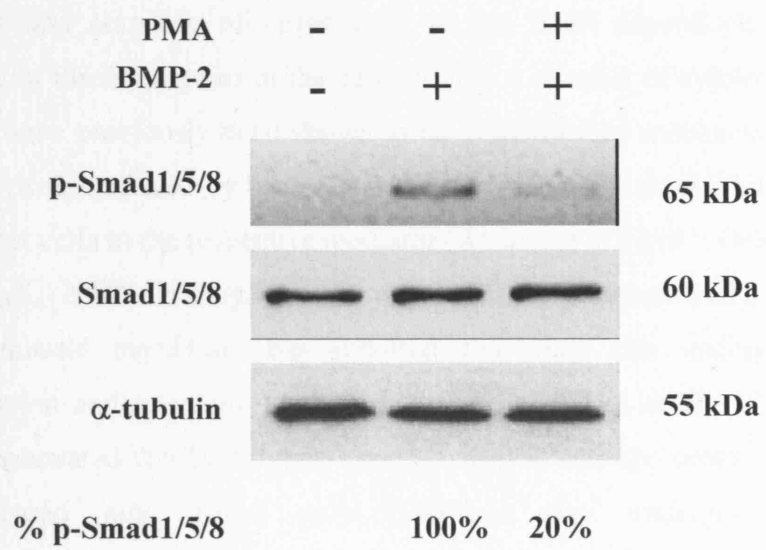


Figure 5.6. Effect of PMA on BMP-2-induced Smad1/5/8 phosphorylation. Cells were cultured alone, treated with 100 ng/ml of BMP-2 for 30 min or treated with 20 ng/ml of PMA for 4 h followed by 100 ng/ml of BMP-2 for 30 min in the continuing presence of PMA. Protein extracts from these cultures were subjected to SDS-PAGE and transferred to PVDF membranes which were then immunoblotted using antibodies against p-Smad1/5/8, Smad1/5/8 and α-tubulin (internal control). The immunoreactive bands show the electrophoretic mobility of the 65, 60 and 55 kDa polypeptides corresponding to these proteins, respectively, and the numbers under the bands show the percentage of Smad1/5/8 phosphorylation, defined as 100% in the BMP-2-treated cells.

5.4. Discussion

The BMPs elicit their biological activities via signal transduction through BMPR-IA, BMPR-IB and BMPR-II (ten Dijke *et al.*, 1994b; Nohno *et al.*, 1995), and thus the sensitivity and response of target cells to the BMP depend on the expression and regulation of these receptors at the cell surface. A number of cytokine and growth factor receptors have previously been shown to be controlled by mechanisms of internalisation via endocytosis and also by proteolytic shedding, thereby modulating the responsiveness of the target cells to the respective mediator (Anders *et al.*, 1997; Dello and Rovida, 2002; Hayes *et al.*, 2002; Di Guglielmo *et al.*, 2003). A previous study of the BMPR in rat skeletal muscle myoblasts has reported that they can undergo ligand-dependent internalisation and recycling to the cell surface (Jortikka *et al.*, 1997). Recently, it has been demonstrated that both types-I and -II BMPR undergo constitutive endocytosis via clathrin-coated pits, while only BMPR-II also undergoes caveolae-mediated internalisation. (Nohe *et al.*, 2005; Hartung *et al.*, 2006). The results of the present study have shown, for the first time, that all three BMPR can also be regulated by shedding as 'soluble' forms from the surface of AB cells. These were identified in culture supernatants by immunoprecipitation and WB using antibodies specific for the extracellular domain of each of the BMPR, after treatment of the cells with PMA, a potent PKC activator previously shown to cause shedding of certain other cell surface proteins (Dello and Rovida, 2002). The presence of these sBMPR was associated with a reduction of approximately 43-54% in the level of surface-associated BMPR, as determined by FCM.

BMP-2 binding assays also showed that the sBMPR-IA and -IB produced by the AB cells in response to PMA treatment were able to bind their cognate ligand BMP-2, suggesting that under the conditions used here, these soluble receptors contain the ligand binding site(s) and are thus potentially biologically active. This observation indicates that type-I BMPR can bind to the ligand without the concomitant presence of type-II BMPR, as has previously been reported (ten Dijke *et al.*, 1994a; Rosenzweig *et al.*, 1995). In contrast, sBMPR-II, which was also induced by PMA, appeared to be incapable of binding to its respective ligand BMP-2, at least in the absence of the type-I receptor. This suggests either that the sBMPR-II does not contain the ligand binding site or that this receptor has very low affinity for the ligand in the absence of type-I BMPR, as has previously been

reported for the binding affinity of different BMPR homo- and hetero-oligomers (ten Dijke *et al.*, 1994a; Rosenzweig *et al.*, 1995). However, further protein sequencing analysis of the sBMPR is required to determine whether the cleavage site for BMPR is located within the extracellular domain or at a membrane-proximal site near the transmembrane region.

In general, the cleavage site of proteolytic shedding of many transmembrane receptors is within the extracellular domain near the transmembrane domain (Rose-John and Heinrich, 1994), but the molecular weight of soluble growth factor receptors generated by shedding has been found to be higher than the expected size of the extracellular domain of the full length receptor. For example, sFGFR-I and sPDGF-αR have a molecular size of 55-60 and 90 kDa (Tiesman and Hart, 1993; Hanneken, 2001), while the expected size of the extracellular domain of FGFR-I and PDGF-αR are approximately 40 and 56 kDa, respectively (Peri *et al.*, 2003). In the present study, molecular weight of sBMPR-IA, -IB and -II were found to be approximately 18, 18 and 20 kDa, respectively, while their respective extracellular domains are expected to be approximately 14, 12 and 14 kDa, respectively (Peri *et al.*, 2003). This is consistent with the pattern reported for sFGFR-I and sPDGF-αR, although the size difference between the soluble forms and the expected extracellular domains of the BMPR is much less than that of FGFR-I and PDGF-αR. Since the larger size of the soluble forms is probably due to post-translational modifications of the receptor such as glycosylation, the differences between the BMPR and FGFR-I/PDGF-αR could possibly due to the shorter extracellular domain of the BMPR, thereby containing less putative glycosylation sites (Peri *et al.*, 2003).

It is notable that some soluble receptors have also been shown to be generated by translation of alternatively spliced mRNA transcripts lacking nucleotide sequences for transmembrane and cytoplasmic regions, which result from either insertion or deletion of certain nucleotide sequences at the transmembrane sequence that leads to a reading frame shift (Rose-John and Heinrich, 1994; Muller-Newen *et al.*, 1996a). Thus, differential splicing forms can be detected by RT-PCR using primers which amplify sequences of the transmembrane domain and a part of the extracellular region, and the PCR product is expected to be smaller in size compared with that of its corresponding membrane-bound counterpart. Although it is possible that sBMPR could be generated by such differential

splicing, the RT- PCR carried out in the present study, using specific primers that amplify sequences of the transmembrane domain and a part of the extracellular region of BMPR, detected only one form of PCR product corresponding to each of the three BMPR transcripts. Thus, the AB cells did not appear to generate alternatively spliced mRNA transcripts which could have generated 'soluble' forms for these receptors. In addition, again in experiments using the transmembrane and extracellular domain amplifying primers of BMPR, it was found that treatment with PMA did not result in the expression of any other BMPR transcripts in these cells (Figure 5.5). It is therefore unlikely that the AB cells generated alternatively spliced mRNA transcripts for these receptors which could have given rise to sBMPR. It is notable that many of the soluble receptors which have been identified are generated by proteolytic cleavage at the cell surface and not by the secretion of the products of alternatively spliced transcripts (Werb and Yan, 1998; Mullberg *et al.*, 2000), such as those of TGFβR-III and PDGF-αR (Lopez-Casillas *et al.*, 1991; Tiesman and Hart, 1993), while the soluble form of FGFR-I is generated by both the shedding and alternative splicing mechanisms (Hanneken, 2001).

Previous studies suggest that the shedding process is mediated mainly by the activation of 'proteases' via both PKC-dependent and -independent signal transduction pathways (Vecchi *et al.*, 1996; Rizoli *et al.*, 1999; Umata *et al.*, 2001; Dello and Rovida, 2002). In the present study PMA, a synthetic PKC activator, generated soluble forms of BMPR-IA, -IB and -II, indicating that shedding of these three BMPR can occur via the PKC pathway although this does not preclude a role for a PKC-independent pathway.

As noted above, a number of surface proteins, including the BMPR, are regulated by internalisation via endocytosis (Anders *et al.*, 1997; Jortikka *et al.*, 1997; Dore, Jr. *et al.*, 2001; Hayes *et al.*, 2002; Di Guglielmo *et al.*, 2003), thereby reducing cell surface expression and function. However, down-regulation of BMPR expression by PMA, as observed here, is unlikely to be mediated by this internalisation process since there was no concomitant increase in the intracellular level of BMPR protein following incubation with PMA, based on FCM analysis of BMPR levels obtained from permeabilised and non-permeabilised cells (Figure 5.4).

The potential functional importance of the PMA-induced loss of surface BMPR expression was investigated by measuring the effect of PMA on the phosphorylation of Smad1/5/8, generally accepted as a very early downstream event in the BMPR signalling pathway (ten Dijke *et al.*, 2003a) and thus the cell response to BMP. The marked decrease in cell surface BMPR levels caused by PMA was found to result in a profound reduction in the level of phosphorylated Smad1/5/8. Since Smad-mediated BMP/BMPR signalling is known to play a key part in BMP-2-induced osteoblast differentiation (Fujii *et al.*, 1999), the loss of this central activation step is likely to be of major importance for the sensitivity of bone cells to BMP-2 and their functional activity. It is also notable that the decreased BMP-induced Smad1/5/8 phosphorylation by PMA treatment is unlikely to be due to the direct suppression of Smad1/5/8 expression since no apparent effect of PMA on these proteins was observed (Figure 5.6).

It has previously been proposed that the soluble receptors can function as endogenous inhibitors of the biological activities of their cognate ligands (Rose-John and Heinrich, 1994; Dello and Rovida, 2002). For example, soluble forms of FGFR-I have been shown to antagonise the biological activities of the FGF family *in vivo*. (Hanneken *et al.*, 1994; Hanneken *et al.*, 1995). Likewise, the present results showed that sBMPR-IA and -IB produced in response to PMA can bind to BMP-2, suggesting that the sBMPR might also act as BMP-2 antagonists and hence possibly reduce cellular responses to BMP-2 in concert with the decrease in surface BMPR expression.

In summary, the present study has provided evidence, for the first time, that BMPR can be modulated at the cell surface by ectodomain shedding which results in rapid down-regulation of the BMPR from the cell surface and the generation of soluble forms of the BMPR. These sBMPR could then act as BMPR antagonists, by competing for the ligand, BMP-2. Moreover, in response to the potent shedding inducer PMA, the cells consequently exhibited a markedly diminished response to the cognate ligand, BMP-2, as shown by the reduction of BMP-2-induced Smad1/5/8 phosphorylation *in vitro*.

CHAPTER 6
EFFECTS OF INFLAMMATORY CYTOKINES ON BMPR EXPRESSION AND FUNCTION

6.1. Introduction

A number of inflammatory cytokines, including IL-1β, IL-6, TNF-α and PGE-2, have been shown to play an important role in bone remodelling, the process involving osteoblast-mediated bone formation following bone resorption by osteoclasts (Kwan *et al.*, 2004). The biological functions of these cytokines in such process have been reviewed in detail in Section 1.2. In addition to their inhibitory effect on bone remodelling through the stimulation of bone resorption, IL-1β, IL-6, TNF-α and PGE-2 have previously been shown to suppress the osteogenic activity of osteoblasts (Li and Stashenko, 1992; Hughes and Howells, 1993; Scharla *et al.*, 1994; Nakase *et al.*, 1997; Kajii *et al.*, 1999; Gilbert *et al.*, 2000; Kumar *et al.*, 2001; Gilbert *et al.*, 2002; Kim *et al.*, 2002; Nanes, 2003; Lukic *et al.*, 2005). While BMP-induced osteogenic function is known to play a central role in osteogenesis, the inhibitory effect of the cytokines IL-1β, IL-6, TNF-α and PGE-2 on bone formation has previously been shown to be associated with ablation of BMP expression and function (Nakase *et al.*, 1997; Virdi *et al.*, 1998; Takiguchi *et al.*, 1999; Lukic *et al.*, 2005). Although the biological activity of these cytokines in BMP-mediated osteogenesis is not yet fully understood, it is possible that these cytokines could inhibit osteoblast activity at least partly via down-regulation of BMPR expression. To test this hypothesis, the present study was carried out to examine the effects of IL-1β, IL-6, TNF-α and PGE-2 on the expression of BMPR-IA,-IB and -II and the functional consequences of these cytokines on AB cell function.

6.2. Materials and Methods

Cell culture, C-ELISA, RT-PCR, Q-PCR, FCM, WB and ALP activity assay were performed as described in Chapter 2. Preparation of cell culture supernatants, immunoprecipitation of concentrated supernatant samples and the BMP-2 binding assay were carried out as previously described (Section 5.2); additional methods used in this chapter are described below.

6.2.1. Treatment of cells

Exponentially growing cultures of AB cells were subjected to serum starvation (1% FCS) for 24 h prior to treatment, then treated for 24 h with 1, 5, 20 and 50 ng/ml each of IL-1β, IL-6, TNF-α and PGE-2 (R&D Systems) in fresh medium containing 1% FCS. In other experiments to examine the effect of IL-1β and TNF-α on BMP-2-induced osteogenic functions, cells were pre-treated with the cytokines (50 ng/ml) for 24 h, followed by the addition of BMP-2 (50-300 ng/ml) for 24-72 h in the continuing presence of the cytokines. In some experiments, cells were first pre-treated for 2 h with SB203580 (10 μM), U0126 (1 μM), SP600125 (10 μM) or wortmannin (0.1 μM) (all from Sigma), inhibitors of p38 MAPK, ERK1/2, JNK1/2/3 and PI3K, respectively, which are major signalling proteins in the TNF-α pathway (Madge and Pober, 2001; Chen and Goeddel, 2002), followed by the addition of 50 ng/ml of TNF-α for 24 h in the continuing presence of the inhibitors. At the concentrations and conditions used here, the cytokines and inhibitors were found to cause less than 5% cell death, as determined by trypan blue exclusion tests performed in duplicate. RNA and protein extractions, C-ELISA, FCM and the ALP activity assay were carried out on untreated and treated cells, and culture media supernatants were collected and stored at -20°C until required.

6.3. Results

6.3.1. Effect of inflammatory cytokines on BMPR gene transcripts

AB cells were treated with IL-1β, IL-6, TNF-α and PGE-2 at 0, 1, 5, 20 and 50 ng/ml for 24 h and then analysed by RT-PCR. The results in Figure 6.1 show that under the experimental conditions used here, IL-1β down-regulated BMPR-IA transcripts by 36-60% whereas treatment of the cells with IL-6 was found to have relatively little if any effect, decreasing transcript levels between 3-10% (Figure 6.1A). In marked contrast to IL-6, all the above doses of TNF-α significantly down-regulated BMPR-IA transcripts by 50-60% ($p<0.05$), while treatment with increasing concentrations of PGE-2 progressively reduced BMPR-IA mRNA, the highest two concentrations causing significant down-regulation of approximately 50% and 63%, respectively ($p<0.05$) (Figure 6.1A).

In contrast to the suppressive effect on BMPR-IA transcripts, progressively increasing concentrations of the cytokines IL-1β and TNF-α significantly up-regulated BMPR-IB mRNA in a dose-dependent manner, increasing transcript levels by between 3- and 16.5-fold in IL-1β-treated cells and approximately 2- and 9-fold in TNF-α-treated cells ($p<0.05$) (Figure 6.1B). The results in Figure 6.1B also show that the expression of BMPR-IB transcripts was unaffected by both IL-6 and PGE-2.

IL-1β, IL-6 and TNF-α were found to suppress BMPR-II gene expression, by approximately 20-30%, 0-15% and 20-30%, respectively (Figure 6.1C). However, the expression of this gene was significantly decreased by PGE-2 in a dose-dependent manner, with approximately 50% and 60% suppression at 20 and 50 ng/ml of PGE-2, respectively ($p<0.05$).

Thus, the results demonstrated that BMPR transcripts were differentially regulated by these cytokines in the AB cells, with BMPR-IA and -II generally being inhibited and BMPR-IB being stimulated.

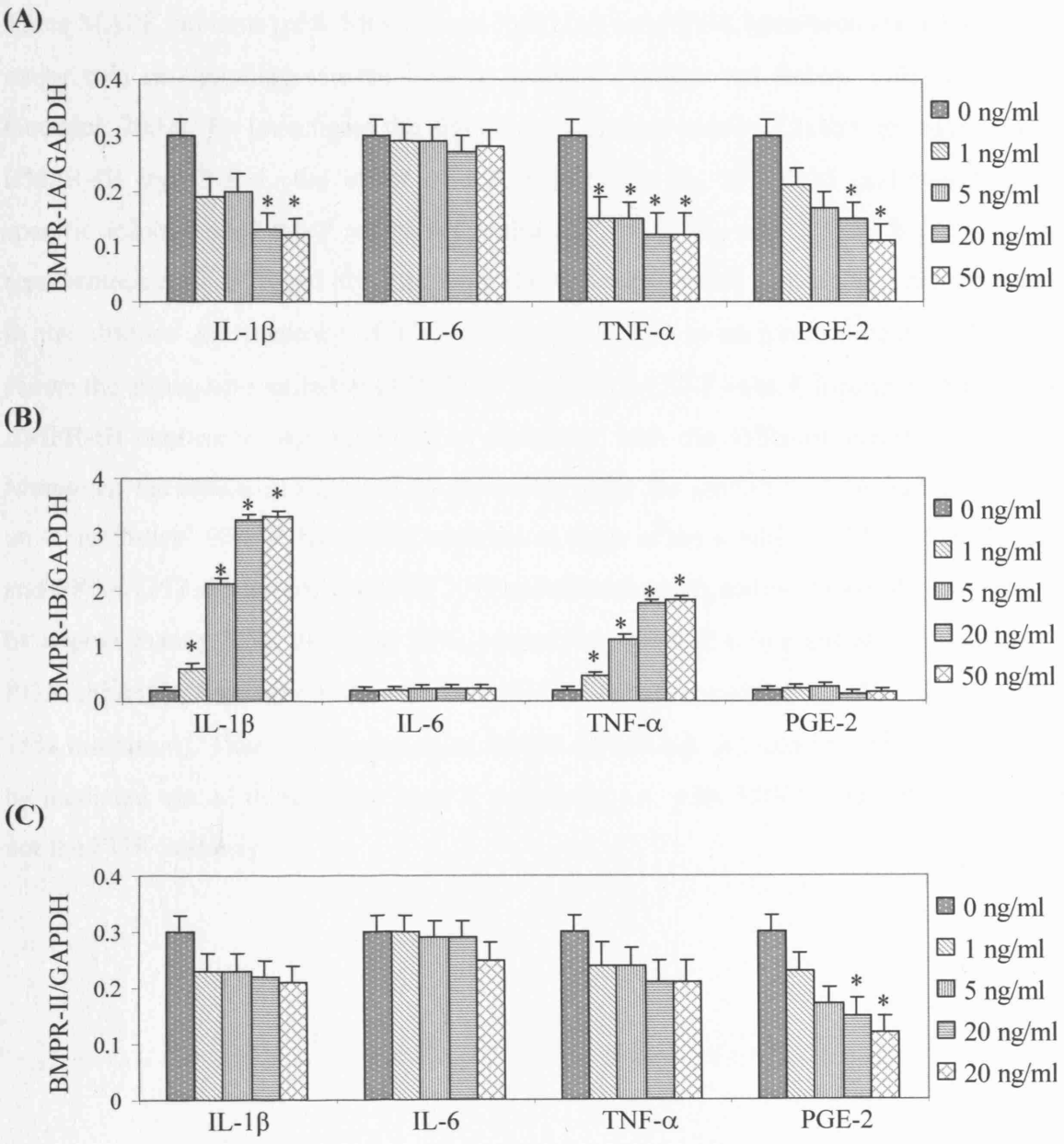


Figure 6.1. Effect of inflammatory cytokines on BMPR-IA (A), -IB (B) and -II (C) mRNA transcripts. Cells were incubated with 0-50 ng/ml of IL-1β, IL-6, TNF-α and PGE-2 for 24 h and the expression of BMPR-IA, -IB and -II transcripts examined by RT-PCR. The results are presented as the mean ± SE of the level of each of BMPR transcript relative to that of GAPDH, obtained from triplicate experiments.

* $p<0.05$ vs untreated cells.

6.3.2. Effect of protein inhibitors on TNF-α induction of BMPR-IB transcripts

Three MAPK proteins (p38, ERK1/2 and JNK1/2/3) and PI3K have been shown to have a major role in signalling via the TNF-α pathway (Madge and Pober, 2001; Chen and Goeddel, 2002). To investigate the signalling pathways involved in the up-regulation of BMPR-IB by TNF-α, the effect of SB203580, U0126, SP600125 and wortmannin, specific inhibitors of these respective pathways, was examined. Figure 6.2A shows a representative RT-PCR gel obtained from the AB cells treated with each of the inhibitors in the absence and presence of TNF-α, using GAPDH as an internal control. This gel shows the strong up-regulation of BMPR-IB mRNA by TNF-α and, further, indicates that BMPR-IB expression was modified by treatment with the different specific inhibitors. Moreover, the results in Figure 6.2B show that while the inhibitors alone had little effect on 'constitutive' BMPR-IB mRNA expression, three of the inhibitors (SB203580, U0126 and SP600125) significantly ablated TNF-α-induced up-regulation of BMPR-IB mRNA, by approximately 85%, 84% and 70%, respectively ($p<0.05$). In contrast, wortmannin, a PI3K inhibitor, had very little effect on BMPR-IB up-regulation by TNF-α (less than 15% inhibition). Thus, up-regulation of BMPR-IB mRNA induced by TNF-α appears to be mediated via all three major MAPK pathways, *i.e.,* p38, ERK1/2 and JNK1/2/3, but not the PI3K pathway.

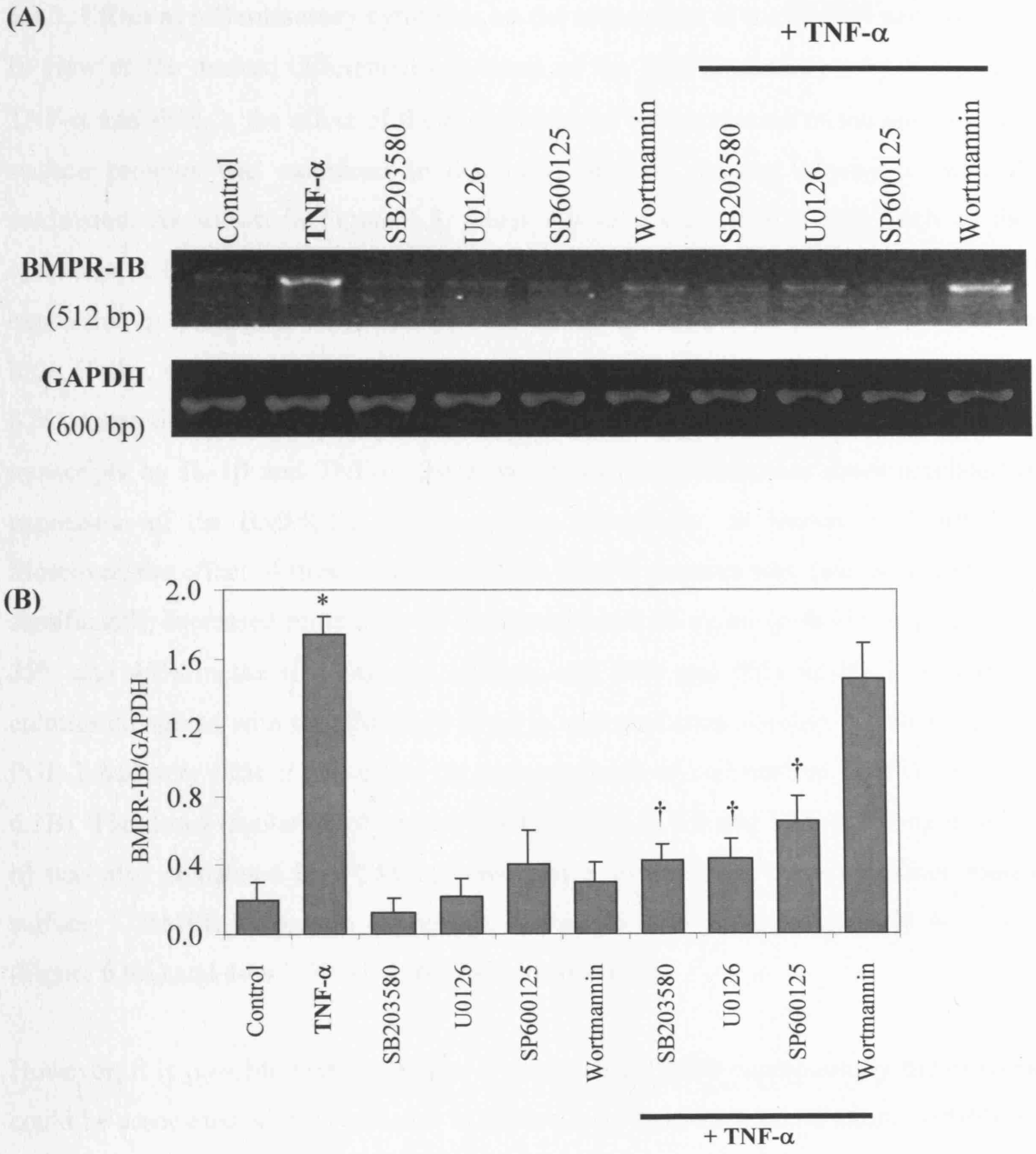


Figure 6.2. Up-regulation of BMPR-IB transcripts by TNF-α is mediated via the MAPK pathway. Cells were pre-treated for 2 h with different inhibitors, then 50 ng/ml of TNF-α added for 24 h in the continuing presence of the inhibitors. The expression of BMPR-IB transcripts was then examined by RT-PCR. (A) shows representative electrophoresis gels of RT-PCR products using primers specific for BMPR-IB and GAPDH, obtained from untreated and treated cells. The results in (B) are presented as the mean ± SE of the level of each of BMPR-IB transcript relative to that of GAPDH, obtained from triplicate experiments. * $p<0.05$ vs untreated cells (control). † $p<0.05$ vs TNF-α-treated culture.

6.3.3. Effect of inflammatory cytokines on the expression of the BMPR proteins

In view of the marked differential regulation of the BMPR transcripts by IL-1β, IL-6, TNF-α and PGE-2, the effect of these cytokines on the expression of the corresponding surface proteins was examined to determine whether receptor expression was also modulated. As shown in Figure 6.3, when AB cells were treated with each of these cytokines at 0, 1, 5, 20 and 50 ng/ml for 24 h and then analysed for surface BMPR expression by C-ELISA, the results showed that IL-1β, IL-6, TNF-α and PGE-2 had very little, if any, effect on either the BMPR-IA or -II cell surface proteins (Figure 6.3A and 6.3C, respectively). In marked contrast, despite the significant up-regulation of BMPR-IB transcripts by IL-1β and TNF-α, these two cytokines nevertheless down-regulated the expression of the BMPR-IB surface protein specifically, as shown in Figure 6.3B. Moreover, the effect of these cytokines on the BMPR proteins was dose-dependent, with significantly decreased protein levels found at 20 and 50 ng/ml ($p<0.05$) (approximately 35% and 45% in the IL-1β-treated cultures and 30% and 40% in the TNF-α-treated cultures compared with the BMPR-IB level in untreated control cells). Notably, IL-6 and PGE-2 had very little if any effect on the expression of cell surface BMPR-IB (Figure 6.3B). The down-regulation of surface BMPR-IB by IL-1β and TNF-α (50 ng/ml for 24 h) was also confirmed by FCM analysis, which showed that these cytokines reduced surface BMPR-IB protein expression in the AB cells by approximately 44 ± 4.2% (Figure 6.4A) and 40 ± 3.9% (Figure 6.4B), respectively.

However, it is possible that a decrease in surface BMPR-IB expression by the cytokines could be associated with an increase in intracellular receptor accumulation, possibly as a result of either decreased surface transport of the newly synthesised receptor or increased internalisation of the surface receptor in response to the cytokines. To examine this possibility, the effect of IL-1β and TNF-α on intracellular BMPR-IB expression was determined by C-ELISA. The results in Figure 6.5 showed that the cytokines had no effect on the intracellular level of BMPR-IB protein, indicating that the loss of BMPR-IB surface expression in response to IL-1β and TNF-α was not due to cytokine-induced intracellular accumulation of this protein specifically.

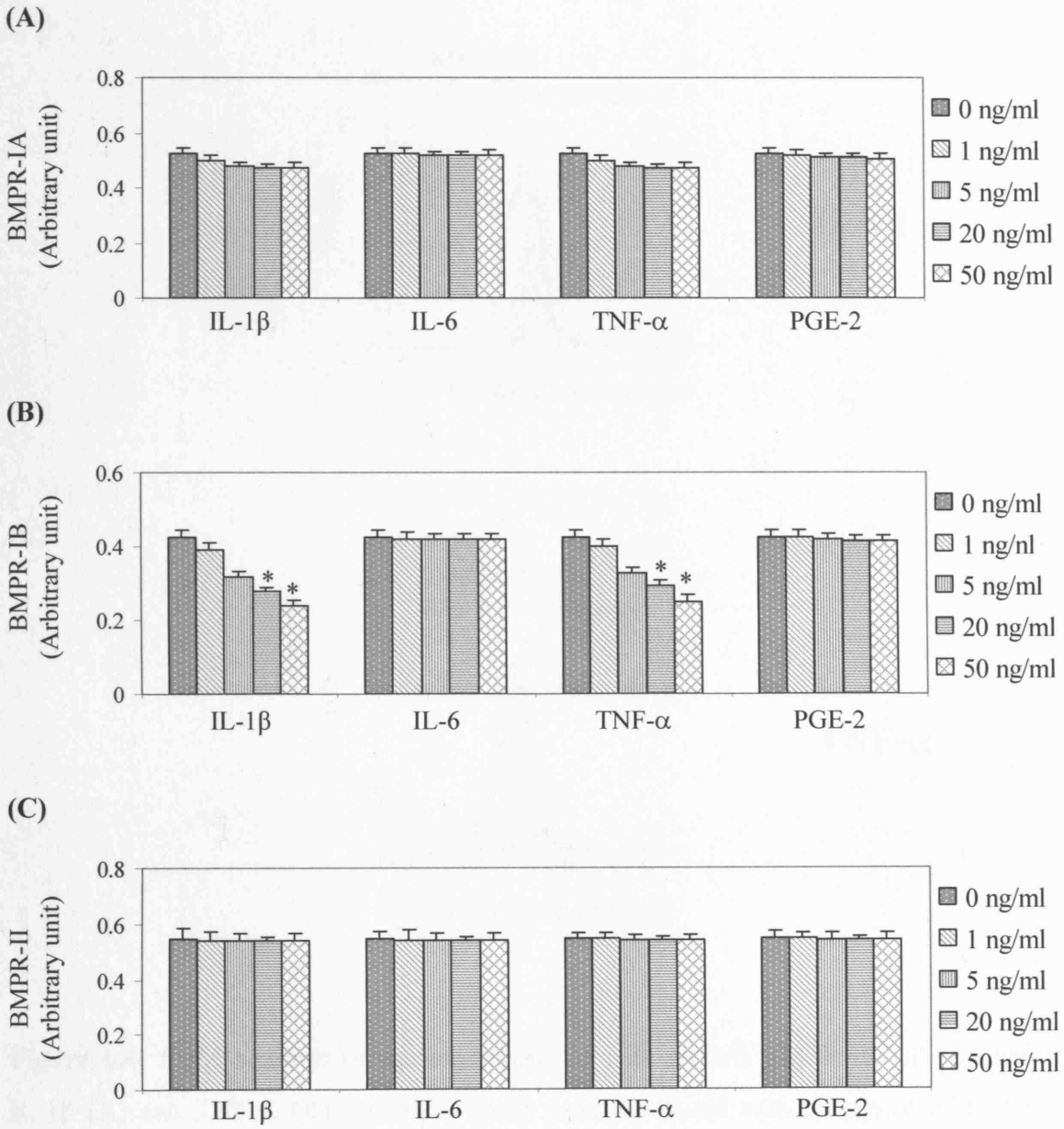
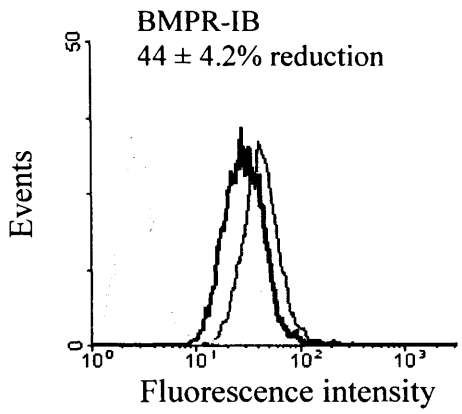


Figure 6.3. Effect of inflammatory cytokines on the surface expression of BMPR-IA (A), -IB (B) and -II (C) proteins. Cells were incubated with 0-50 ng/ml of IL-1β, IL-6, TNF-α and PGE-2 for 24 h and the surface expression of BMPR-IA (A), -IB (B) and -II (C) proteins examined by C-ELISA. The results are presented as the mean ± SE of the relative level of each of BMPR protein obtained from triplicate experiments.

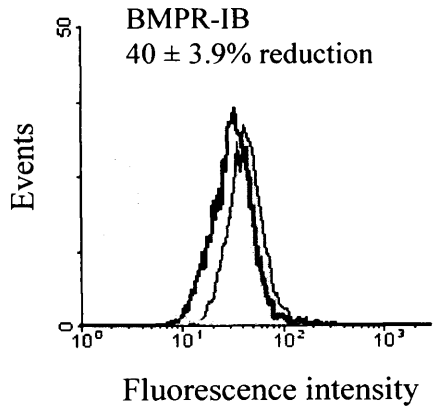
* $p<0.05$ vs untreated cells.

(A)



IL-1β

(B)



TNF-α

Figure 6.4. FCM analysis of the expression of cell surface BMPR-IB in response to IL-1β (A) and TNF-α (B) treatment. Cells were incubated with 50 ng/ml of IL-1β or TNF-α for 24 h and the surface expression of BMPR-IB determined by FCM. The decrease in the relative level of BMPR-IB is shown by the shift of the clear (solid line) histograms (cytokine-treated cultures) to the left compared with the shaded histogram (control untreated cultures). The numbers show the mean percentage reduction (± SE) from three different experiments. The dotted histogram is the fluorescence of the isotype-matched sample used as a control.

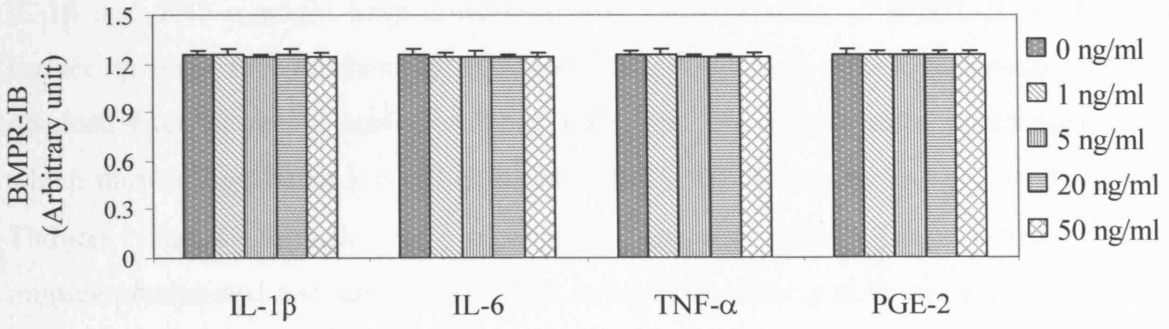


Figure 6.5. Effect of inflammatory cytokines on the intracellular expression of BMPR-IB protein. Cells were incubated with 0-50 ng/ml of IL-1β, IL-6, TNF-α and PGE-2 for 24 h and the expression of BMPR-IB analysed by C-ELISA. The results represent the relative level of intracellular BMPR calculated from the relative levels of surface and total cellular BMPR, as described previously, and are presented as the mean ± SE obtained from triplicate experiments.

6.3.4. IL-1β- and TNF-α-induced shedding of a soluble form of BMPR-IB by AB cells

It has previously been reported that IL-1β and TNF-α are able to induce shedding of certain cell surface proteins (Tanida *et al.*, 2004; Franchimont *et al.*, 2005a). Moreover, the results in Chapter 5 have shown that BMPR-IB can be shed from the cell surface by PMA and the presence of sBMPR-IB has been described. To examine the possibility that IL-1β and TNF-α might have down-regulated the expression of BMPR-IB at the cell surface through this mechanism of protein 'clipping', cell culture supernatants were obtained from untreated control cultures and from AB cells cultured under conditions which showed significant loss of cell surface BMPR-IB (24 h with 50 ng/ml of IL-1β and TNF-α). Equal amounts of supernatant protein from these cultures were immunoprecipitated and subjected to WB, using antibodies specific for the extracellular domain of BMPR-IB, as described previously. The results showed that concurrently with the decrease in cell surface expression of BMPR-IB in response to IL-1β and TNF-α, 'soluble' forms of BMPR-IB were detected on SDS-PAGE, as approximately 18 and 18 kDa polypeptides (Figure 6.6A and 6.6B, respectively). Culture supernatants obtained from cells in the absence of the cytokines contained no comparable immunoreactive band corresponding to 'sBMPR-IB', suggesting that no shedding of sBMPR-IB had occurred 'spontaneously' in the cultured AB cells. These results thus demonstrate that IL-1β and TNF-α are able to down-regulate the expression of BMPR-IB, at least partly, by causing shedding of a low molecular weight soluble form of the protein from the cell surface.

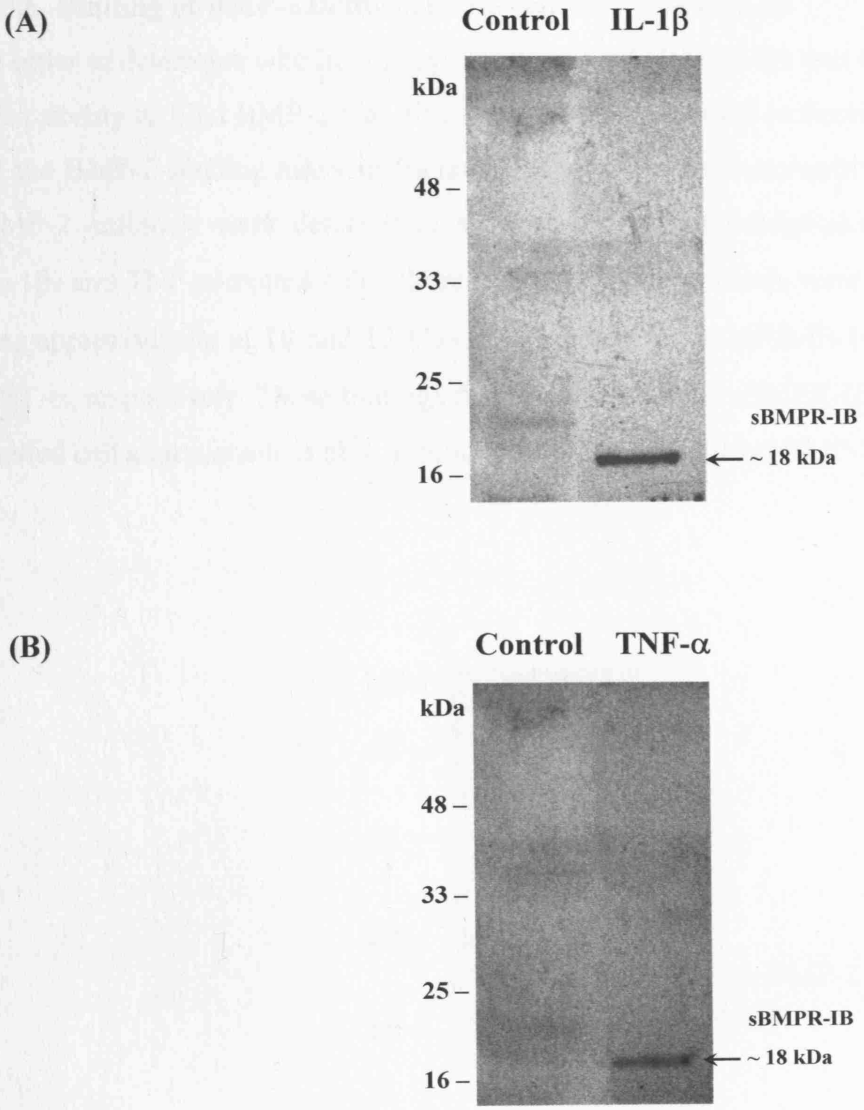


Figure 6.6. Identification of a soluble form of BMPR-IB in IL-1β- and TNF-α-treated AB cells. Cells were incubated with 50 ng/ml of IL-1β (A) and TNF-α (B) for 24 h and the expression of a soluble BMPR-IB in cell culture supernatants examined by immunoprecipitation and WB. The immunoreactive band shows the electrophoretic migration of an approximately 18 kDa polypeptide on SDS-PAGE corresponding to the soluble form of BMPR-IB (sBMPR-IB). No immunoreactive band is detected in the untreated (control) culture supernatant.

6.3.5. Binding of BMP-2 to the cytokine-induced sBMPR-IB

In order to determine whether the cytokine-induced sBMPR-IB was functionally relevant, their ability to bind BMP-2 was also examined as described in Section 5.2.4. The results of the BMP-2 binding assay in Figure 6.7 show that immunoreactive bands of the anti-BMP-2 antibody were detected in sBMPR-IB immunoprecipitates obtained from the IL-1β- and TNF-α-treated cell cultures. Moreover, these bands were in the same position, *i.e.,* approximately at 18 and 18 kDa, as observed for sBMPR-IB induced by IL-1β and TNF-α, respectively. These findings thus indicate that the sBMPR-IB present in cytokine-treated cell supernatants is able to bind to their cognate ligand BMP-2.

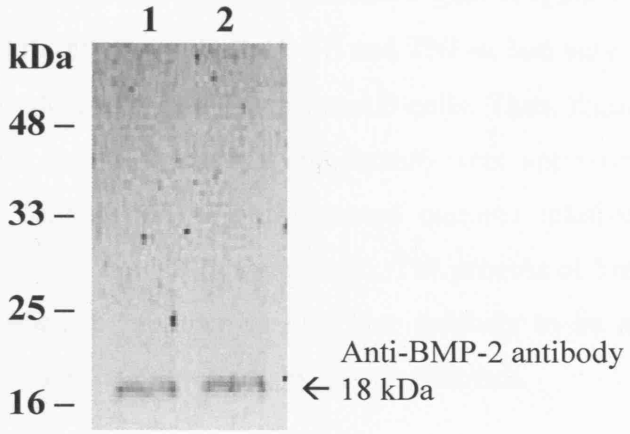


Figure 6.7. BMP-2 binding to the sBMPR-IB obtained from cytokine-treated cell cultures. Cells were incubated with 50 ng/ml of IL-1β (lane 1) and TNF-α (lane 2) for 24 h and the cell culture supernatants were concentrated and immunoprecipitated using the BMPR-IB antibody. The immunoprecipitated samples were then transferred to a PVDF membrane and subjected to the BMP-2 binding assay, as described in Section 5.2.4. The immunoreactive bands detected in lane 1 and lane 2 show the presence of BMP-2 binding to the sBMPR-IB obtained from IL-1β- and TNF-α-treated cultures, respectively.

6.3.6. Effect of IL-1β and TNF-α on Smurf1/Smad6-induced BMPR-IB breakdown

It has previously been shown that type-I BMPR can be targeted to undergo intracellular breakdown by the Smurf1 and Smad6, which are both involved in the ubiquitination-dependent degradation of type-I BMPR (Imamura *et al.*, 1997; Murakami *et al.*, 2003). To determine whether IL-1β and TNF-α may have down-regulated BMPR-IB through the stimulation of Smurf1 and Smad6 expression, the cells were treated with IL-1β and TNF-α (0, 1, 5, 20, 50 ng/ml) for 24 h and the expression of Smurf1 and Smad6 transcripts examined by RT-PCR.

Using primer sequences specific for the Smurf1 and Smad6 genes, RT-PCR products corresponding to the expected molecular sizes of 203 and 204 bp, respectively, were visualised under UV light, as shown in the representative gels (Figure 6.8 and 6.9). However, with the doses used in this study, both IL-1β and TNF-α had very little, if any, effect on Smurf1 and Smad6 mRNA expression in the AB cells. Thus, regardless of the cytokine treatment, the mRNA levels of Smurf1 and Smad6 were approximately 0.81-0.82 and 0.63-0.65 in both control and cytokine-treated cultures relative to that of GAPDH, defined as 1.0 (Figure 6.8 and 6.9, respectively). The process of Smurf1/Smad6 mediated-ubiquitination-dependent breakdown is therefore unlikely to be an important mechanism in the control of BMPR-IB expression by these cytokines.

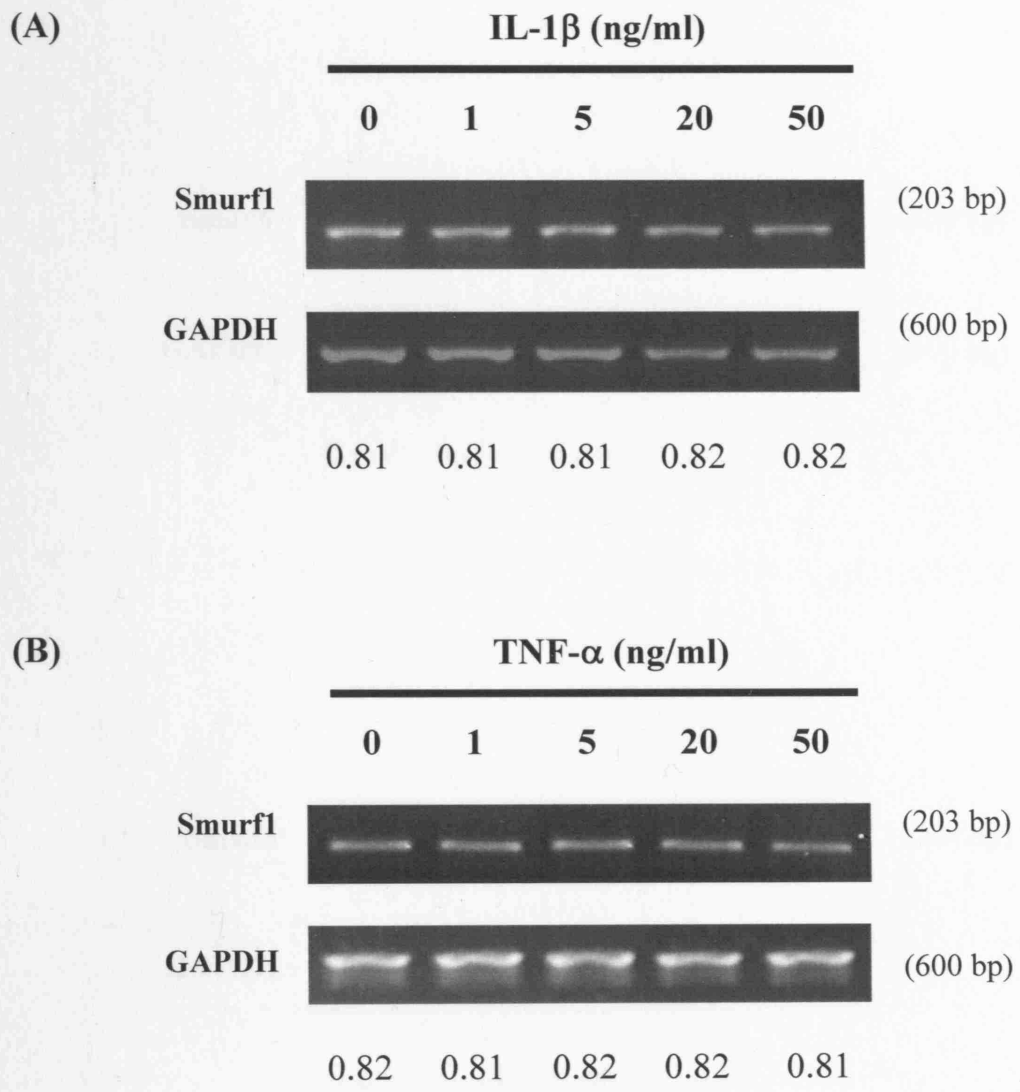


Figure 6.8. Effect of IL-1β (A) and TNF-α (B) on the expression of Smurf1 transcripts. Cells were incubated with 0-50 ng/ml of IL-1β (A) and TNF-α (B) for 24 h and the expression of Smurf1 transcripts examined by RT-PCR. The figure shows representative electrophoresis gels of the RT-PCR products using primers specific for Smurf1 and GAPDH, obtained from untreated and cytokine-treated cells. The numbers under the bands show the means of the levels of Smurf1 mRNA transcripts relative to that of GAPDH, obtained from triplicate experiments.

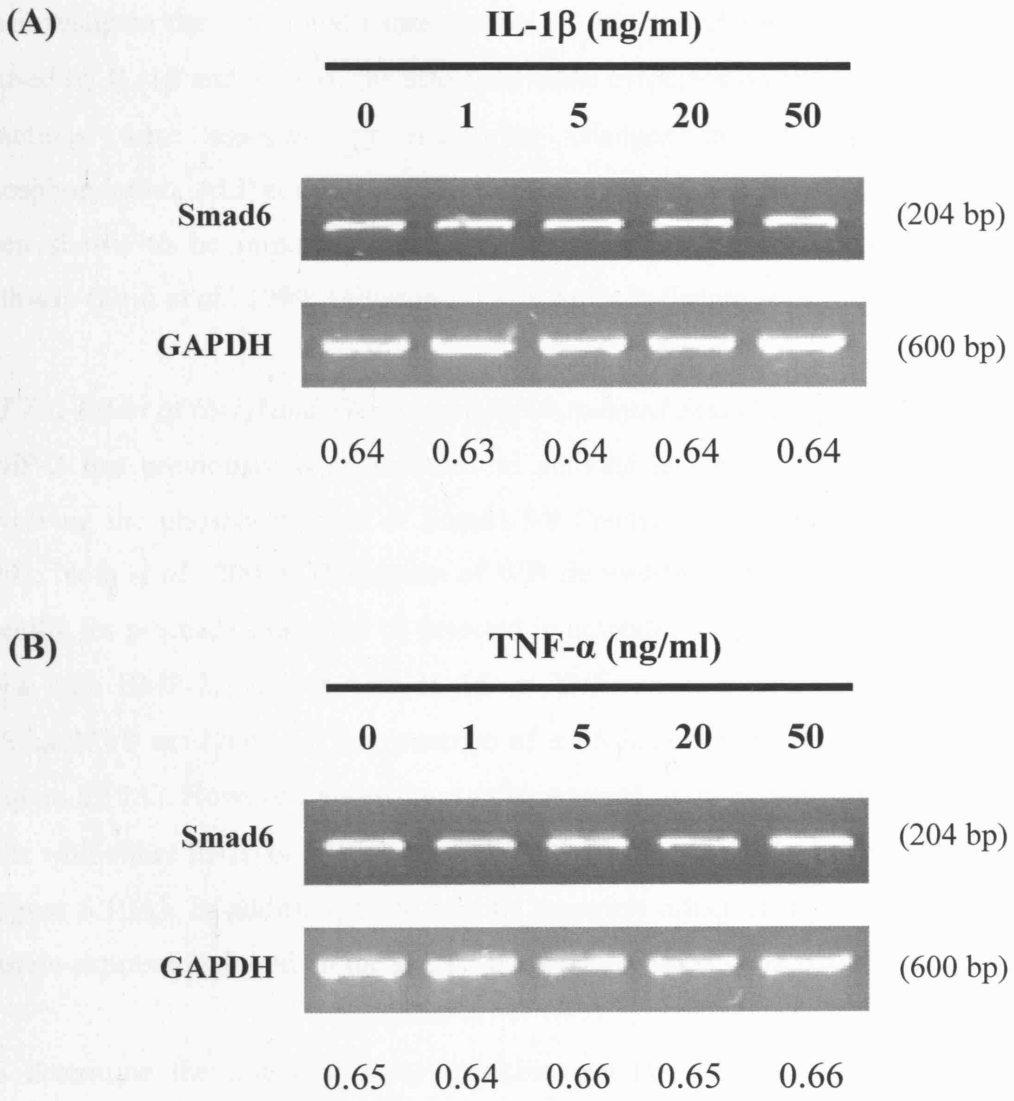


Figure 6.9. Effect of IL-1β (A) and TNF-α (B) on the expression of Smad6 transcripts. Cells were incubated with 0-50 ng/ml of IL-1β (A) and TNF-α (B) for 24 h and the expression of Smad6 transcripts examined by RT-PCR. The figure shows representative electrophoresis gels of the RT-PCR products using primers specific for Smad6 and GAPDH, obtained from untreated and cytokine-treated cells. The numbers under the bands show the means of the levels of Smad6 mRNA transcripts relative to that of GAPDH, obtained from triplicate experiments.

6.3.7. Effects of IL-1β and TNF-α on BMP-2-induced osteoblast functions of AB cells

To investigate the functional consequences of the loss of surface BMPR-IB expression caused by IL-1β and TNF-α, the effects of these cytokines on BMP-2-induced osteoblast functions were assessed by measuring changes in the levels of Smad1/5/8 phosphorylation, ALP activity and OC transcript expression. All of these have previously been shown to be important functional consequences of the BMP/BMPR signalling pathway (Fujii *et al.*, 1999; Miyazono, 1999; Spinella-Jaegle *et al.*, 2001).

6.3.7.1. Effect of IL-1β and TNF-α on BMP-2-induced Smad1/5/8 phosphorylation

BMP-2 has previously been reported to activate an intracellular signalling pathway involving the phosphorylation of Smad1/5/8 (Yamamoto *et al.*, 1997; Lai and Cheng, 2002; Noth *et al.*, 2003). The results of WB showed that while no immunoreactive band specific for p-Smad1/5/8 could be detected in untreated control cells, treatment of the AB cells with BMP-2, but not with IL-1β or TNF-α alone, resulted in the formation of p-Smad1/5/8 as shown by the presence of a 65 kDa polypeptide band on SDS-PAGE (Figure 6.10A). However, under the conditions used in the present study, treatment of the cells with either IL-1β or TNF-α alone did not result in the phosphorylation of Smad1/5/8 (Figure 6.10A). In addition, there was no apparent effect on the amount of Smad1/5/8 protein expression, based on the similar band intensities (Figure 6.10A).

To determine the effect of these cytokines on BMP-2-induced osteogenic function, Smad1/5/8 phosphorylation was measured after 30 min of exposure of the cells to BMP-2 (100 ng/ml), as previously described. The results in Figure 6.10B show that while no immunoreactive band specific for p-Smad1/5/8 could be detected in untreated control cells, treatment with BMP-2 alone resulted in the formation of p-Smad1/5/8 as shown by the presence of a 65 kDa polypeptide band on SDS-PAGE, with no apparent effect on Smad1/5/8 protein expression, as noted above. However, exposure of the AB cells to either IL-1β or TNF-α for 24 h, prior to BMP-2 treatment, markedly reduced the level of BMP-2-induced Smad1/5/8 phosphorylation, by approximately 65% and 50% compared with that in cells treated with BMP-2 alone, defined as 100%, respectively (Figure 6.10B). In addition, the apparently lower level of BMP-2-induced p-Smad1/5/8 by the cytokines was not due to a decrease in the level of Smad1/5/8 protein since the cytokines

had no apparent effect on the amount of Smad1/5/8 protein expression, as shown in Figure 6.10A.

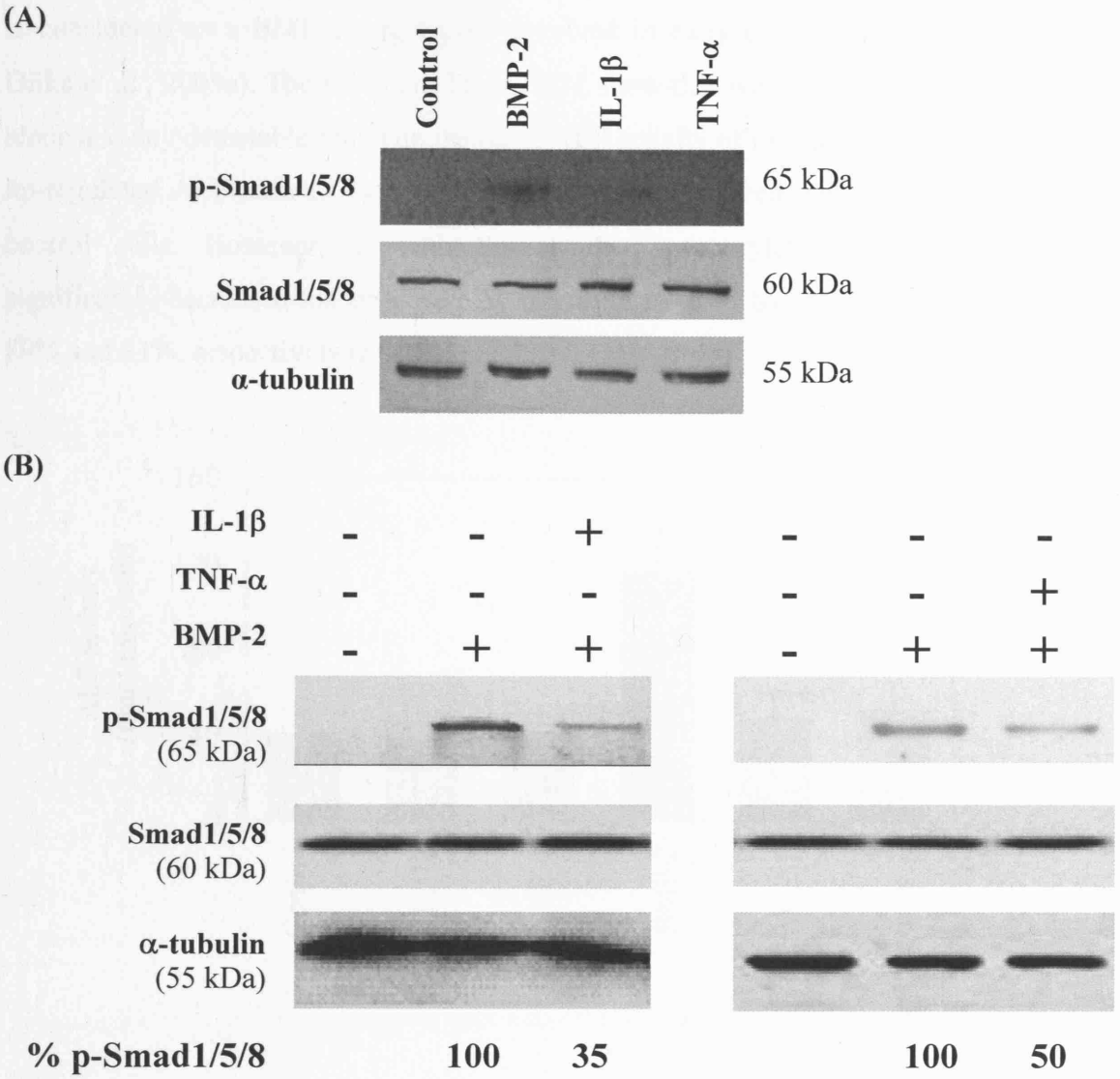


Figure 6.10. Effects of IL-1β and TNF-α on Smad1/5/8 phosphorylation (A) and on BMP-2-induced Smad1/5/8 phosphorylation (B). Cells were cultured alone (control), incubated with BMP-2 (100 ng/ml for 30 min), IL-1β (50 ng/ml for 24 h) and TNF-α (50 ng/ml for 24 h) alone or incubated with IL-1β and TNF-α (50 ng/ml for 24 h) followed by BMP-2 (100 ng/ml for 30 min) in the continuing presence of the cytokines. Protein extracts from these cultures were prepared and analysed by WB. The numbers under the immunoreactive bands show the percentage of Smad1/5/8 phosphorylation, defined as 100% of that in the BMP-2-treated cells.

6.3.7.2. Effect of IL-1β and TNF-α on BMP-2-induced ALP activity

The functional consequence of down-regulation of BMPR-IB surface expression by IL-1β and TNF-α was further examined by their effects on BMP-2-induced ALP activity, which is considered as a BMP-2 target gene involved in early osteoblast differentiation (ten Dijke *et al.*, 2003a). The results in Figure 6.11 show that while neither IL-1β nor TNF-α alone had any detectable effect on the basal ALP activity of the AB cells, BMP-2 strongly up-regulated ALP activity by approximately 3-fold compared with that of the untreated control cells. However, as with Smad1/5/8 phosphorylation, IL-1β and TNF-α significantly decreased the enhanced ALP activity induced by BMP-2, by approximately 89% and 83%, respectively ($p<0.05$) (Figure 6.11).

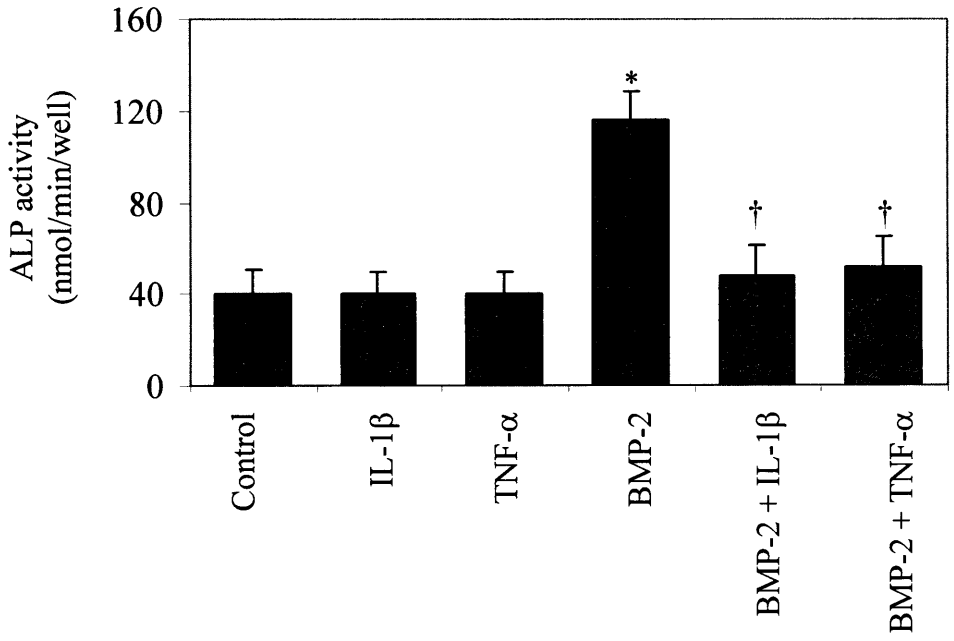


Figure 6.11. Effects of IL-1β and TNF-α on BMP-2-induced ALP activity. Cells were cultured alone (control), incubated with IL-1β and TNF-α (50 ng/ml for 96 h), incubated with BMP-2 (300 ng/ml for 72 h), or incubated with IL-1β and TNF-α (50 ng/ml for 24 h) followed by BMP-2 (300 ng/ml for 72 h) in the continuing presence of the cytokines. ALP activity was then assayed. The results are presented as the mean ± SE of *p*-nitrophenol produced/min/well from triplicate experiments. * $p<0.05$ vs control culture. † $p<0.05$ vs BMP-2-treated culture.

6.3.7.3. Effect of IL-1β and TNF-α on BMP-2-induced OC mRNA expression

To investigate the biological consequences of the loss of surface BMPR-IB expression caused by IL-1β and TNF-α, the effect of these cytokines on BMP-2-induced OC mRNA expression, a marker associated with the late stage of osteoblast differentiation (ten Dijke *et al.*, 2003a), was also examined. In addition to Smad1/5/8 phosphorylation and ALP activity, the effect of IL-1β and TNF-α on BMP-2-mediated OC gene expression was measured quantitatively using Q-PCR. The results showed that while IL-1β and TNF-α had little effect on the expression of OC mRNA, the BMP-2-stimulated increase in OC transcript expression (which was approximately 75% higher compared with that of the untreated control cells) was significantly suppressed by IL-1β and TNF-α, by approximately 75% and 88%, respectively ($p<0.05$) (Figure 6.12).

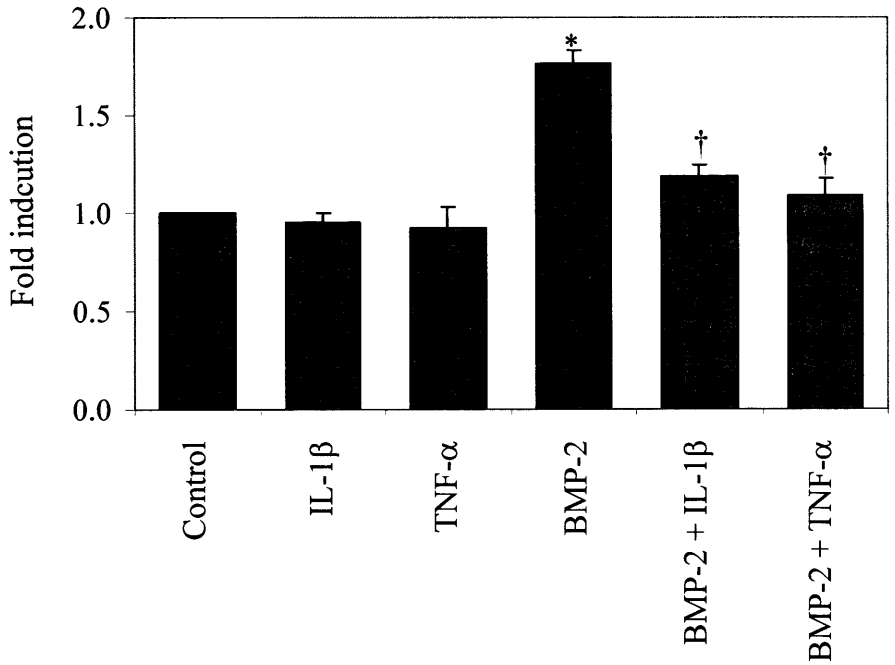


Figure 6.12. Effects of IL-1β and TNF-α on BMP-2-induced OC mRNA expression. Cells were cultured alone (control), incubated with IL-1β or TNF-α (50 ng/ml for 48 h), incubated with BMP-2 (50 ng/ml for 24 h), or incubated with IL-1β or TNF-α (50 ng/ml for 24 h) followed by BMP-2 (50 ng/ml for 24 h) in the continuing presence of the cytokines. Q-PCR analysis of OC mRNA expression was then carried out. The results are presented as the mean fold induction (± SE) of the level of OC mRNA transcript compared with that in the control culture, defined as 1.0. The results were obtained from three separate experiments.

* $p<0.05$ vs control culture. † $p<0.05$ vs BMP-2-treated culture.

6.4. Discussion

A number of studies of the effects of inflammatory cytokines and BMP on osteoblast differentiation have suggested that cytokines such as IL-1β, IL-6, TNF-α and PGE-2 can inhibit osteogenic activity and the responses of bone cells to BMP (Nakase *et al.*, 1997; Virdi *et al.*, 1998; Takiguchi *et al.*, 1999; Gilbert *et al.*, 2002; Nakashima *et al.*, 2002; Nanes, 2003; Yagi *et al.*, 2003; Fukui *et al.*, 2003; Toyoda *et al.*, 2005). Since the BMPs elicit their biological activities via signal transduction through BMPR-IA, BMPR-IB and BMPR-II (ten Dijke *et al.*, 1994b; Nohno *et al.*, 1995), the sensitivity and response of target cells to the BMP depend to some extent on the expression and regulation of the receptors at the cell surface. In this study, the results have provided evidence, for the first time, that such responses may be mediated, at least partly, by the differential regulation of the BMPR by inflammatory cytokines.

The present results have shown that the expression of each of BMPR genes in the AB cells was differentially controlled by the inflammatory cytokines studied. Unlike a previous report in human chondrocytes which showed the lack of effects of IL-1β and TNF-α on BMPR-IA and -II expression (Fukui *et al.*, 2003), in the present study these cytokines as well as PGE-2 were found to decrease transcript expression of BMPR-IA. In marked contrast, the present study also showed that IL-1β and TNF-α had a pronounced stimulatory effect on the BMPR-IB gene specifically, which has previously been reported to play a major role in osteoblast differentiation of bone-derived mesenchymal progenitor cells (Chen *et al.*, 1998).

Although the inflammatory cytokine IL-6 was found to have very little effect on any of the BMPR in the AB cells studied here, a previous report has shown that IL-6 (plus sIL-6R) stimulated BMPR-II mRNA expression in fetal rat calvarial cells (Yeh *et al.*, 2002). Since this IL-6 effect was dependent at least in part on the expression of functional cell surface IL-6R and the generation of functionally active sIL-6R (Ernst and Jenkins, 2004; Franchimont *et al.*, 2005b), the lack of IL-6 effect on BMPR expression observed in the AB cells could also possibly be attributed to low levels of IL-6R and/or sIL-6R expression by the AB cells, which still remain to be determined.

Since the results showed that certain cytokines such as TNF-α were capable of stimulating the expression of the BMPR-IB gene, the signalling pathways leading to increased BMPR-IB gene expression by TNF-α were initially explored using inhibitors of the TNF-α signalling proteins. The results suggested that at least three major subfamilies of the MAPK proteins (p38, ERK1/2 and JNK1/2/3) (Pearson *et al.*, 2001), but not PI3K, could be involved in TNF-α-mediated up-regulation of the BMPR-IB transcripts. Further studies of specific agonists for these MAPK proteins associated with BMPR-IB gene expression would help to clarify the precise components involved in BMP-mediated osteogenesis.

Notably, it was also found that while the cytokines IL-1β, TNF-α and PGE-2 decreased transcript expression of BMPR-IA and, to a lesser extent, BMPR-II, the expression of the corresponding surface proteins was unaffected by these cytokines at the time period examined. Although the mechanism(s) involved in maintaining a constant steady state level of these two plasma membrane proteins is unknown, it is possible that this may involve increased protein half-life and/or increased translational efficiency, as reported for the p53 protein after DNA damage in irradiated cells (Price and Calderwood, 1993; Takagi *et al.*, 2005). It is also possible that modulation of the expression of the BMPR-IA and -II proteins might be time-dependent. Thus, at later time periods (longer than the 24 h examined here) the decreased expression of such proteins in response to the cytokines might have been observed, which would ultimately result in a reduction in the response of the AB cells to the BMP, as observed for the reduced BMP-2-mediated AB cell functions by IL-1β and TNF-α in the present study.

A major finding of the present study has been the observation that despite the marked up-regulation of BMPR-IB mRNA by IL-1β and TNF-α, the corresponding surface protein was significantly decreased. Post-translational regulation of cell surface receptors has previously been shown to occur both by proteolytic shedding of cell surface receptors (Lum *et al.*, 1999; Philip *et al.*, 1999; Hanneken, 2001; Dello and Rovida, 2002; Garton *et al.*, 2003; Hu *et al.*, 2004) and by internalisation via endocytosis of receptors from the cell surface (Anders *et al.*, 1997; Hayes *et al.*, 2002; Di Guglielmo *et al.*, 2003). Analysis of the effects of IL-1β and TNF-α on BMPR-IB reported here has demonstrated, for the first time, that 'soluble' forms of BMPR-IB were present in culture supernatants of cells

incubated with IL-1β and TNF-α. Moreover, these were found to occur concurrently with the loss of surface BMPR-IB expression, suggesting that down-regulation of BMPR-IB by the naturally-occurring cytokines IL-1β and TNF-α is mediated by shedding of the surface protein, a process which may at least partly explain the negative control of osteogenic activity by IL-1β and TNF-α generally observed in bone (Scharla *et al.*, 1994; Nakase *et al.*, 1997; Gilbert *et al.*, 2000; Kumar *et al.*, 2001; Gilbert *et al.*, 2002; Kim *et al.*, 2002; Nanes, 2003; Lukic *et al.*, 2005). While it has previously been reported that IL-1β and TNF-α are able to elicit proteolytic shedding of certain plasma membrane proteins (Tanida *et al.*, 2004; Franchimont *et al.*, 2005a), the current study is the first to demonstrate that these cytokines induce the release of soluble forms of BMPR-IB specifically from the surface of primary human bone cells *in vitro*. In addition, it was found that the sBMPR-IB identified in the present study is likely to be biologically active since it was shown to be able to bind to BMP-2 (Figure 6.7). A number of studies have also shown that soluble forms of other transmembrane receptors play a major part in the regulation of many essential cellular functions (Kotake *et al.*, 1996; Black *et al.*, 1997; Pinckard *et al.*, 1997; Jones *et al.*, 2001; Franchimont *et al.*, 2005a). For example, the presence of sIL-6R in the synovial fluids has been shown to be responsible for osteoclastogenesis in rheumatoid arthritis patients (Kotake *et al.*, 1996), while sTNFR-I and -II can inhibit TNF-induced production of IL-6 *in vivo* (Pinckard *et al.*, 1997). Moreover, certain soluble receptors such as those for IL-1, IL-2, IL-6 and TNF-α have been considered as markers to define certain disease activity, and have previously been used for neutralisation of their respective ligands involved in disease processes (Muller-Newen *et al.*, 1996a).

Although some soluble receptors have also been shown to be generated by the formation of alternatively spliced mRNA transcripts (Rose-John and Heinrich, 1994; Muller-Newen *et al.*, 1996a), for example the sIL-6R and sFGFR-I isoforms (Jones *et al.*, 1998; Hanneken, 2001), only one form of PCR product corresponding to the BMPR-IB transcript was detected in RT-PCR experiments carried out on cells cultured in the absence and presence of IL-1β and TNF-α, using primers that amplify the transcript sequences corresponding to the transmembrane domain and a part of the extracellular region of BMPR-IB (Appendix III-Figure 1). It is therefore unlikely that AB cells

generated other forms of differentially spliced mRNA transcripts for this receptor, even in the presence of the cytokines.

Although the molecular mechanisms involved in cytokine-induced shedding of the BMPR observed in the present study are not yet known, previous studies suggest that this process is mediated mainly by the activation of 'proteases' via both PKC-dependent and -independent signal transduction pathways (Reiland *et al.*, 1996; Vecchi *et al.*, 1996; Vecchi and Carpenter, 1997; Izumi *et al.*, 1998; Vecchi *et al.*, 1998; Rizoli *et al.*, 1999; Umata *et al.*, 2001; Dello and Rovida, 2002). The results presented here (Chapter 5) have demonstrated that treatment of the AB cells with a PKC activator also generated a soluble form of BMPR-IB, suggesting that shedding of this osteogenesis-associated receptor by IL-1β and TNF-α might also occur via the PKC pathway. In addition, it has been reported that these two naturally-occurring cytokines mediate IL-6R shedding through the activation of a distinct metalloprotease TACE in osteoblast-like cells (Franchimont *et al.*, 2005a), suggesting that this mechanism could also be involved in BMPR-IB shedding induced by these cytokines.

As noted above, a number of surface proteins, including the BMPR, are regulated by internalisation via endocytosis (Anders *et al.*, 1997; Jortikka *et al.*, 1997; Dore, Jr. *et al.*, 2001; Hayes *et al.*, 2002; Di Guglielmo *et al.*, 2003), thus resulting in the loss of cell surface expression and function. However, the down-regulation of BMPR-IB expression by IL-1β and TNF-α observed here is unlikely to have been mediated by this internalisation process since no concomitant increase was observed in the intracellular level of BMPR-IB protein following incubation with IL-1β and TNF-α as shown by C-ELISA analysis (Figure 6.5). In addition, although the present study did not directly examine the effect of the cytokines on changes in the levels and/or intracellular localisation of the Smurf1 and Smad6 proteins, which have previously been reported to be involved in targeting type-I BMP receptors for their degradation (Imamura *et al.*, 1997; Murakami *et al.*, 2003), it was found that IL-1β and TNF-α had very little effect on the relative level of expression of Smurf1 and Smad6 transcripts in the AB cells. These findings thus suggest that the down-regulation of surface BMPR-IB expression elicited by IL-1β and TNF-α is not likely to be associated with Smurf1/Smad6-mediated BMPR-IB degradation.

Smad1/5/8 are now generally considered to be very early downstream effectors in the BMPR signalling pathway, and the activation of these proteins is thus fundamental in the responsiveness of cells to BMP (Yamamoto *et al.*, 1997; Lai and Cheng, 2002; Noth *et al.*, 2003). The present study showed that the marked decrease in cell surface BMPR-IB levels following incubation with IL-1β and TNF-α was also associated with a profound reduction in Smad1/5/8 phosphorylation. The loss of this central activation step in the BMP-2-induced signal transduction pathway is undoubtedly of major importance for the functional activity of bone cells and their sensitivity to BMP-2. Moreover, ALP and OC are also widely recognised as early and late markers for osteoblast differentiation, respectively (Ebara and Nakayama, 2002; van der Horst *et al.*, 2002; Noth *et al.*, 2003), and their induction by BMP is closely related to osteoblast differentiation (Spinella-Jaegle *et al.*, 2001; ten Dijke *et al.*, 2003a). It is therefore notable that the shedding of surface BMPR-IB by the naturally-occurring cytokines IL-1β and TNF-α reported in the present study was found not only to strikingly reduce p-Smad1/5/8 levels but also to significantly inhibit BMP-2-induced ALP activity and OC mRNA expression. Since the BMPR-IA and -II surface proteins were largely unaffected by IL-1β and TNF-α under the conditions used here, these data suggest that BMPR-IB could play a major part in osteogenic signalling via BMP-2 in the AB cells, which is negatively regulated by the cytokine-induced shedding of this receptor specifically.

It has previously been suggested that IL-1β and TNF-α initially engage distinct signalling pathways that converge with the activation of the transcription factor NF-κB and stimulation of the MAPK system, resulting in suppression of bone matrix genes and differentiation of osteoblast progenitors (Weitzmann and Pacifici, 2005). Shedding of a 'soluble form' of the BMPR-IB by IL-1β and TNF-α, as demonstrated here *in vitro*, suggests an additional mechanism by which these two major inflammatory cytokines could inhibit osteoblast differentiation and function *in vivo.* Moreover, a previous study of bone reactions following bone marrow ablation in mice has suggested that TNF-α could act on osteoblasts to reduce BMPR expression (Shimizu *et al.*, 1998), and it is therefore possible that shedding of BMPR-IB induced by TNF-α could also represent an important mechanism underlying decreased expression of BMPR-IB *in vivo*.

In conclusion, the present study has provided evidence, for the first time, that BMPR can be differentially regulated by the inflammatory cytokines IL-1β, IL-6, TNF-α and PGE-2 at both the post-transcriptional and post-translational levels. In addition, the shedding of a soluble form of BMPR-IB from AB cells was found to be induced by IL-1β and TNF-α and was closely associated with a markedly diminished response to the cognate ligand BMP-2, as shown by the reduction of BMP-2-induced Smad1/5/8 phosphorylation, ALP activity and OC expression *in vitro*.

CHAPTER 7
CONTROL OF BMPR EXPRESSION AND FUNCTION BY GROWTH FACTORS AND HORMONES

7.1. Introduction

BMPs are generally regarded as the most potent growth factors with respect to osteogenesis and bone remodelling, having the ability to effectively promote differentiation of mesenchymal stem cells/osteoprogenitors into mature osteoblasts, as detailed in Section 1.5.4. A wide range of other growth factors and hormones also play an important part in the proliferation and differentiation of osteoprogenitor cells and the formation of mature bone. These include TGF-β, FGF, PDGF, IGF, androgen, hydrocortisone, Dex and RA (Section 1.2), and although the mechanisms by which these mediators regulate osteogenic activity are not yet fully understood, it is possible that modulation of the BMP/BMPR signalling pathway is fundamental. The aim of the present study was therefore to examine the specific biological effects of the growth factors BMP-2, TGF-β1, FGF-2 and PDGF-AB and IGF-1, and of the hormones DHT, hydrocortisone, Dex and RA, on BMPR expression and BMP-2-induced osteogenic responses (*i.e.,* Smad 1/5/8 phosphorylation, Dlx5 expression and ALP activity). Moreover, an RNAi approach (Amarzguioui, 2004; Ovcharenko *et al.*, 2005) was used, by transfecting an siRNA targeting BMPR-IB, to determine the functional importance of this receptor as a target for growth factor-induced osteogenic activity.

7.2. Materials and Methods

Cell culture, RT-PCR, Q-PCR, FCM, WB, ALP activity assay and siRNA transfection were performed as described in Chapter 2. Additional methods used in this chapter are noted below.

7.2.1. Treatment of cells

Exponentially growing AB cells obtained 48 h after plating were incubated in 1% FCS-DMEM for 24 h and then incubated with BMP-2, TGF-β1, FGF-2, PDGF-AB, IGF-1, DHT, hydrocortisone, Dex and RA (the concentrations and exposure times of each factor are noted in each experiment below). In some experiments to examine the effect of the growth factors on BMP-2-induced osteogenic functions, cells were pre-treated with the growth factors for 24 h, followed by the addition of BMP-2 (50-300 ng/ml) for 24-72 h in the continuing presence of the growth factors. The optimal concentrations and incubation times used for BMP-2 treatment are indicated below for each particular assay. For experiments using chemical inhibitors of signalling proteins, cells were pre-treated (2 h) with SB203580 (10 μM), U0126 (1 μM), SP600125 (10 μM), Bis IX (2 μM), U73122 (0.5 μM) and wortmannin (0.1 μM) (all from Sigma), which are inhibitors of p38 MAPK, ERK1/2, JNK1/2/3, PKC, phospholipase C (PLC) and PI3K, respectively, followed by the addition of 50 ng/ml of BMP-2, 0.1 ng/ml of TGF-β1, 0.1 ng/ml of FGF-2 and 1 ng/ml of PDFG-AB for 24 h in the continuing presence of the inhibitors. These are major signalling proteins in growth factor signalling pathways (Heldin *et al.*, 1998; Massague and Chen, 2000; Miyazono *et al.*, 2005; Webber *et al.*, 2005). At the concentrations and conditions used here, these inhibitors were found to cause less than 5% cell death, as determined by trypan blue exclusion tests performed in duplicate. RNA and protein extraction, FCM and the ALP activity assay were carried out on untreated and treated cells.

7.3. Results

7.3.1. Effect of growth factors and hormones on BMPR transcript expression

Cells were treated with BMP-2, TGF-β1, FGF-2, PDGF-AB, IGF-1, DHT, hydrocortisone, Dex and RA, and the expression of each of the BMPR transcripts examined by semi-quantitative RT-PCR. The results in Figure 7.1A show that none of the mediators significantly affected BMPR-IA mRNA expression ($p>0.05$), although treatment with Dex and particularly RA appeared to down-regulate this receptor. In marked contrast to BMPR-IA, BMPR-IB mRNA was markedly and differentially affected by the growth factors and a hormone. Thus, with the concentrations and conditions used in these experiments, the ligand BMP-2, TGF-β1, FGF-2, PDGF-AB, IGF-1 and DHT significantly up-regulated BMPR-IB transcripts by between 1.8- and 3.8-fold ($p<0.05$), whereas hydrocortisone, Dex and RA had no statistically significant effect ($p>0.05$) (Figure 7.1B). As with BMPR-IA, none of the mediators significantly affected BMPR-II mRNA expression, despite the slight inhibitory effect of RA, as also observed for BMPR-IA ($p>0.05$) (Figure 7.1C).

Using Q-PCR to quantitatively measure the apparent up-regulation of BMPR-IB mRNA by some of the osteogenic factors, the results in Figure 7.2 show that the ligand BMP-2 (10, 50 and 100 ng/ml) significantly increased transcript expression between 2- and 5-fold ($p<0.05$). All concentrations of the growth factor TGF-β1 also significantly up-regulated BMPR-IB mRNA, with a concentration of 0.1 ng/ml eliciting an approximately 12-fold increase compared with the untreated control ($p<0.05$). The optimal concentration of FGF-2, PDGF-AB, IGF-1 and DHT were found to be at 0.1, 1.0, 50 and 100 ng/ml, which increased transcript levels approximately 2.5-, 2.4-, 2.5- and 5.1-fold, respectively (Figure 7.2). Thus, all the five osteogenic factors as well as the ligand BMP-2 were found to exert a significantly positive effect on the activity of the BMPR-IB gene.

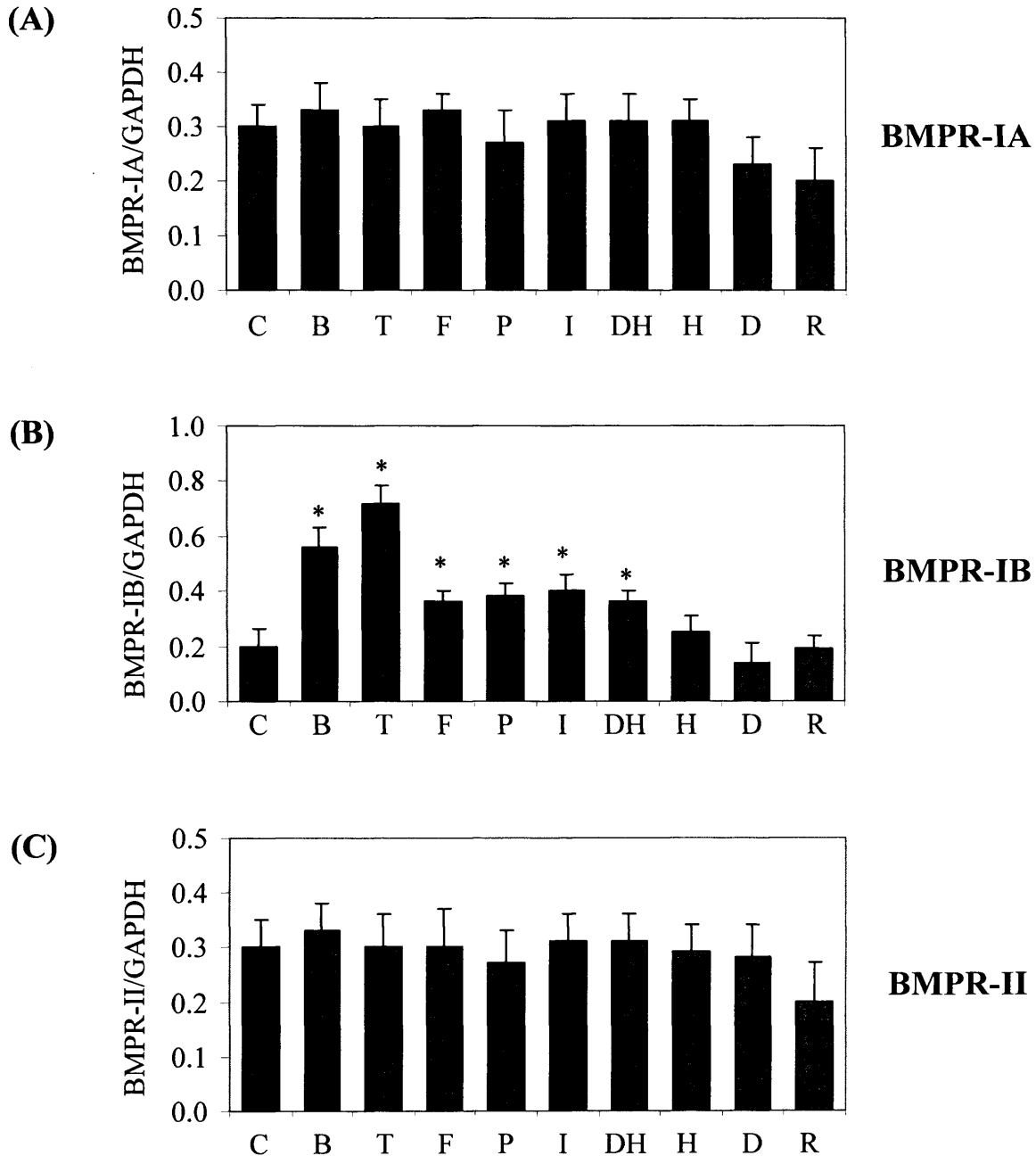


Figure 7.1. Effect of the ligand BMP-2, growth factors and hormones on BMPR-IA (A), -IB (B) and -II (C) mRNA expression. Cells were treated with either BMP-2, TGF-β1, FGF-2, PDGF-AB, IGF-1, DHT (all 10 ng/ml), hydrocortisone (0.2 μM), Dex (10 nM) or RA (10 nM) for 24 h and the relative expression of BMPR transcripts examined by RT-PCR. Data are presented as the mean (± SE) in three separate experiments.
C: untreated control cells; B: BMP-2; T: TGF-β1; F: FGF-2; P: PDGF-AB; I: IGF-1; DH: DHT; H: hydrocortisone; D: Dex; R: RA. * $p<0.05$ vs untreated control cells.

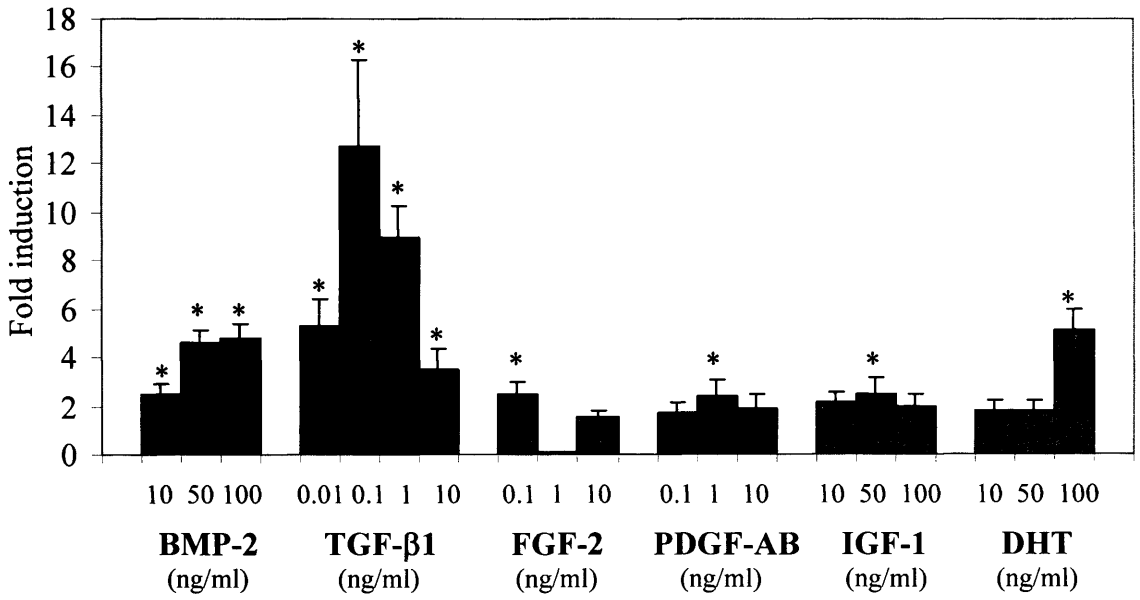
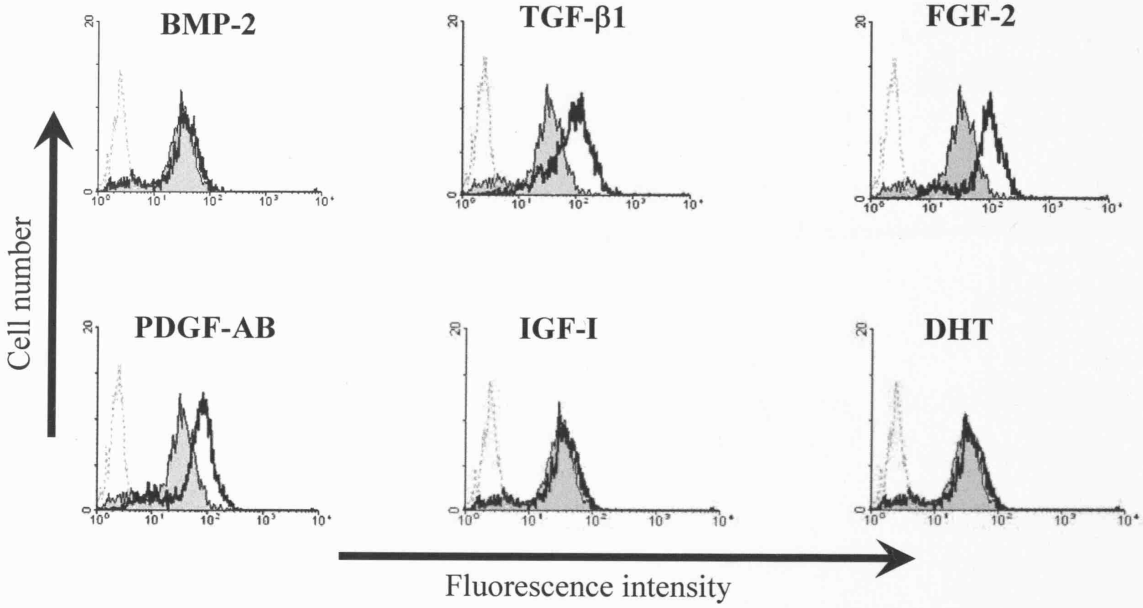


Figure 7.2. Q-PCR analyses of the effect of the ligand BMP-2, growth factors and a hormone on BMPR-IB mRNA expression. Cells were treated with either BMP-2 (10-100 ng/ml), TGF-β1 (0.01-10 ng/ml), FGF-2 (0.1-10 ng/ml), PDGF-AB (0.1-10 ng/ml), IGF-1 (10-100 ng/ml) or DHT (10-100 ng/ml) for 24 h and the expression of BMPR-IB mRNA examined by Q-PCR. Data are presented as the mean fold induction of BMPR-IB transcripts (± SE) in the treated cells compared with untreated control cells, defined as 1.0, performed in three separate experiments. * $p<0.05$ vs untreated control cells.

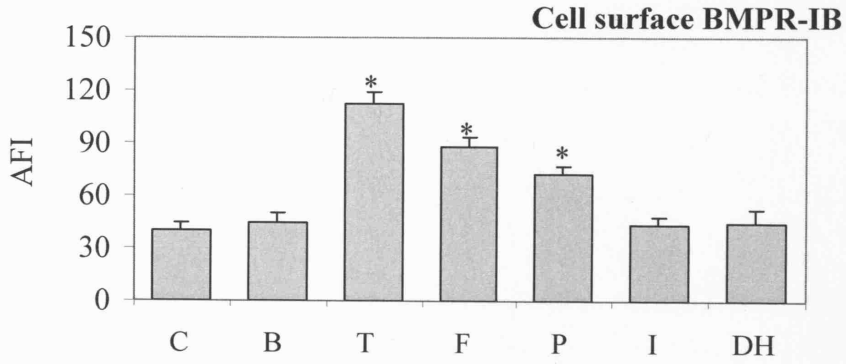
7.3.2. Effects of growth factors and a hormone on cell surface expression of the BMPR-IB protein

Using the optimal concentrations found to up-regulate BMPR-IB transcript levels at 24 h, the effect of BMP-2, TGF-β1, FGF-2, PDGF-AB, IGF-1 and DHT on BMPR-IB protein expression was measured by FCM. Since hydrocortisone, Dex and RA had no significant effect on the expression of BMPR-IB mRNA ($p>0.05$) (Figure 7.1B), their effect on the corresponding protein was not further examined. The representative histograms in Figure 7.3A show that TGF-β1, FGF-2 and PDGF-AB, but not the ligand BMP-2, IGF-1 and DHT, increased BMPR-IB expression at the cell surface, as demonstrated by the shift of the histograms of the treated cultures (clear histograms) to the right of the control cultures (shaded histograms). Analysis of three separate experiments established that the three growth factors, but not BMP-2, IGF-1 and DHT, significantly up-regulated cell surface BMPR-IB protein expression by 2.8-, 2.2- and 1.8-fold, respectively, compared with the control culture ($p<0.05$) (Figure 7.3B). In addition, the results in Figure 7.3C show that TGF-β1, FGF-2 and PDGF-AB also significantly increased the level of intracellular BMPR-IB protein by approximately 2- to 3-fold compared with the control culture ($p<0.05$). Moreover, as with the respective mRNA levels, BMP-2 and the osteogenic factors had little if any effect on the cell surface levels of the BMPR-IA and -II proteins (Appendix IV-Figure 1). Thus, only the growth factors TGF-β1, FGF-2 and PDGF-AB elicited a corresponding increase in the expression of the surface receptor.

(A)


BMP-2
TGF-β1
FGF-2
PDGF-AB
IGF-I
DHT
Cell number
Fluorescence intensity

(B)


Cell surface BMPR-IB
AFI
150
120
90
60
30
0
C
B
T
F
P
I
DH

(C)

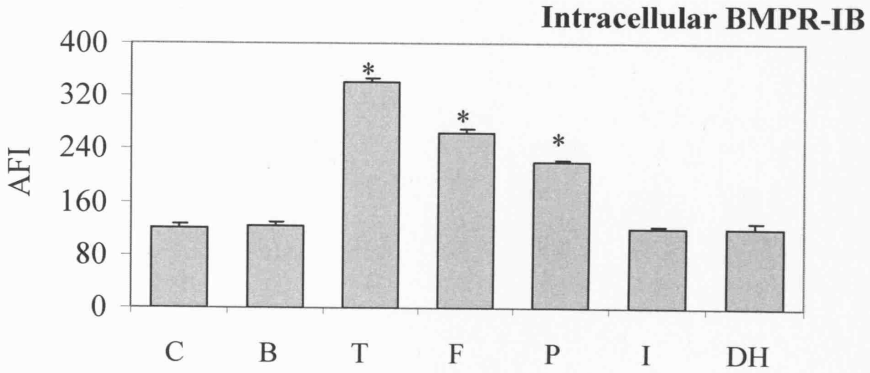

Intracellular BMPR-IB
AFI
400
320
240
160
80
0
C
B
T
F
P
I
DH

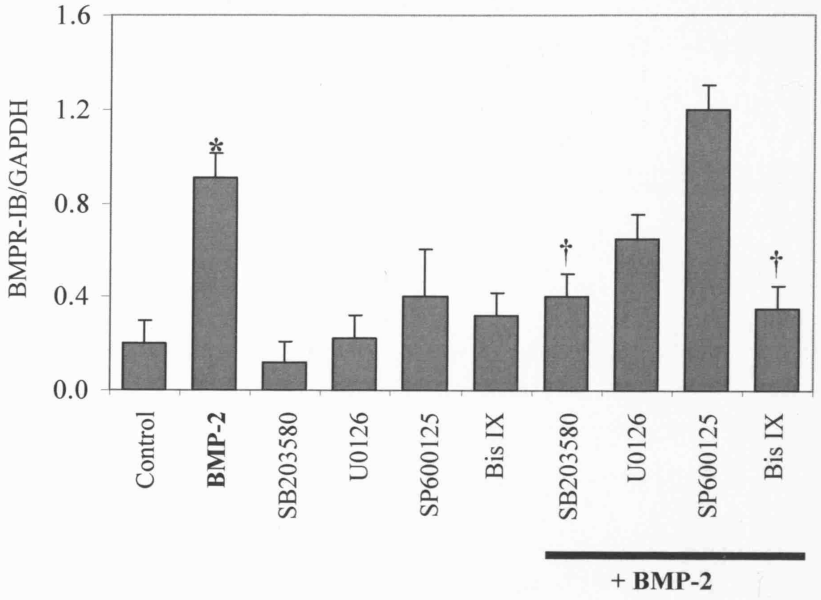
Figure 7.3. Effects of the ligand BMP-2 and osteogenic growth factors and a hormone on the expression of the BMPR-IB protein. Cells were incubated with BMP-2 (50 ng/ml), TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml), PDGF-AB (1 ng/ml), IGF-1 (50 ng/ml) and DHT (100 ng/ml) for 24 h and the expression of BMPR-IB analysed by FCM. (A) shows representative FCM histograms in which up-regulation of the relative surface protein level in the growth factor-treated cultures (clear histograms, solid lines) is shown by a shift of fluorescence to the right compared with the untreated control cultures (shaded histograms). The dotted histograms are the background fluorescence profiles of the isotype control staining. The effects of these mediators on the expression of cell surface and intracellular BMPR-IB protein are summarised in (B) and (C), respectively. Data are presented as the mean AFI (± SE) of untreated and treated cultures of triplicate experiments. C: untreated control cells; B: BMP-2; T: TGF-β1; F: FGF-2; P: PDGF-AB; I: IGF-1; DH: DHT. * $p<0.05$ vs untreated control cells.

7.3.3. Signalling pathways in growth factor-induced BMPR-IB gene expression

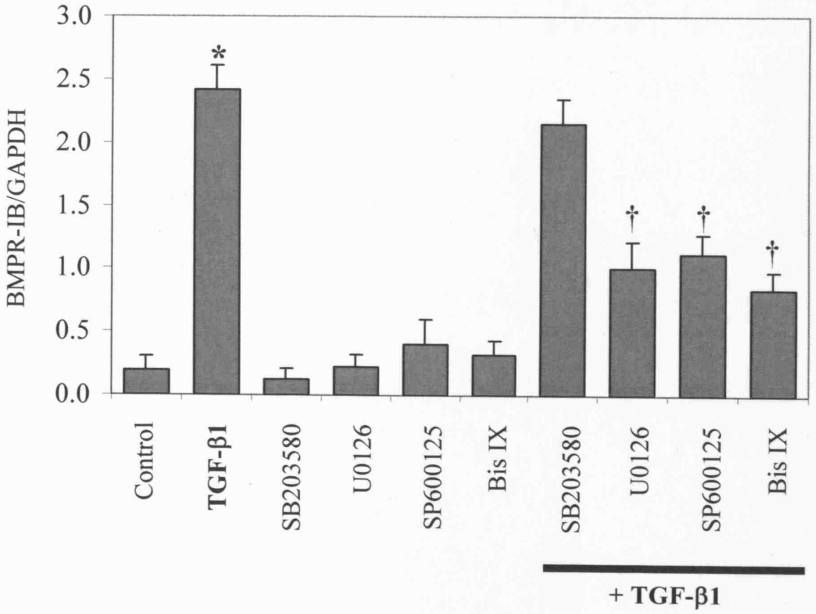
To investigate the signalling pathways involved in the up-regulation of BMPR-IB by growth factors, the effect of inhibitors of major signalling proteins on growth factor induction of BMPR-IB mRNA was examined. The inhibitors used were SB203580, U0126, SP600125, Bis IX, U73122 and wortmannin, inhibitors of p38 MAPK, ERK1/2, JNK1/2/3, PKC, PLC and PI3K, respectively, which have previously been shown to play a central part in growth factor signalling pathways (Heldin *et al.*, 1998; Massague and Chen, 2000; Miyazono *et al.*, 2005; Webber *et al.*, 2005).

The results in Figure 7.4 show that the inhibitors alone had no significant effect on 'constitutive' BMPR-IB mRNA expression, as measured by RT-PCR ($p>0.05$), while each of the growth factors alone significantly stimulated BMPR-IB transcript levels ($p<0.05$). However, treatment of the AB cells with SB203580 or Bis IX significantly ablated BMP-2-induced up-regulation of BMPR-IB mRNA, by approximately 71% and 78% compared with that in cells treated with BMP-2 alone, respectively ($p<0.05$) (Figure 7.4A). U0126 treatment also suppressed the induction effect of BMP-2, although to a lesser degree (less than 40%). In contrast, BMP-2-enhanced up-regulation of BMPR-IB was further increased by SP600125, although not significantly ($p>0.05$), as shown in Figure 7.4A. The results in Figure 7.4B show that TGF-β1-induced BMPR-IB mRNA expression was significantly abrogated by either U0126, SP600125 or Bis IX (by approximately 63%, 58% and 71% suppression, respectively) ($p<0.05$), although SB203580 had no inhibitory effect. FGF-2-induced BMPR-IB up-regulation was found to be significantly diminished by U0126 treatment ($p<0.05$), but strongly enhanced by U73122 treatment ($p<0.05$) (Figure 7.4C), while Bis IX and wortmannin had no significant effect on BMPR-IB up-regulation induced by FGF-2 ($p>0.05$). However, Bis IX and wortmannin both appeared to potentiate BMPR-IB up-regulation induced by PDGF-AB, by more than 200% (Figure 7.4D) whereas U73122 treatment resulted in suppression of PDGF-AB-induced BMPR-IB up-regulation. Thus, up-regulation of BMPR-IB gene expression by osteogenic growth factors was mediated by various signalling pathways, with p38 MAPK involved in up-regulation of BMPR-IB induced by BMP-2, ERK1/2 by TGF-β1 and FGF-2, JNK1/2/3 by TGF-β1, PKC by BMP-2 and TGF-β1 and PLC by PDGF-AB, while the PI3K pathway may not play a significant role in the increase of BMPR-IB gene expression induced by the growth factors.

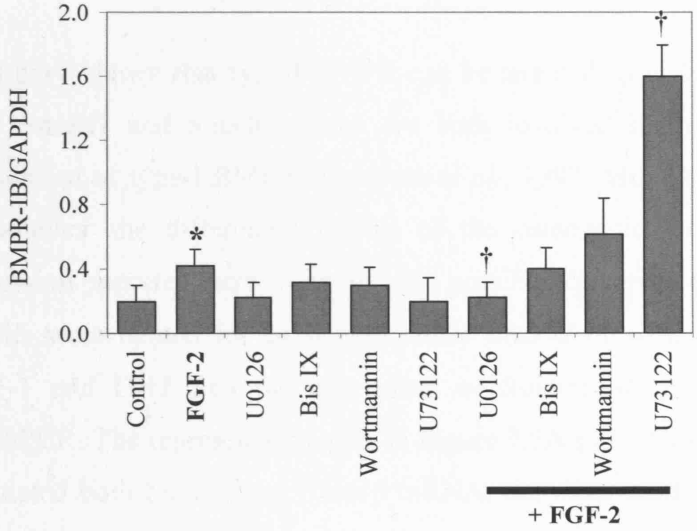
(A)


BMPR-IB/GAPDH
1.6
1.2
0.8
0.4
0.0
*
†
†
Control
BMP-2
SB203580
U0126
SP600125
Bis IX
SB203580
U0126
SP600125
Bis IX
+ BMP-2

(B)


BMPR-IB/GAPDH
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
†
†
†
Control
TGF-β1
SB203580
U0126
SP600125
Bis IX
SB203580
U0126
SP600125
Bis IX
+ TGF-β1

(C)



(D)

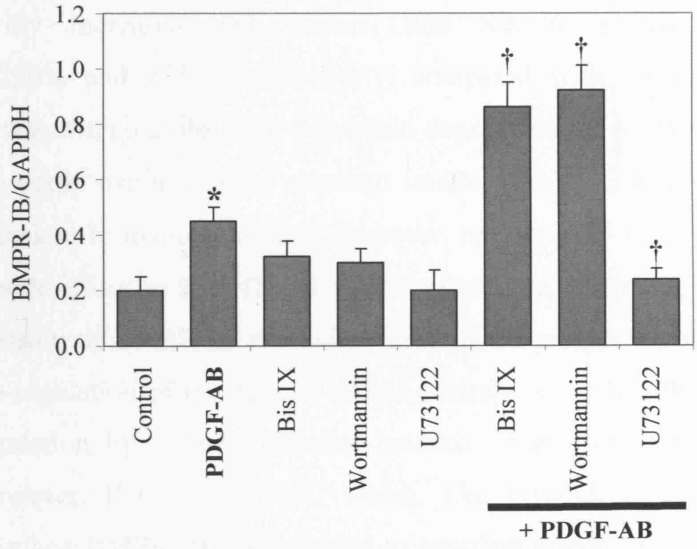


Figure 7.4. Summary of RT-PCR results showing the effect of inhibitors of signalling pathways on BMPR-IB transcript expression induced by either BMP-2 (A), TGF-β1 (B), FGF-2 (C) or PDGF-AB (D). Cells were pre-treated (2 h) with SB203580, U0126, SP600125, Bis IX, U73122 and wortmannin, which are inhibitors of p38 MAPK, ERK1/2, JNK1/2/3, PKC, PLC and PI3K, respectively, followed by the addition of 50 ng/ml of BMP-2, 0.1 ng/ml of TGF-β1, 0.1 ng/ml of FGF-2 and 1 ng/ml of PDFG-AB for 24 h in the continuing presence of the inhibitors. The expression of BMPR-IB mRNA was then examined by RT-PCR. The results are presented as the mean ± SE of the level of BMPR-IB mRNA transcript relative to that of GAPDH, obtained from triplicate experiments.

* $p<0.05$ vs untreated control cells. † $p<0.05$ vs cells treated with the growth factor alone.

7.3.4. Effect of the ligand BMP-2 and osteogenic factors on Smurf1 and Smad6 expression

Previous studies have shown that type-I BMPR can be targeted to undergo intracellular turnover by the Smurf1 and Smad6, which are both involved in the ubiquitination-dependent degradation of type-I BMPR (Imamura *et al.*, 1997; Murakami *et al.*, 2003). To determine whether the differential effects of the osteogenic factors on surface BMPR-IB expression reported here were due to possible changes in Smurf1/Smad6 pathway, the cells were treated for 24 h with either BMP-2 or with TGF-β1, FGF-2, PDGF-AB, IGF-1 and DHT and the expression of Smurf1 and Smad6 transcripts examined by RT-PCR. The representative gels in Figure 7.5A indicate that while BMP-2 strongly up-regulated both Smurf1 and Smad6 mRNA, the other mediators appeared to have little effect. The results in Figure 7.5B further demonstrate that the ligand BMP-2 by itself significantly increased both Smurf1 and Smad6 mRNA expression (by approximately 290% and 475%, respectively, compared with the untreated control) ($p<0.05$), indicating the possible BMP-2-induced degradation of surface BMPR-IB, and possibly at least partly explaining the apparent failure of BMP-2 to up-regulate surface BMPR-IB expression. In marked contrast, however, neither TGF-β1, FGF-2 nor PDGF-AB had any notable effect on Smurf1 and Smad6 transcripts, suggesting that the increase in surface expression of BMPR-IB elicited by these three growth factors was associated directly with up-regulation of the activity of the corresponding BMPR-IB gene and did not involve regulation by a Smurf1/Smad6-induced ubiquitination-related breakdown mechanism. However, IGF-1 and DHT, which, like BMP-2, failed to increase the expression of surface BMPR-IB, were found to have no effect on Smurf1 and Smad6 (Figure 7.5) and thus likely to have no effect on the Smurf1/Smad6-mediated BMPR-IB breakdown process, as did BMP-2.

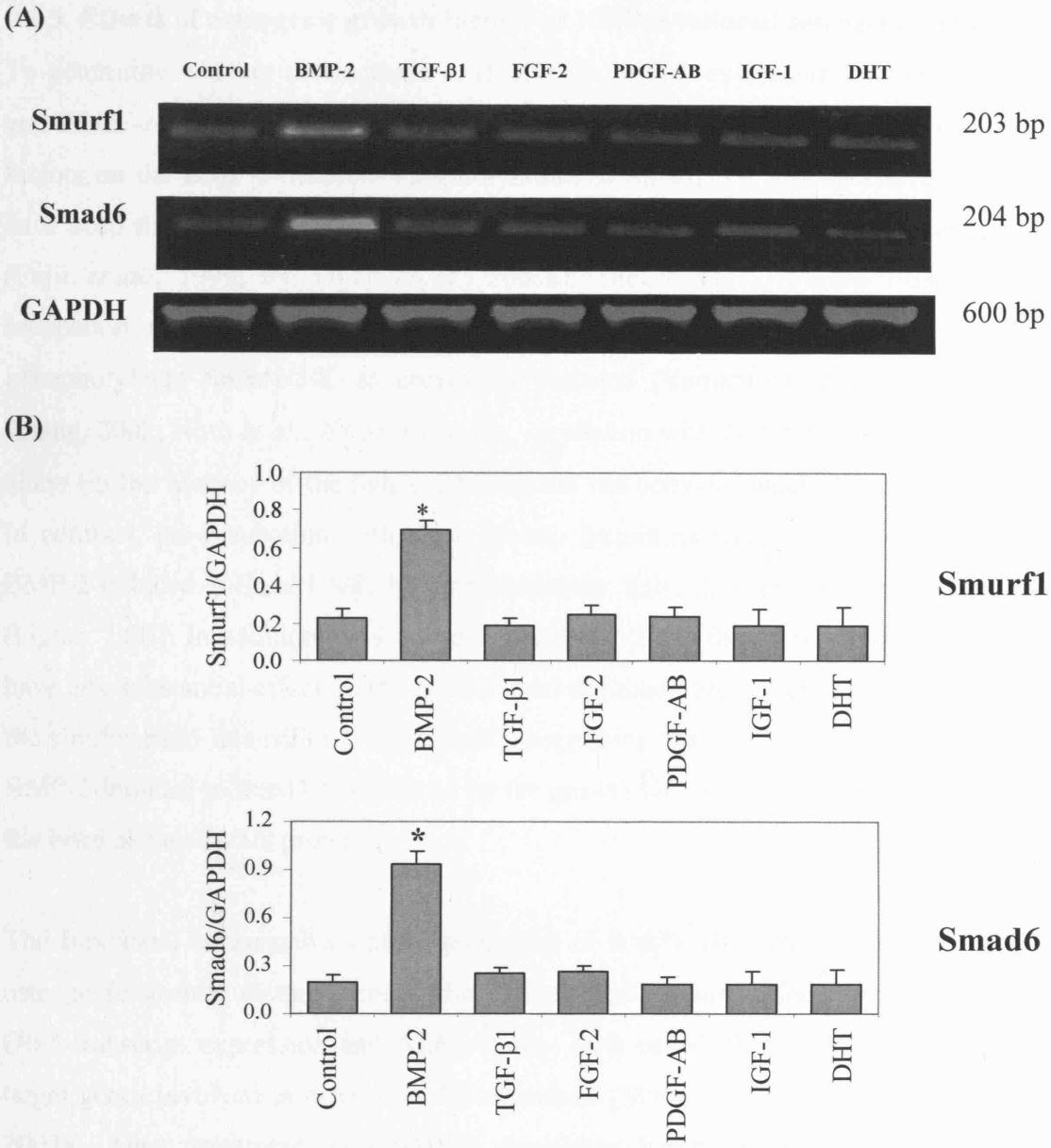


Figure 7.5. Effect of the ligand BMP-2 and osteogenic factors on Smurf1 and Smad6 expression. Cells were treated with BMP-2 (50 ng/ml), TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml), PDGF-AB (1 ng/ml), IGF-1 (50 ng/ml) and DHT (100 ng/ml) for 24 h and the expression of Smurf1 and Smad6 mRNA analysed by RT-PCR, using GAPDH as an internal control. (A) shows representative electrophoresis gels, and the results in (B) are presented as the mean ± SE of the level of Smurf1 and Smad6 mRNA transcripts relative to that of GAPDH, obtained from triplicate experiments.

* $p<0.05$ vs untreated control cells.

7.3.5. Effects of osteogenic growth factors on BMP-2-induced osteogenic functions

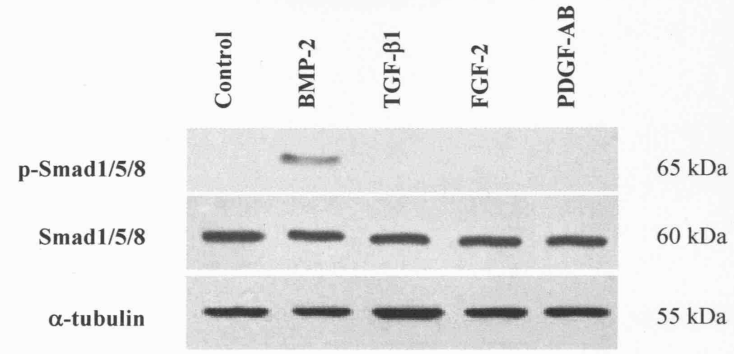
To determine whether the increase in BMPR-IB surface expression by TGF-β1, FGF-2 and PDGF-AB was functionally significant, the effects of these three osteogenic growth factors on the BMP-2-induced phosphorylation of Smad1/5/8 was measured since these have been shown to be immediate downstream effectors in the BMPR signalling pathway (Fujii *et al.*, 1999; ten Dijke *et al.*, 2003a). The results in Figure 7.6A show that incubation of the cells with BMP-2 alone resulted in readily detectable levels of phosphorylated Smad1/5/8, as previously reported (Yamamoto *et al.*, 1997; Lai and Cheng, 2002; Noth *et al.*, 2003). However, incubation with TGF-β1, FGF-2 or PDGF-AB alone (in the absence of the ligand BMP-2) did not activate Smad1/5/8 phosphorylation. In contrast, pre-incubation with these growth factors markedly enhanced the levels of BMP-2-induced p-Smad1/5/8, by approximately 2.9-, 2.1- and 1.8-fold, respectively (Figure 7.6B). In addition, neither the ligand BMP-2 nor the growth factors appeared to have any substantial effect on the overall level of Smad1/5/8 protein expression, based on the similar band intensities (Figure 7.6B), suggesting that the apparently higher level of BMP-2-induced p-Smad1/5/8 induced by the growth factors was not due to an increase in the level of Smad1/5/8 protein.

The functional consequences of up-regulation of BMPR-IB surface expression by these osteogenic growth factors were further determined by their effects on BMP-2-induced Dlx5 transcript expression and ALP activity, both of which are recognised as BMP-2 target genes involved in osteoblast differentiation (Miyama *et al.*, 1999; ten Dijke *et al.*, 2003a). Thus, treatment with BMP-2 alone was found to significantly increase the expression of Dlx5 mRNA, by approximately 165% compared with untreated cells, defined as 100% ($p<0.05$) (Figure 7.6C). Notably, when the cells were first incubated with either TGF-β1, FGF-2 or PDGF-AB and then subsequently exposed to BMP-2, the expression of Dlx5 transcripts was significantly enhanced (by approximately 150-154% compared with cells treated with BMP-2 alone and no growth factors, defined as 100%) ($p<0.05$) (Figure 7.6C). As shown in Figure 7.6D, the ALP activity was also significantly increased, by approximately 3-fold, when the cells were cultured for 72 h with BMP-2 ($p<0.05$). As with Dlx5, pre-treatment of the three growth factors TGF-β1, FGF-2 or PDGF-AB and then BMP-2, significantly increased ALP activity by approximately 140-

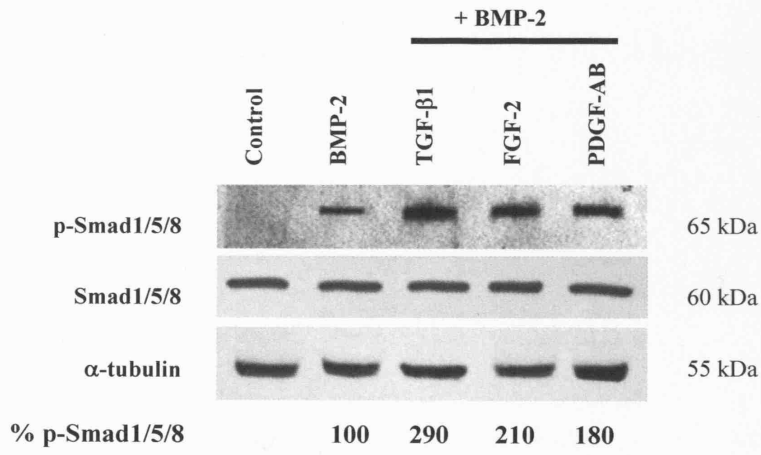
165 % compared with cells treated with BMP-2 alone (and no growth factors) ($p<0.05$) (Figure 7.6D).

These experiments show that the up-regulation of BMPR-IB expression by TGF-β1, FGF-2 and PDGF-AB markedly enhances the level of BMP-2-induced Smad1/5/8 phosphorylation, Dlx5 transcript expression and ALP activity and suggest that these growth factors could potentially have a pronounced osteogenic effect on bone cells.

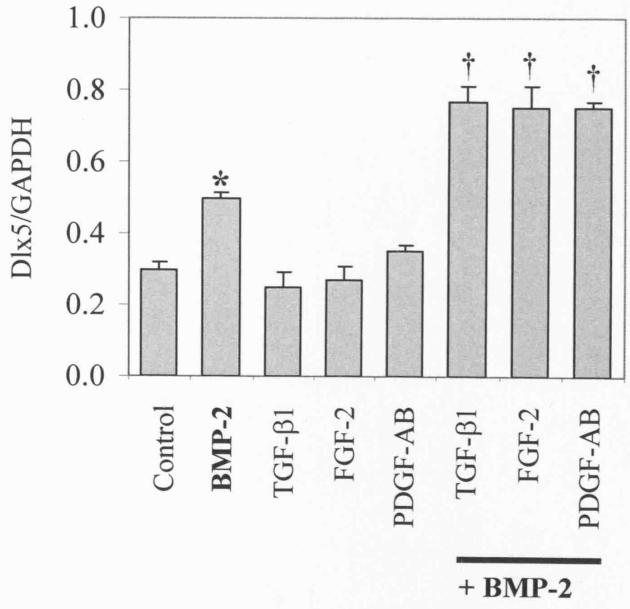
(A)


Control
BMP-2
TGF-β1
FGF-2
PDGF-AB
p-Smad1/5/8
Smad1/5/8
α-tubulin
65 kDa
60 kDa
55 kDa

(B)


+ BMP-2
Control
BMP-2
TGF-β1
FGF-2
PDGF-AB
p-Smad1/5/8
Smad1/5/8
α-tubulin
65 kDa
60 kDa
55 kDa
% p-Smad1/5/8
100
290
210
180

(C)


Dlx5/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
*
†
†
†
Control
BMP-2
TGF-β1
FGF-2
PDGF-AB
TGF-β1
FGF-2
PDGF-AB
+ BMP-2

(D)

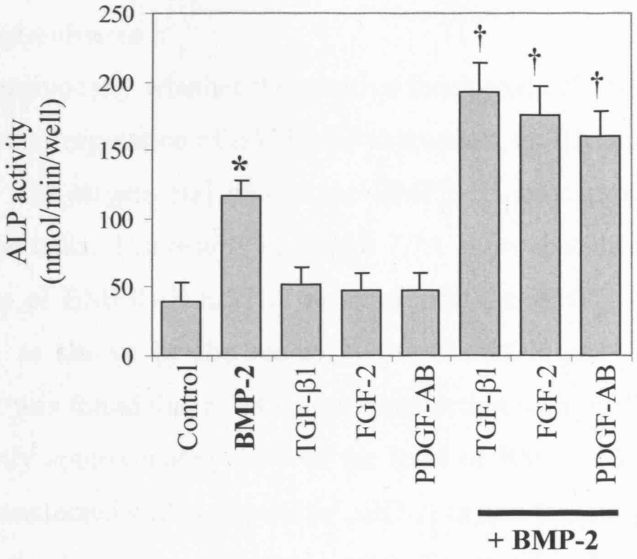


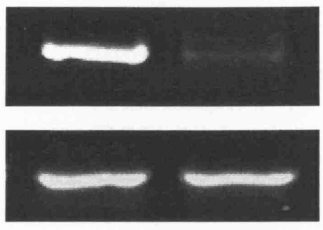
Figure 7.6. Effect of growth factors on BMP-2-induced osteogenic functions. Cells were cultured alone (control), incubated with BMP-2 (100 ng/ml) alone for 30 min and TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) alone for 24 h (A), or pre-treated with TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h, followed by BMP-2 (100 ng/ml) for 30 min in the continuing presence of the growth factors (B). Protein extracts from these cultures were prepared and analysed by WB. The numbers under the immunoreactive bands in (B) show the percentage of Smad1/5/8 phosphorylation, defined as 100% in the cells treated with BMP-2 alone. In (C), cells were cultured alone (control), incubated with BMP-2 (50 ng/ml) alone for 24 h, TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) alone for 48 h, or pre-treated with TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h, followed by BMP-2 (50 ng/ml) for 24 h in the continuing presence of the growth factors. The expression of Dlx5 transcripts was then determined by RT-PCR. The results are presented as the mean ± SE of the level of Dlx5 transcripts relative to that of GAPDH obtained from triplicate experiments. In (D), cells were cultured alone (control), incubated with BMP-2 (300 ng/ml) alone for 72 h, TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) alone for 96 h, or pre-treated with TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h, followed by BMP-2 (300 ng/ml) for 72 h in the continuing presence of the growth factors. ALP activity is presented as the mean ± SE of *p*-nitrophenol produced/min/well from triplicate experiments. * $p<0.05$ vs untreated control culture. † $p<0.05$ vs cells treated with BMP-2 alone.

7.3.6. Effect of BMP-2 and growth factors on BMPR-IB expression in BMPR-IB siRNA-transfected cultures

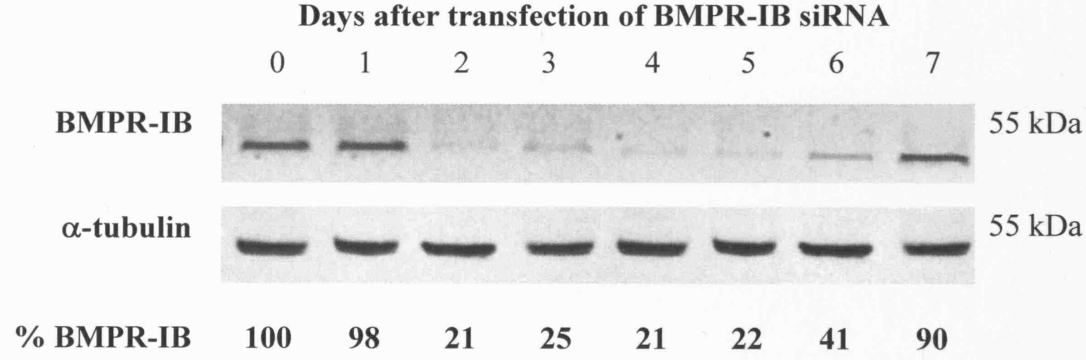
To determine unequivocally whether the positive functional effects of the growth factors were dependent on up-regulation of BMPR-IB expression, an RNAi approach was used in which an siRNA that targets and blocks the BMPR-IB gene product specifically was transfected into the cells. The results in Figure 7.7A show that there was, consequently, strong suppression of BMPR-IB mRNA expression in the BMPR-IB siRNA-transfected AB cell cultures, as shown by the representative RT-PCR gel. Using GAPDH as an internal control, it was found that at 48 h after transfection with the BMPR-IB siRNA, the cells contained only approximately 12% of the level of BMPR-IB transcripts present in control cultures transfected with a 'scramble' siRNA (a non-targeting non-specific siRNA with limited sequence homology to known genes). To establish that the BMPR-IB siRNA also blocked expression of the BMPR-IB protein, WB was carried out on cells incubated between 0 and 7 days after transfection with the BMPR-IB siRNA. The results in Figure 7.7B show that although the level of BMPR-IB protein at one day post-transfection appeared to be unaffected by the siRNA treatment, thereafter expression of the receptor protein was strongly inhibited from day 2 to day 5 after transfection, with only approximately 21-25% of the BMPR-IB protein found in the control culture prior to transfection (at day 0). Subsequently, BMPR-IB protein levels increased and by day 7 was 90% of the original control level. Thus, transfection with BMPR-IB siRNA substantially ablated both BMPR-IB mRNA and protein expression in the AB cell cultures.

The results in Figure 7.7C show the effect of the BMP-2 ligand and each of the growth factors on BMPR-IB transcript levels in the siRNA-transfected cultures. Compared with the relative levels of BMPR-IB mRNA in the scramble siRNA-transfected control cultures treated with BMP-2 and the growth factors, transcript levels in the respective BMPR-IB siRNA-transfected cultures were still all significantly reduced, with only less than 15% BMPR-IB expression remaining (Figure 7.7C). Thus, incubation with BMP-2 and the growth factors did not overcome the suppressive effect of the siRNA on BMPR-IB expression.

(A)


BMPR-IB
(512 bp)
GAPDH
(600 bp)
Scramble siRNA
BMPR-IB siRNA
100%
12%
% BMPR-IB

(B)


Days after transfection of BMPR-IB siRNA
0 1 2 3 4 5 6 7
BMPR-IB
55 kDa
α-tubulin
55 kDa
% BMPR-IB 100 98 21 25 21 22 41 90

(C)

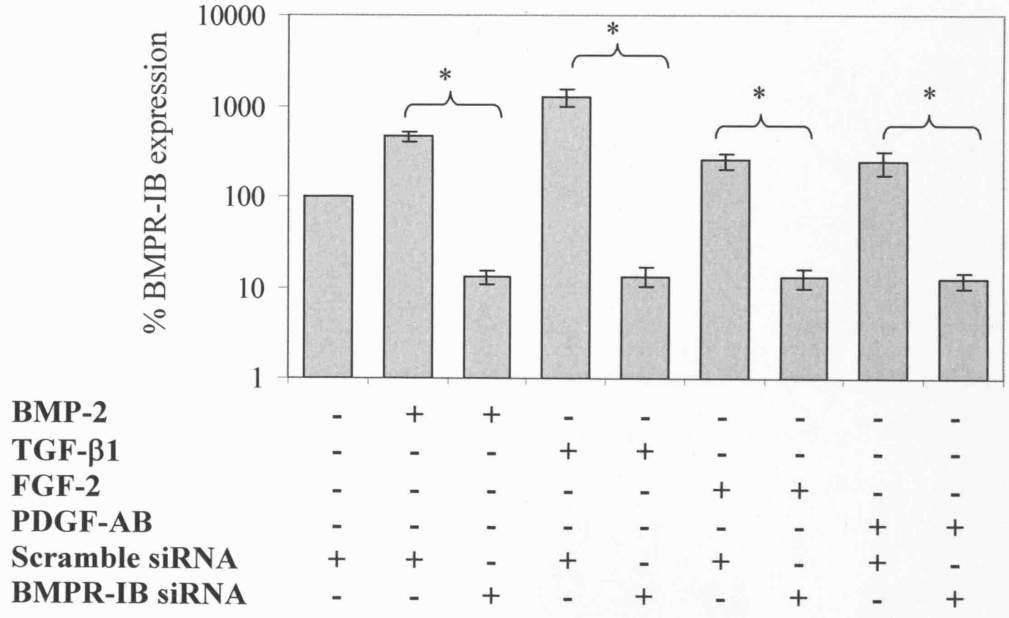

% BMPR-IB expression
10000
1000
100
10
1
*
*
*
*
BMP-2 - + + - - - - - -
TGF-β1 - - - + + - - - -
FGF-2 - - - - - + + - -
PDGF-AB - - - - - - - + +
Scramble siRNA + + - + - + - + -
BMPR-IB siRNA - - + - + - + - +

Figure 7.7. Effect of the ligand BMP-2 and osteogenic growth factors on BMPR-IB expression in BMPR-IB siRNA-transfected cultures. In (A), cells were transfected with the scramble siRNA and with the BMPR-IB siRNA (100 nM) and, 48 h post-transfection, the expression of BMPR-IB transcripts analysed by RT-PCR, using GAPDH as an internal control. The numbers under the RT-PCR gel show the percentage of BMPR-IB transcripts, defined as 100% in the scramble siRNA-transfected culture. In (B), cells were transfected with BMPR-IB siRNA (100 nM) and the expression of BMPR-IB protein analysed between days 0 and 7 by WB, using α-tubulin as an internal control. The numbers under the immunoreactive bands show the percentage of BMPR-IB protein, defined as 100% in the culture at day 0. In (C), cells were transfected with scramble and BMPR-IB siRNA (100 nM) and, 48 h post-transfection, were treated with BMP-2, TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h. The expression of BMPR-IB transcripts was examined by Q-PCR and the data obtained from three separate experiments presented (in log scale) as the percentage of BMPR-IB transcripts (± SE) in the growth factor-treated transfected cultures compared with that of untreated scramble siRNA-transfected cultures, defined as 100%. * $p<0.05$.

7.3.7. Effect of osteogenic growth factors on BMP-2-induced osteogenic functions in BMPR-IB siRNA-transfected cultures

The functional consequences of BMPR-IB 'knock down' in the BMPR-IB siRNA-transfected cultures were determined by measuring the effects of BMP-2 and the growth factors on the levels of Smad1/5/8 phosphorylation, Dlx5 mRNA transcript and ALP activity. As shown in Figure 7.8A, treatment of the 'control' non-specific scramble siRNA-transfected cultures with BMP-2 alone was able to activate Smad1/5/8 phosphorylation, whereas in cultures which had been transfected with the specific BMPR-IB siRNA, BMP-2 induced Smad1/5/8 phosphorylation by only approximately 20%. In addition, pre-incubation of the BMPR-IB siRNA-transfected cultures with TGF-β1, FGF-2 or PDGF-AB resulted in only 22%, 22% and 24% of the Smad1/5/8 phosphorylation levels of that in the scramble siRNA-transfected cultures treated with BMP-2 alone, respectively (Figure 7.8A). Thus, in the BMPR-IB siRNA-transfected cultures, the three osteogenic growth factors did not enhance BMP-2-induced Smad1/5/8 phosphorylation.

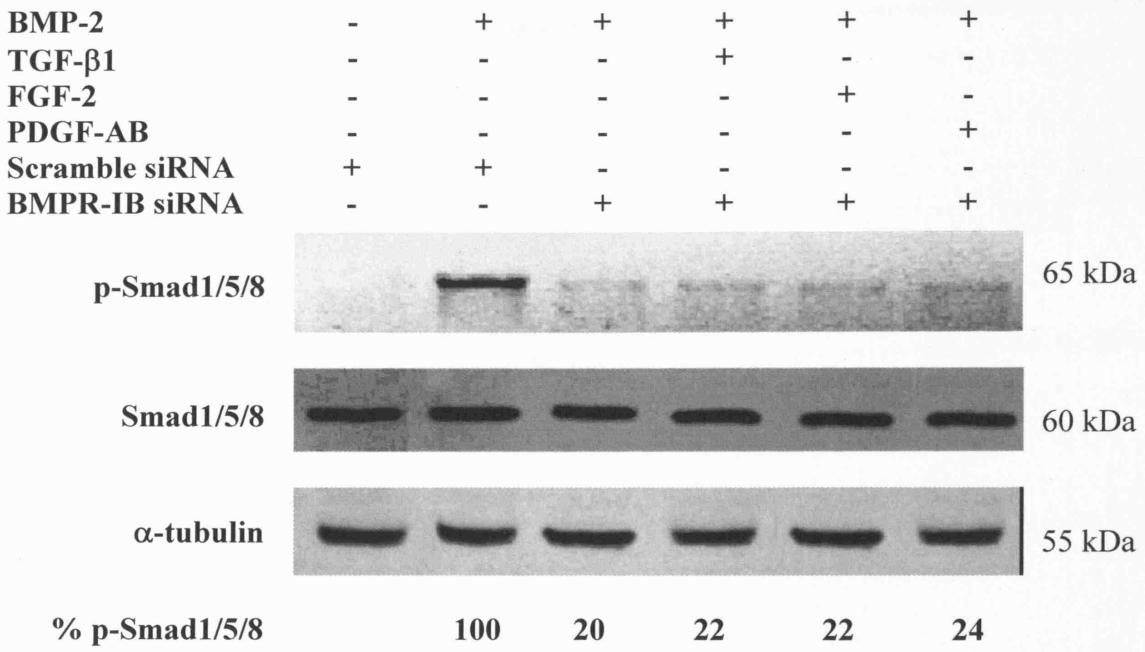
The results in Figure 7.8B show that treatment of scramble siRNA-transfected cultures with BMP-2 alone also significantly stimulated the expression of Dlx5 mRNA transcripts, by approximately 165% compared with that found in the scramble siRNA-transfected cultures which were not treated with BMP-2 ($p<0.05$). In contrast, treatment of the BMPR-IB siRNA-transfected cultures with BMP-2 induced Dlx5 transcript expression by only approximately 115% of that in the scramble siRNA-transfected cultures, defined as 100%. Moreover, pre-incubation of the BMPR-IB siRNA-transfected cultures with TGF-β1, FGF-2 or PDGF-AB also resulted in only 113%, 116% and 120% of the Dlx5 mRNA level observed in the scramble siRNA-transfected cultures, respectively (Figure 7.8B). Thus, the three osteogenic growth factors were not able to potentiate BMP-2-induced expression of Dlx5 mRNA in the BMPR-IB siRNA-transfected cultures.

Treatment of scramble siRNA-transfected cultures with BMP-2 alone also significantly stimulated ALP activity, by approximately 280% compared with that found in the scramble siRNA-transfected cultures which were not treated with BMP-2 ($p<0.05$) (Figure 7.8C). In marked contrast, treatment of the BMPR-IB siRNA-transfected cultures with BMP-2 induced ALP activity by only approximately 128% of that in the scramble

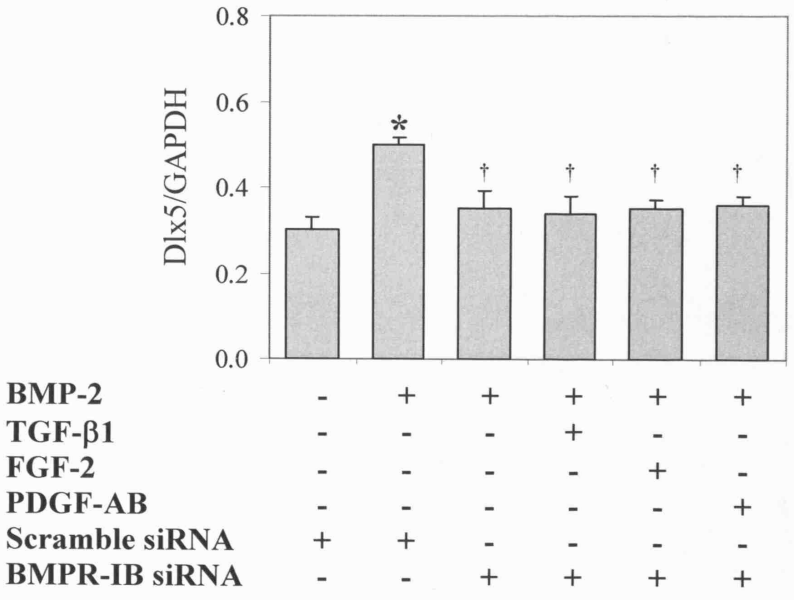
siRNA-transfected cultures, defined as 100%. Furthermore, pre-incubation of the BMPR-IB siRNA-transfected cultures with TGF-β1, FGF-2 or PDGF-AB also resulted in only 130%, 125% and 128% of the ALP activity level found in the scramble siRNA-transfected cultures, respectively (Figure 7.8C). Thus, none of the three osteogenic growth factors enhanced BMP-2-induced ALP activity in the BMPR-IB siRNA-transfected cultures.

Taken together, these data demonstrate that ablation of BMPR-IB protein expression by transfection with BMPR-IB-specific siRNA abolished the ability of the growth factors to augment BMP-2-induced osteogenic functions in the AB cells.

(A)


BMP-2 - + + + + +
TGF-β1 - - - + - -
FGF-2 - - - - + -
PDGF-AB - - - - - +
Scramble siRNA + + - - - -
BMPR-IB siRNA - - + + + +
p-Smad1/5/8
65 kDa
Smad1/5/8
60 kDa
α-tubulin
55 kDa
% p-Smad1/5/8 100 20 22 22 24

(B)


Dlx5/GAPDH
0.8
0.6
0.4
0.2
0.0
*
†
BMP-2 - + + + + +
TGF-β1 - - - + - -
FGF-2 - - - - + -
PDGF-AB - - - - - +
Scramble siRNA + + - - - -
BMPR-IB siRNA - - + + + +

(C)

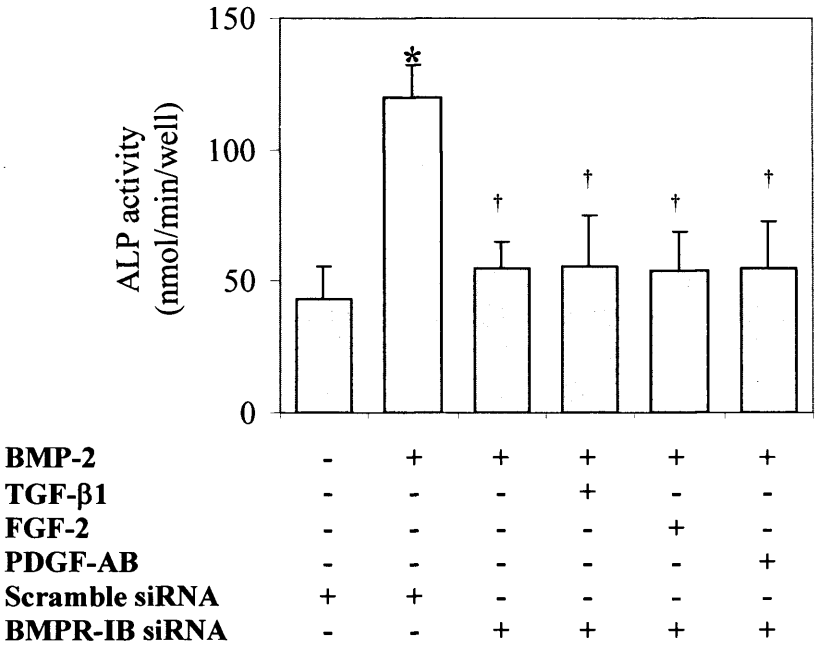


Figure 7.8. Effect of growth factors on BMP-2-induced osteogenic functions in BMPR-IB siRNA-transfected cultures. In (A), the BMPR-IB siRNA-transfected cultures were treated with BMP-2 alone (100 ng/ml for 30 min) or pre-incubated with TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h, followed by BMP-2 (100 ng/ml) for 30 min in the continuing presence of the growth factors. Scramble siRNA-transfected cultures were also cultured with BMP-2 (100 ng/ml for 30 min) as a baseline control. Protein extracts were prepared from these cultures and analysed by WB. The numbers under the immunoreactive bands show the percentage of Smad1/5/8 phosphorylation, defined as 100% in the scramble siRNA-transfected culture treated with BMP-2. In (B), the BMPR-IB siRNA-transfected cultures were treated with BMP-2 alone (50 ng/ml for 24 h) or pre-incubated with TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h, followed by BMP-2 (50 ng/ml) for 24 h in the continuing presence of the growth factors. The expression of Dlx5 transcripts was then determined by RT-PCR. The results are presented as the mean ± SE of the level of Dlx5 transcripts relative to that of GAPDH obtained from triplicate experiments. In (C), the BMPR-IB siRNA-transfected cultures were treated with BMP-2 alone (300 ng/ml for 72 h) or pre-incubated with TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml) and PDGF-AB (1 ng/ml) for 24 h, followed by BMP-2 (300ng/ml) for 72 h in the continuing presence of the growth factors. ALP activity was then measured and the results presented as the mean ± SE of *p*-nitrophenol produced/min/well from triplicate experiments. * $p<0.05$ vs scramble siRNA-transfected culture (without BMP-2 treatment). † $p<0.05$ vs scramble siRNA-transfected culture treated with BMP-2.

7.4. Discussion

A number of osteogenesis-associated factors, including TGF-β1, FGF-2, PDGF-AB, IGF-1, DHT, hydrocortisone, Dex and RA, have previously been shown to affect bone formation and/or the biological activity of BMP (Section 1.2). Such factors have also been shown to regulate differentiation along the osteoblast lineage (Aubin, 1998a; Yamaguchi *et al.*, 2000; Aubin, 2001), and the present study has demonstrated, for the first time, that the growth factors TGF-β1, FGF-2 and PDGF-AB significantly up-regulate BMPR-IB and are thus likely to have a considerable impact on osteoblast differentiation (Chen *et al.*, 1998). Notably, the elevated levels of surface BMPR-IB expression elicited by the growth factors were functionally significant, since they enhanced Smad1/5/8 phosphorylation and BMP-2-induced Dlx5 transcript expression and ALP activity, delineating early stages in the BMP signalling cascade (Fujii *et al.*, 1999; ten Dijke *et al.*, 2003a) and osteoblast differentiation (Miyama *et al.*, 1999; ten Dijke *et al.*, 2003a; Kim *et al.*, 2004), respectively.

The results of the present study also showed that BMPR-IB was the only BMPR which was significantly responsive to the ligand BMP-2 and the osteogenic factors studied (*i.e.*, TGF-β1, FGF-2, PDGF-AB, IGF-1 and DHT) in the AB cells. Although it has previously been reported that BMP-2 induced the expression of BMPR-IA and -II but not BMPR-IB transcripts, these findings were obtained using myoblastic cells *in vitro* and skeletal muscle tissue *in vivo* (Akiyama *et al.*, 1997; Nakamura *et al.*, 2003; Nakamura *et al.*, 2005b). Thus, while BMPR-IA and -II may have an important role in muscle-derived cells, the present results are consistent with other findings that BMPR-IB plays a major part in osteoblast differentiation during embryonic bone development and bone fracture healing processes (Ishidou *et al.*, 1995; Kloen *et al.*, 2002; Kloen *et al.*, 2003). Moreover, the present observation that BMPR-IB mRNA expression was induced by FGF-2 *in vitro* is also consistent with a previous study of ectopic bone formation *in vivo* (Nakamura *et al.*, 2005a), while all the three BMPR transcripts have previously been shown to be induced by TGF-β1 during periosteal chondrogenesis *in vitro* (Sanyal *et al.*, 2002). In addition, the present results have shown, for the first time, that PDGF-AB and IGF-1 are also capable of inducing BMPR-IB mRNA expression. Unlike other steroid hormones tested in this study, DHT, which is a potent derivative form of testosterone, was found to be the only hormone that significantly stimulated the expression of BMPR-IB mRNA in

AB cells. This finding is also consistent with the effect of androgen stimulation in the prostate cancer LNCaP cell line (Ide *et al.*, 1997a). Moreover, the inhibitory effect of RA on BMPR-IA and -II transcripts observed here was consistent with a recent report of RA-induced suppression of BMP-mediated chondrogenesis (Hoffman *et al.*, 2006). Hydrocortisone and Dex had very little effect on the expression of BMPR transcripts, although these hormones have previously been shown to stimulate osteoblast differentiation and bone nodule formation (Bellows *et al.*, 1987; Ireland *et al.*, 2004), suggesting the possibility that the osteogenic activity of these two hormones is likely to be mediated via a pathway distinct from BMPR signalling. Thus, the present results suggest that the ligand BMP-2 and the osteogenic factors TGF-β1, FGF-2, PDGF-AB, IGF-1 and DHT have a significant stimulatory effect on the regulation of the BMPR-IB gene specifically in AB cells.

Although previous studies have reported that growth factors enhance the cellular levels of the BMPR (Nakamura *et al.*, 2003; Nakamura *et al.*, 2005a; Nakamura *et al.*, 2005b), the present study is the first to demonstrate that TGF-β1, FGF-2 and PDGF-AB up-regulate the BMPR-IB protein at the cell surface specifically, a key component in initiating signal transduction from the extracellular BMP ligand to intracellular secondary messengers. In addition, this study has also shown that under the conditions used here, the increase in this cell surface protein was not due to its translocation from the cytoplasm, but rather to an increase in BMPR-IB gene activity, resulting in higher levels of intracellular protein and a corresponding proportional increase in the level of surface-associated BMPR-IB (Figure 7.3B and 7.3C).

Although the downstream signalling cascades induced by BMPR have been extensively studied (Miyazono, 1999; ten Dijke *et al.*, 2003a; Chen *et al.*, 2004a; Miyazono *et al.*, 2005), the regulatory pathways that control BMPR expression are unclear. By using inhibitors to block specific pathways, it was found that BMPR-IB mRNA expression was up-regulated by the growth factors examined through a number of signalling molecules. Thus, the results here have shown that BMPR-IB transcripts were stimulated by BMP-2 via the p38 MAPK and PKC cascades, by TGF-β1 via ERK1/2, JNK1/2/3 and PKC, by FGF-2 via ERK1/2 and by PDGF-AB via PLC (Figure 7.4). It is noteworthy that in FGF-2 up-regulation of BMPR-IB, U73122, a specific inhibitor of PLC, not only failed to

block FGF-2-induced BMPR-IB mRNA, but it markedly potentiated BMPR-IB transcript expression, suggesting a negative regulatory signal in FGF-2/BMPR-IB crosstalk via the PLC pathway. This was also found for specific inhibitors of PKC and PI3K in the PDGF-AB-induced up-regulation of BMPR-IB expression, which were enhanced by Bis IX and wortmannin, respectively (Figure 7.4D). These data suggest that up-regulation of an apparently key receptor for osteoblast differentiation, BMPR-IB, induced by growth factors involves a number of different signalling cascades.

Notably, although the ligand BMP-2 as well as the osteogenic factors TGF-β1, FGF-2, PDGF-AB, IGF-1 and DHT were all found to be able to up-regulate BMPR-IB transcripts, the surface expression of the corresponding protein was increased only by TGF-β1, FGF-2 and PDGF-AB and not by the cognate ligand BMP-2, IGF-1 or DHT. A number of cellular processes could be responsible for this finding, including BMP-2-induced intracellular degradation of this receptor, as has previously been reported in COS7 and 293T cells, in which the nuclear protein Smurf1 was found to form a complex with Smad6 and then recruited to type-I BMPR for their degradation (Murakami *et al.*, 2003). Similarly, BMP-2 has been shown to induce Smad6 mRNA expression in mouse myoblast C2C12, mesenchymal C3H10T1/2 and bone marrow stromal ST2 cells (Takase *et al.*, 1998; Li *et al.*, 2003a), while overexpression of Smurf1 in osteoblasts appears to inhibit postnatal bone formation in mice *in vivo* (Zhao *et al.*, 2004). In the present study, only the ligand BMP-2 was found to induce both Smurf1 and Smad6 mRNA expression in the AB cells, suggesting that surface BMPR-IB degradation mediated by BMP-2 up-regulation of the Smurf1/Smad6 pathway may at least partly explain the apparent anomaly between BMPR-IB mRNA and surface protein expression in the BMP-2-treated AB cells. In contrast to BMP-2, it is unlikely that IGF-1 and DHT, which failed to increase the surface expression of BMPR-IB, modulate BMPR-IB surface expression through Smurf1/Smad6-mediated BMPR-IB turnover mechanism since these two factors had very little effect on Smurf1 and Smad6 expression (Figure 7.5). However, it is possible that other post-transcriptional processes could play a part in the regulation of the BMPR-IB surface protein, such as rapid breakdown of mRNA transcripts and also shedding of a soluble form of the cell surface protein, as has previously been reported for a number of other growth factor receptors (Philip *et al.*, 1999; Hanneken, 2001; Hu *et al.*, 2004). Moreover, it is also possible that regulation of BMPR-IB expression is time-

dependent. Thus, at later time points (longer than 24 h) up-regulation of surface BMPR-IB expression in response to IGF-1 and DHT might have been observed, thereby possibly resulting in an increase in the responsiveness of the AB cells to the BMP, as currently shown by the increased BMP-2-induced osteogenic activities mediated by the three growth factors (*i.e.,* TGF-β1, FGF-2, PDGF-AB), although further studies are required to examine this.

BMP-2 has previously been shown to induce phosphorylation of the Smad1/5/8, immediate downstream effectors in the BMPR signalling pathway (Yamamoto *et al.*, 1997; Fujii *et al.*, 1999; Noth *et al.*, 2003), and to stimulate osteoblast differentiation via up-regulation of the Dlx5 gene, an indispensable mediator in the BMP-2-induced osteoblast differentiation pathway (Miyama *et al.*, 1999; Kim *et al.*, 2004), and increased ALP activity, an early marker of osteoblast differentiation (ten Dijke *et al.*, 2003a). The present study has provided evidence that three osteogenic growth factors, *i.e.,* TGF-β1, FGF-2 and PDGF-AB, which enhanced both transcript and cell surface protein levels of BMPR-IB specifically, augmented the osteogenic activity of BMP-2 by up-regulating Smad 1/5/8 phosphorylation, Dlx5 mRNA expression and ALP activity. Previous reports have demonstrated that other modulators are also capable of acting synergistically with BMP-2 *in vitro* (Chan *et al.*, 2003; Li *et al.*, 2003b; Kugimiya *et al.*, 2005b; Lin *et al.*, 2005), for example the RA-induced stimulation of chondrocyte differentiation mediated via Smad1 and Smad5 (Li *et al.*, 2003b) and the PTH-related peptide-enhanced induction of osteoblastogenesis in pluripotent C3H10T1/2 mesenchymal cells mediated via up-regulation of the BMPR-IA gene (Chan *et al.*, 2003).

The results in the present study showed that similar responses of AB cells to BMP-2 were induced by three different growth factors, which have previously been shown to initiate distinct signalling pathways (Heldin *et al.*, 1998; Massague and Chen, 2000; Webber *et al.*, 2005). However, these pathways comprise a wide range of signalling molecules, and have also been reported to share some common signalling proteins and thus contribute to some similar transcriptional events in the nucleus (Pearson *et al.*, 2001; Lee *et al.*, 2002; Celil and Campbell, 2005). The present data that signalling via the ERK1/2 pathway is required for both TGF-β1- and FGF-2-induced up-regulation of BMPR-IB gene expression also support the important role of overlapping signalling processes.

In addition to the induction of BMPR-IB expression, it is also possible that the osteogenic growth factors TGF-β1, FGF-2 and PDGF-AB could have increased the responsiveness of the AB cells to BMP-2 by a direct effect on extracellular BMP antagonists. Previous studies have shown that noggin and other cystine knot-containing proteins can act as BMP antagonists by binding to BMP-2 and thereby blocking BMP signalling (Balemans and Van Hul, 2002). Thus, noggin overexpression in mature osteoblasts has been reported to cause osteoporosis in mice (Devlin *et al.*, 2003; Wu *et al.*, 2003), presumably by control of the level of biologically active BMP-2. Although the effect of the growth factors on extracellular BMP antagonists has not been examined in the present study, it has previously been reported that TGF-β1, FGF-2 and PDGF do not suppress noggin transcripts in osteoblastic cells (Gazzerro *et al.*, 1998), suggesting that these growth factors did not augment BMP-2 action by suppression of noggin.

In the present study, transfection of an siRNA which targets the BMPR-IB gene product specifically was used to suppress endogenous BMPR-IB expression in primary human AB cells. The results showed that there was a pronounced decrease in BMPR-IB protein expression which was evident between day 2 and day 5 after the transfection of siRNA. The reason for the apparent failure of siRNA to suppress BMPR-IB at day 1 after transfection is not clear, although this could be due to the presence of residual, pre-existing BMPR-IB protein which disappeared as a result of normal intracellular turnover by day 2. It is also noteworthy that BMPR-IB protein expression recovered by day 6 after transfection, indicating the loss of an siRNA effect at this time point, possibly as a result of the progressive endogenous breakdown of the siRNA (Rao and Sockanathan, 2005). Experiments in which BMPR-IB signalling was blocked using siRNA specific for this receptor was found to result in ablation of the synergistic effect of the growth factors on BMP-2-induced Smad1/5/8 phosphorylation, Dlx5 mRNA expression and ALP activity. This observation strongly suggests that TGF-β1, FGF-2 and PDGF-AB significantly enhanced BMP-2 activity at least partly via up-regulation of BMPR-IB in the primary human AB cells, and demonstrates the importance of growth factor-induced BMPR-IB expression in osteogenic function.

In conclusion, the present study shows that the BMPR are differentially modulated by growth factors and hormones, with BMPR-IB the most responsive at both the transcript and surface protein levels. Moreover, osteogenic growth factors TGF-β1, FGF-2, and

PDGF-AB were able to enhance certain BMP-2-induced functional activities of AB cells, such as Smad1/5/8 phosphorylation, Dlx5 expression and ALP activity, which were found to be mediated at least partly via BMPR-IB *in vitro*, suggesting that these positive modulators might also contribute to bone repair and regeneration *in vivo*.

CHAPTER 8
ROLE OF BMPR-IB
IN OSTEBLAST-RELATED GENE REGULATION

8.1. Introduction

The biological activity of the BMP is known to be mediated through signal transduction via heteromeric complexes of BMPR types-I and -II, with BMPR-IA and -IB functioning as the main determinants for the specificity of intracellular signalling and thus their respective target genes (Feng and Derynck, 1997; Persson *et al.*, 1998; Chen and Massague, 1999; ten Dijke *et al.*, 2003a). Although both BMPR-IA and -IB have previously been shown to be involved in the differentiation of cells of mesenchymal lineage, their precise roles in osteoblast differentiation remain largely inconclusive (Section 1.6.9) However, the results in previous chapters (Chapter 6 and 7) have demonstrated that the level of BMPR-IB expression is closely associated with the sensitivity and responsiveness of the AB cells to the BMP, at least with respect to the central osteogenic responses of BMP-2-induced Dlx5 and OC expression and ALP activity. In addition, it has previously been demonstrated that BMPR-mediated BMP signalling also stimulates many osteogenic factors involved in osteoblast differentiation and function, including bone matrix proteins (*e.g.,* ALP, Col I, OP, BSP and OC), cytokines related to osteoblast and osteoclast formation (*e.g.,* BMP-2, -4, and -7 and OPG and RANKL), BMP inhibitory factors (*e.g.,* Smurf1, Smad6 and noggin) and osteogenic transcription factors (*e.g.,* Dlx5, Msx2 and Cbfa1) (Ducy *et al.*, 1997; Hofbauer *et al.*, 1998; Takase *et al.*, 1998; de Jong *et al.*, 2002; Diefenderfer *et al.*, 2003; Otsuka *et al.*, 2003; de Jong *et al.*, 2004; Takagi *et al.*, 2004; Celil *et al.*, 2005). These factors play an important role in bone remodelling, which involves the dynamic processes of osteoblast-mediated bone formation and osteoclast-mediated bone resorption; their osteogenic activities have previously been reviewed in Chapter 1. In addition, BMP/BMPR signalling has also been shown to regulate bone resorption via up-regulation of OPG and RANKL expression by osteoblasts (Hofbauer *et al.*, 1998; Otsuka *et al.*, 2003). RANKL stimulates osteoclast formation by activation of osteoclast differentiation of osteoclast precursor cells through signal transduction via interaction with its cognate receptor (receptor activator of nuclear factor-κB; RANK) on the precursor cells, whereas OPG acts

as a soluble receptor antagonist for RANKL that prevents it from binding to and activating RANK (Kwan *et al.*, 2004; Theoleyre *et al.*, 2004). Moreover, homozygous mutations for OPG in mice induce osteoporosis with enhanced bone resorption, while homozygous RANKL mutant mice exhibit osteopetrosis caused by the absence of osteoclasts (Yamamoto *et al.*, 2006).

To determine BMPR-IB-mediated functions in AB cells, the present study examined changes in the expression of the BMP-induced osteoblast-associated genes noted above using a BMPR-IB siRNA, which has been shown in the present study to significantly ablate both BMPR-IB mRNA and protein expression in the AB cell cultures (Section 7.3.6), and the effect of BMP-2 on the expression of these key genes measured in siRNA-transfected cultures by semi-quantitative RT-PCR.

8.2. Materials and Methods

Cell culture, siRNA transfection and RT-PCR were performed as described in Chapter 2. Additional methods used in this chapter are noted below.

8.2.1. Treatment of cells

AB cell cultures were transfected with siRNA (scramble or BMPR-IB siRNA), as described in Section 2.11.2. Forty-eight hours after transfection, siRNA-transfected cultures were treated with or without BMP-2 (50 ng/ml) for 24 h and RNA extraction carried out on BMP-2-treated and -untreated siRNA-transfected cultures.

8.3. Results

8.3.1. Role of BMPR-IB signalling in bone matrix gene expression

The functional importance of BMPR-IB signalling in the AB cells was investigated by determining the effect of BMP-2 on the expression of ALP, Col I, OP, BSP and OC mRNA in siRNA-transfected cultures. The results in Figure 8.1A show that in the scramble siRNA-transfected cultures, the expression of ALP transcripts was significantly up-regulated by BMP-2 compared with that in the control scramble siRNA-transfected cultures without BMP-2 treatment ($p<0.05$). However, BMP-2 induced a much lower level of ALP mRNA in the BMPR-IB siRNA-transfected cultures (Figure 8.1A). In contrast to ALP, BMP-2 appeared to have little effect on the expression of Col I in either the scramble or BMPR-IB siRNA-transfected cultures ($p>0.05$) (Figure 8.1B). BMP-2 was found to have a significant stimulatory effect on the expression of OP and BSP in scramble siRNA-transfected cultures ($p<0.05$), although no significant differences between those in scramble and BMPR-IB siRNA-transfected cultures were observed ($p>0.05$) (Figure 8.1C and 8.1D), suggesting that in those cultures which were responsive to BMP-2, the stimulatory effect is unlikely to be mediated primarily through BMPR-IB signalling. As with ALP, the results in Figure 8.1E show that BMP-2 significantly stimulated the expression of OC transcripts in scramble siRNA-transfected cultures ($p<0.05$), but BMP-2 stimulation of OC was significantly reduced in the BMPR-IB siRNA-transfected cultures ($p<0.05$). These results thus suggest that BMPR-IB-dependent signalling contributes, at least partly, to the BMP-2 up-regulation of the ALP and OC but not the Col I, OP and BSP genes.

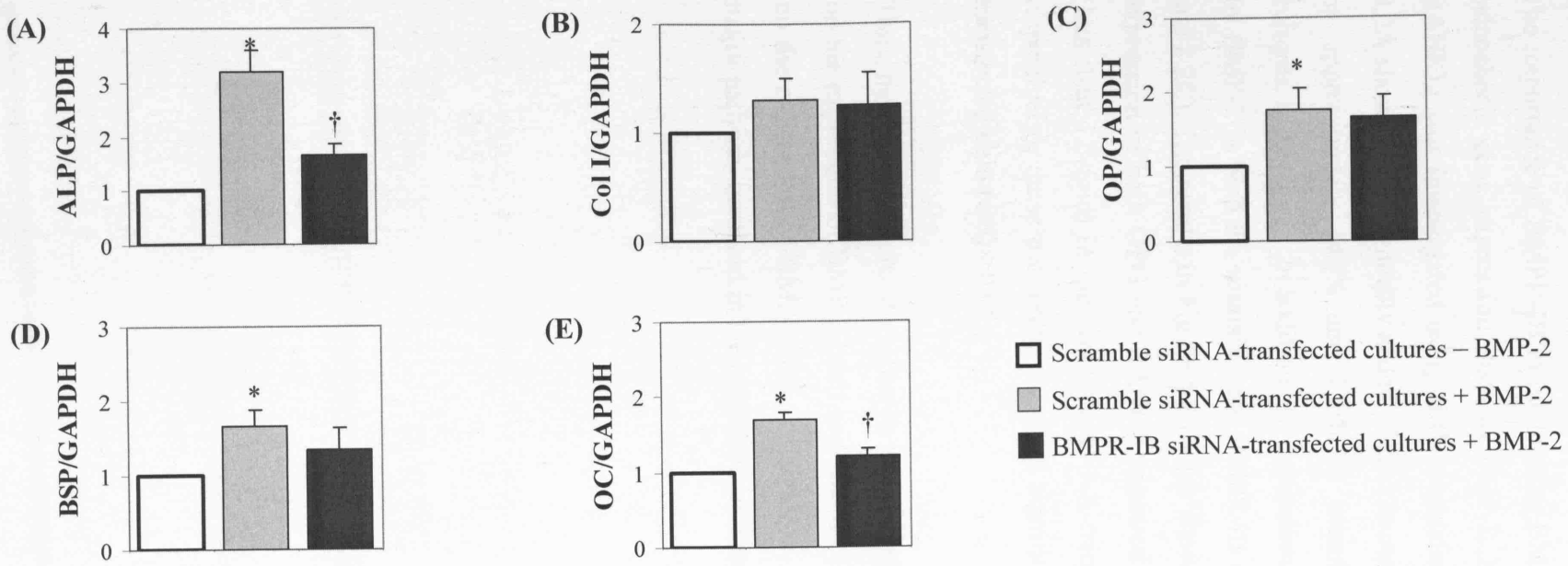


Figure 8.1. Effect of BMP-2 on the transcript expression of the ALP, Col I, OP, BSP and OC genes in siRNA-transfected cultures. Cultures were transfected with siRNA (scramble or BMPR-IB siRNA) and were subsequently treated with or without BMP-2 (50 ng/ml for 24 h). The expression of ALP, Col I, OP, BSP and OC transcripts was examined by RT-PCR. The results are presented as the mean ± SE of the level of each transcript relative to that of GAPDH, defined as 1.0 in the scramble siRNA-transfected cultures without BMP-2 treatment, obtained from triplicate experiments. * $p<0.05$ vs scramble siRNA-transfected cultures – BMP-2. † $p< 0.05$ vs BMP-2-treated scramble siRNA-transfected cultures.

8.3.2. Role of BMPR-IB signalling in the expression of osteogenic regulatory genes

The importance of BMPR-IB in modulating BMP-2-induced signals for osteoblastic and osteoclastic gene expression, such as BMP-2, BMP-4 and BMP-7, and also OPG and RANKL, was investigated using siRNA-transfected cell cultures. The results in Figure 8.2A show that exogenously added BMP-2 increased the expression of BMP-2 transcripts by approximately 140% and 120% in scramble and BMPR-IB siRNA-transfected cultures, respectively. In addition, the expression of BMP-4 and -7 was even less affected by BMP-2 in both the scramble and BMPR-IB siRNA-transfected cultures (Figure 8.2B and 8.2C). The results in Figure 8.2 further show that as with BMP-2, although transcript expression of both OPG and RANKL appeared to be up-regulated by BMP-2 treatment (less than 1.5-fold) in the scramble siRNA-transfected cultures (Figure 8.2D and 8.2E, respectively), these transcripts were not significantly reduced in the BMPR-IB siRNA-transfected cultures ($p>0.05$).

Thus, the present results show that in the AB cells, BMP-2 had some up-regulatory effect on the expression of BMP-2 itself and also on OPG and RANKL, but had a lesser effect on the expression of BMP-4 and -7. However, BMPR-IB signalling is unlikely to be a major pathway involved in the regulation of these mediators by BMP-2.

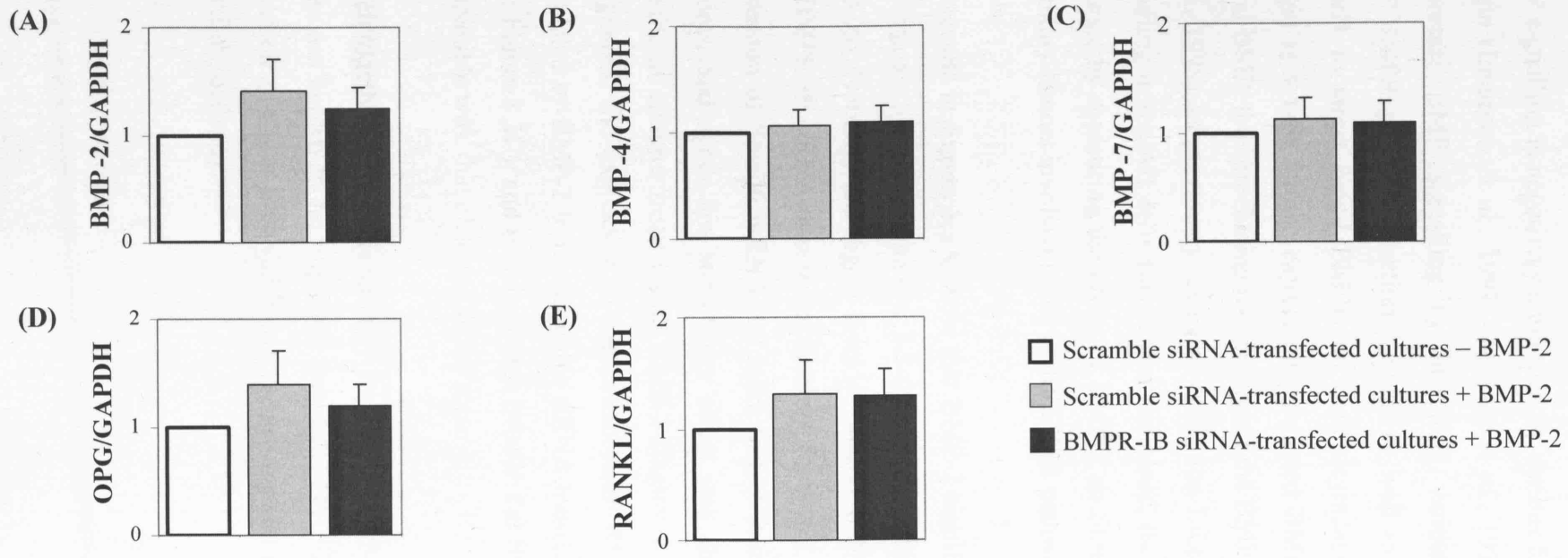


Figure 8.2. Effect of BMP-2 on the transcript expression of the BMP-2, BMP-4 and BMP-7, OPG and RANKL genes in siRNA-transfected cultures. Cultures were transfected with siRNA (scramble or BMPR-IB siRNA) and were subsequently treated with or without BMP-2 (50 ng/ml for 24 h). The expression of BMP-2, BMP-4 and BMP-7, OPG and RANKL transcripts was examined by RT-PCR. The results are presented as the mean ± SE of the level of each transcript relative to that of GAPDH, defined as 1.0 in the scramble siRNA-transfected cultures without BMP-2 treatment, obtained from triplicate experiments.

8.3.3. Role of BMPR-IB signalling in the expression of BMP-inhibitory genes

BMP signalling is negatively controlled by various factors including Smurf1, Smad6 and noggin (Imamura *et al.*, 1997; Gazzerro *et al.*, 1998; Murakami *et al.*, 2003). Smad6 suppresses BMP signalling by inhibiting various important steps throughout the BMP/BMPR pathway (Section 1.6.6.2) as well as by cooperatively functioning with Smurf1 to target type-I BMPR for their degradation (Murakami *et al.*, 2003), while noggin is a well known extracellular secreted BMP antagonist which can bind to the ligand BMP, thus precluding the binding of the BMP to the cell surface BMPR (Gazzerro *et al.*, 1998), as previously reviewed in Section 1.6.6.1. The functional role of BMPR-IB signalling in the AB cells was further examined, using the BMPR-IB siRNA-transfected cultures, by measuring the effect of BMP-2 on Smurf1, Smad6 and noggin, three main inhibitory factors involved in the BMP/BMPR pathway.

The results in Figure 8.3A show that BMP-2 significantly up-regulated Smurf1 mRNA expression, by approximately 2.8-fold in the scramble siRNA-transfected cultures ($p<0.05$). Notably, this higher level of Smurf1 mRNA expression was also observed in the BMPR-IB siRNA-transfected cultures. Similarly, BMP-2 was also found to induce the expression of Smad6 mRNA, by more than 4-fold in the scramble siRNA-transfected cultures, and again this stimulatory effect was also found in the BMPR-IB siRNA-transfected cultures treated with BMP-2 (Figure 8.3B). As with the intracellular BMP antagonists, the expression of the extracellular BMP antagonist noggin was markedly stimulated by BMP-2 in the scramble siRNA-transfected cultures (by approximately 3.5-fold; Figure 8.3C), and remained high even in the BMPR-IB siRNA-transfected cultures, comparable with that observed in the scramble siRNA-transfected cultures (Figure 8.3C).

Taken together, the results strongly suggest that although the Smurf1, Smad6 and noggin genes are known to be important targets of BMP-2, signal transduction leading to the activation of these BMP-inhibitory genes appears to be independent of the BMPR-IB signalling pathway.

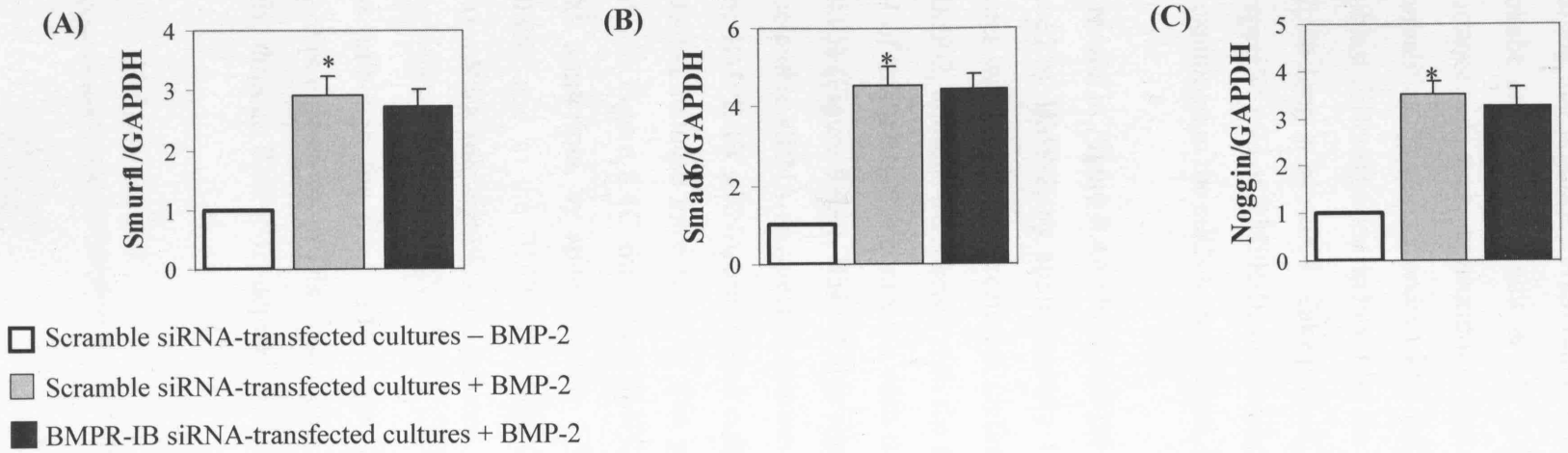


Figure 8.3. Effect of BMP-2 on expression of Smurf1, Smad6 and noggin mRNA transcripts in the siRNA-transfected cultures. Cultures were transfected with siRNA (scramble or BMPR-IB siRNA) and were subsequently treated with or without BMP-2 (50 ng/ml for 24 h). The expression of Smurf1, Smad6 and noggin transcripts was examined by RT-PCR. The results are presented as the mean ± SE of the level of each transcript relative to that of GAPDH, defined as 1.0 in the scramble siRNA-transfected cultures with no BMP-2 treatment, obtained from triplicate experiments. * $p<0.05$ vs scramble siRNA-transfected cultures – BMP-2.

8.3.4. Role of BMPR-IB signalling in the expression of osteogenic transcription factor genes

Osteogenic transcription factors Dlx5, Msx2, and Cbfa1 are targets of BMP/BMPR signalling that regulate a wide range of osteogenesis-associated genes (Ducy *et al.*, 1997; Shirakabe *et al.*, 2001; Tadic *et al.*, 2002; Barnes *et al.*, 2003; Kim *et al.*, 2004). The importance of such transcription factors has been demonstrated in both skeletal abnormalities *in vivo* (Korchynskyi and ten Dijke, 2000; ten Dijke *et al.*, 2003a) and osteoblast differentiation induced by the BMP/BMPR signalling *in vitro* (de Jong *et al.*, 2002; de Jong *et al.*, 2004; Takagi *et al.*, 2004), as previously reviewed in Section 1.6.5. The specific role of BMPR-IB on the expression of the Dlx5, Msx2 and Cbfa1 transcripts was examined in the siRNA-transfected AB cell cultures.

The results in Figure 8.4A show that the expression of Dlx5 transcripts was significantly induced by BMP-2, by approximately 180% of that in the scramble siRNA-transfected cultures without BMP-2 treatment (defined as 100%) ($p<0.05$). However, in the presence of BMP-2, cultures transfected with the BMPR-IB siRNA expressed a significantly lower level of this transcript compared with the cultures transfected with the scramble siRNA ($p>0.05$) (Figure 8.4A). BMP-2 also significantly stimulated Msx2 mRNA expression in the scramble siRNA-transfected cultures ($p<0.05$), but disruption of BMPR-IB signalling in the BMPR-IB siRNA-transfected cultures had little effect on the relatively high level of BMP-2-induced expression of this gene ($p>0.05$) (Figure 8.4B). As with Dlx5, the results in Figure 8.4C show that BMP-2 also significantly stimulated the expression of Cbfa1 transcripts, by approximately 170% in the scramble siRNA-transfected cultures ($p<0.05$), and in the BMPR-IB siRNA-transfected cultures the BMP-2 stimulation of Cbfa1 mRNA was significantly reduced, by approximately 70% ($p<0.05$).

Thus, although the Dlx5, Msx2 and Cbfa1 genes were all up-regulated by BMP-2, transcript expression of Dlx5 and Cbfa1 but not Msx2 was found to be mediated, at least partly, through BMPR-IB-dependent signalling.

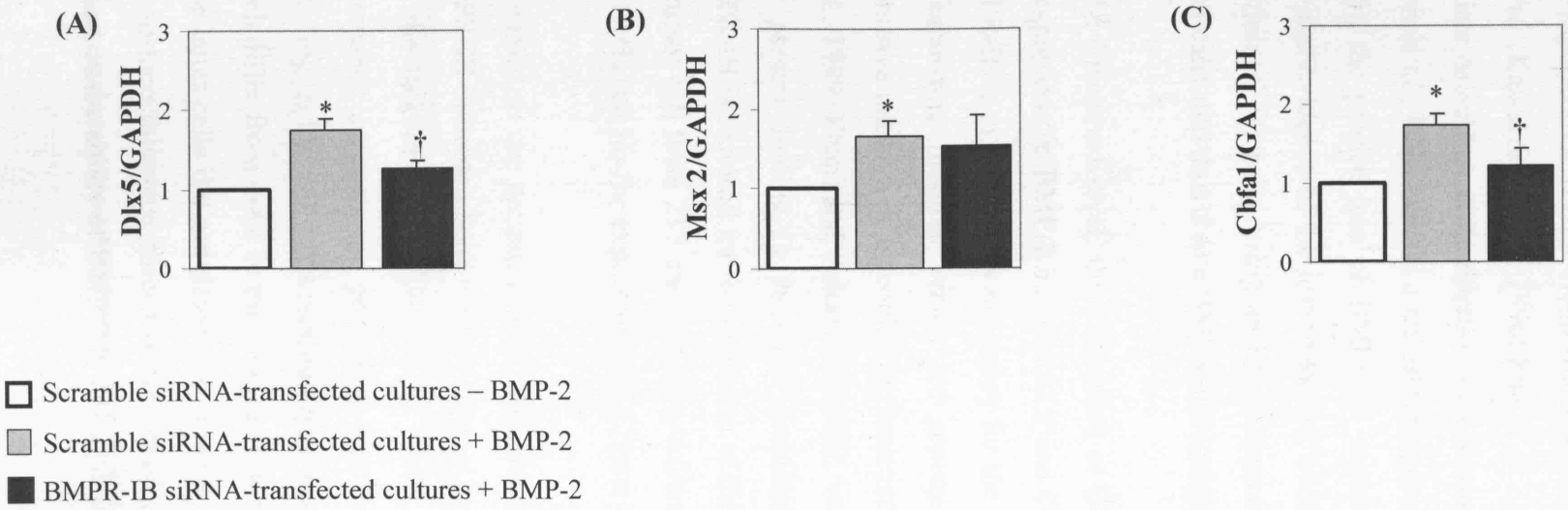


Figure 8.4. Effect of BMP-2 on the transcript expression of the osteogenic transcription factor Dlx5, Msx2 and Cbfa1 genes in siRNA-transfected cultures. Cultures were transfected with siRNA (scramble or BMPR-IB siRNA) and were subsequently treated with or without BMP-2 (50 ng/ml for 24 h). The expression of Dlx5, Msx2 and Cbfa1 transcripts was examined by RT-PCR. The results are presented as the mean ± SE of the level of each transcript relative to that of GAPDH, defined as 1.0 in the scramble siRNA-transfected cultures with no BMP-2 treatment, obtained from triplicate experiments. * $p<0.05$ vs scramble siRNA-transfected cultures – BMP-2. † $p< 0.05$ vs BMP-2-treated scramble siRNA-transfected cultures.

8.4. Discussion

BMPR-IB signalling has previously been shown to play an important role in osteoblast differentiation of cells of mesenchymal lineage (Section 1.6.9). Although previous studies have reported that BMPR-IB-mediated signalling is fundamental for skeletal development *in vivo* (Kawakami *et al.*, 1996; Zou *et al.*, 1997; Yi *et al.*, 2000) and *in vitro* is able to mediate osteoblast differentiation in a murine bone-derived mesenchymal cell line, 2T3 (Chen *et al.*, 1998) and in a rat osteoprogenitor-like cell line, ROB-C26 (Nishitoh *et al.*, 1996), the precise role of BMPR-IB remains unclear. The results in this study have provided evidence of the importance of BMPR-IB-mediated signalling in the osteogenic function of AB cells, using an RNAi approach to suppress the expression of BMPR-IB specifically and thus ablate BMPR-IB signalling.

At the functional level, this disruption of BMPR-IB signalling significantly suppressed the expression of BMP-2 induced ALP and OC transcripts, providing strong evidence that BMPR-IB is at least partly necessary for the expression of these two genes and thus their corresponding proteins, which are generally regarded as key early and late BMP-responsive markers of osteoblast differentiation, respectively (Lecanda *et al.*, 1997; Hay *et al.*, 1999; Ebara and Nakayama, 2002; van der Horst *et al.*, 2002; Noth *et al.*, 2003). The present finding is therefore consistent with other studies which showed that BMPR-IB is required for the induction of these osteoblast marker genes by BMP-2 in the precursor cell lines 2T3 and C2C12 (Akiyama *et al.*, 1997; Namiki *et al.*, 1997; Chen *et al.*, 1998) and for the expression of OC gene *in vivo* (Zhao *et al.*, 2002).

In contrast to the present results, Osyczka *et al.* (2004) reported that overexpression of BMPR-IB in human bone marrow stromal cells had no effect on ALP activity, suggesting that the lack of ALP regulation by BMP-2 was not due to lack of BMPR-IB. This discrepancy suggests the possibility that the induction of ALP activity via BMPR-IB signalling in the AB cells reported here may involve downstream signalling molecules which differ from those in the bone marrow stromal cells. However, the previous study of these latter cells did not determine whether they expressed the BMPR-IB protein at the cell surface following forced overexpression of BMPR-IB, which would have a major impact on the ability of BMP-2 to up-regulate ALP via BMP/BMPR signalling.

In the present study, knock-down of BMPR-IB was found to have no significant effect on the expression of Col I gene at the transcriptional level, consistent with previously reported results using human bone cells (Fromigue *et al.*, 1998; Hay *et al.*, 1999). It is possible, however, that BMPR-IB-mediated signalling could nevertheless be involved in post-transcriptional regulation of Col I, since it has previously been reported that in cultures of murine 2T3 cells overexpressing truncated (non-functional) BMPR-IB, there was a significant decrease in collagen fibrils in the bone matrix (Chen *et al.*, 1998).

Previous studies have implicated both OP and BSP as essential components in bone matrix mineralisation (Hunter and Goldberg, 1993; Denhardt and Noda, 1998), and their expression has been shown to be stimulated by BMP-2 in human marrow stromal cells and human osteoblasts (Lecanda *et al.*, 1997; Diefenderfer *et al.*, 2003). The results of the present study have also demonstrated that the expression of OP and BSP mRNA by AB cells was increased by BMP-2 treatment, but was not mediated by BMPR-IB-dependent signalling. Further studies are required to examine whether other pathways, for example BMPR-IA-mediated signalling, might be responsible for this in the AB cells.

A number of naturally-occurring mediators associated with bone remodelling, including BMPs, OPG and RANKL, have also been shown to be controlled by BMP-2. For example, BMP-2 has been reported to stimulate the expression of its own transcripts in human marrow stromal cells (Diefenderfer *et al.*, 2003), of OPG transcripts in human osteoblastic cells (Hofbauer *et al.*, 1998) and of RANKL transcripts in C2C12 cells (Otsuka *et al.*, 2003). However, the present results have shown that BMP-2 had no significant effect on the expression of BMP-2, -4, -7, OPG and RANKL transcripts, possibly because of limited or absent expression of some of the signalling components downstream of BMP-2 that are required for the expression of these genes in the AB cells specifically. However, unlike ALP and OC, the results here suggest that the regulation of BMP-2, -4, -7, OPG and RANKL by BMP-2 does not involve BMPR-IB-mediated signalling.

BMPR-mediated BMP signal transduction is also known to be negatively controlled by previously identified BMP inhibitory molecules, including Smurf1, Smad6 and noggin (Imamura *et al.*, 1997; Gazzerro *et al.*, 1998; Murakami *et al.*, 2003). In Chapter 7, the

results showed that the expression of Smurf1 transcripts was up-regulated by BMP-2, and it has previously been reported that the genes coding for Smad6 and noggin are transcriptionally activated in response to BMP-2 treatment *in vitro* (Takase *et al.*, 1998; Diefenderfer *et al.*, 2003; Li *et al.*, 2003a; Osyczka *et al.*, 2004). Consistent with the previous studies, the present results demonstrated that BMP-2 markedly induced the expression of Smurf1, Smad6 and noggin transcripts in the AB cells, thereby possibly leading to down-regulation of the BMP/BMPR signal. This is consistent with other data reported here that in response to prolonged BMP-2 treatment, the level of p-Smad1/5/8 decreased (Appendix II-Figure 1), possibly reflecting increased expression of such BMP inhibitors. However, BMP-2 induction of these BMP inhibitory proteins was shown here to be unlikely to involve BMPR-IB signaling, in contrast to a previous study showing induction of noggin by transfection of human marrow stromal cells with caBMPR-IB (Osyczka *et al.*, 2004). Although the reason for this discrepancy remains obscure, the role of BMPR-IB signalling in the expression of noggin might depend on the cell type, the stage of differentiation of the cells and the dose of BMP-2 used.

The osteoblast-associated transcription factors Dlx5, Msx2 and Cbfa1 play a pivotal part in osteoblast differentiation and bone formation (Ducy *et al.*, 1997; Komori *et al.*, 1997; Shirakabe *et al.*, 2001; Tadic *et al.*, 2002). However, while Dlx5 and Cbfa1 have been shown to stimulate osteogenesis-related functions such as ALP activity and the expression of Col I, OP, BSP and OC genes, Msx2 down-regulates these genes (Ducy *et al.*, 1997; Shirakabe *et al.*, 2001; Tadic *et al.*, 2002; Barnes *et al.*, 2003; Kim *et al.*, 2004). In addition, the specific role of the BMPR in mediating BMP-induced changes in these transcription factors is still unclear, although these factors have previously been reported to be regulated by BMP (Ducy *et al.*, 1997; Miyama *et al.*, 1999; Ashique *et al.*, 2002; Lee *et al.*, 2003). In the present study, while BMP-2 up-regulation of Dlx5 transcript expression via BMPR-IB signalling was observed, BMP-2-mediated up-regulation of Msx2 was found not to involve signalling cascades initiated by BMPR-IB in the AB cells, consistent with a previous *in vivo* study reporting that Msx2 expression was not up-regulated in the caBMPR-IB transgenic mice (Zhang *et al.*, 2000). Further studies are therefore required to determine whether pathways other than BMPR-IB, for example BMPR-IA, might have this role in the cells used here. Additionally, the present results showed that as with Dlx5, the expression of Cbfa1 gene was up-regulated by BMP-2 and

was mediated at least in part by BMPR-IB signalling, consistent with previous studies showing that increased expression of Cbfa1 mRNA by BMP-2 was blocked specifically by overexpression of truncated BMPR-IB in murine 2T3 cells (Chen *et al.*, 1998) and that the expression of this transcription factor was strongly ablated in homozygous truncated BMPR-IB transgenic mice (Zhao *et al.*, 2002).

The present results have provided evidence that BMPR-IB is required, at least partly, for BMP-2 induction of ALP, OC, Dlx5 and Cbfa1 gene expression. Although the role of BMPR-IB in BMP-2-induced mineralised bone matrix formation was not examined here, a previous study has reported that BMP-2-induced mineralisation was completely blocked by overexpression of truncated BMPR-IB in 2T3 cells (Chen *et al.*, 1998). Therefore, BMPR-IB signalling appears to be a key factor in BMP-2-induced osteoblast functions including mineralisation, while this receptor appears to play only a minor part in BMP-2 up-regulation of certain inhibitory components in the BMP signaling pathway, *e.g.*, Smurf1, Smad6, noggin and Msx2.

In summary, the present study has shown, for the first time, that BMPR-IB-dependent signalling plays a crucial role in BMP-2 stimulation of the expression of the ALP, OC, Dlx5 and Cbfa1 genes in AB cells. These findings have provided important insight into the osteogenic role of BMPR-IB in BMP-2-induced osteoblast differentiation of these cells *in vitro*, and highlight the possibility that this receptor could be a therapeutic target for enhancing bone formation *in vivo*.

CHAPTER 9
GENERAL DISCUSSION

9.1. General discussion

The BMPs play a fundamental role in osteogenesis by promoting differentiation of uncommitted mesenchymal cells as well as differentiation of committed progenitor cells toward cells of the osteoblastic lineage, which enhance bone growth and bone healing following disease and injury (Rosen *et al.*, 1996b; van der Horst *et al.*, 2002; Nakashima and Reddi, 2003). Since the BMPs elicit their osteogenic activities via signal transduction through cognate transmembrane BMPR, the response of bone cells to these factors thus depends at least partly on the expression and function of the specific BMPR, as shown by the lack of BMP effect in the absence of BMPR expression *in vivo* (Hamdy *et al.*, 2003). Understanding of the regulation of these receptors in primary human bone cells is therefore fundamental for enhancing bone healing processes and identifying potential new targets for therapeutic intervention. However, detailed studies on the expression of the BMPR and the mechanisms by which they are regulated in human osteogenic cells are limited. The present study has therefore examined the hypothesis that the expression, localisation and function of the BMPR are controlled by a number of biologically active mediators. The studies carried out in this thesis have shown that the expression and function of the BMPR, especially BMPR-IB, can be differentially modulated *in vitro* by a number of cytokines, growth factors and hormones. Moreover, the expression level of BMPR-IB has been shown to be of fundamental importance to the sensitivity and responsiveness of the AB cells to the BMP and thus the osteogenic activity of these potent osteogenic factors. A summary of the key findings in this thesis is shown in Figure 9.1.

The present study has used AB as the source of human cells of osteoblast lineage (Doglioli and Scortecci, 1991; Salih *et al.*, 2001; Pradel *et al.*, 2005), from which primary cell cultures can differentiate into mature osteoblasts having the ability to form bone-like mineralised structures *in vitro*. The use of such human bone cells is advantageous over rodent cells for the study of BMPR since the regulatory mechanisms, transcription factors and BMPR-associated signalling pathways may differ between these two species, as has

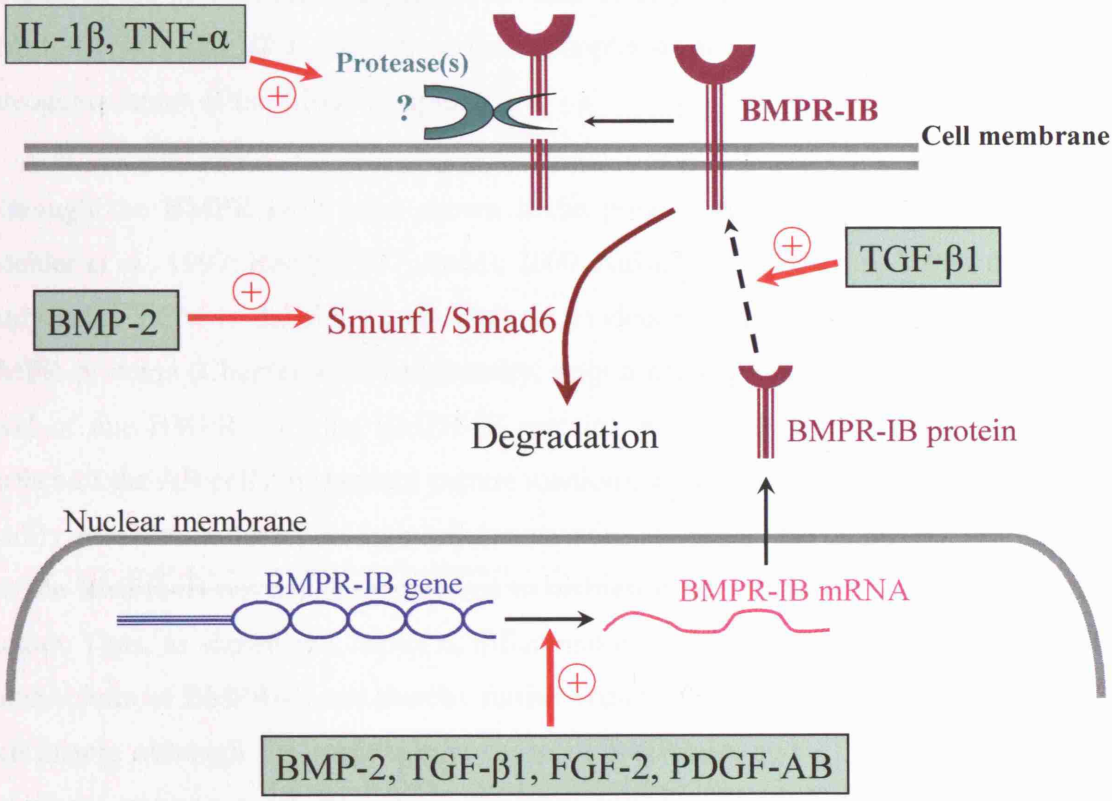


Figure 9.1. Regulation of BMPR-IB by cytokines and growth factors. The expression of cell surface BMPR-IB was found to be negatively controlled by proteolytic shedding, induced by two inflammatory cytokines IL-1β and TNF-α, although the protease(s) involved are yet to be investigated. In contrast, a number of osteogenic growth factors, *i.e.,* BMP-2, TGF-β1, FGF-2 and PDGF-AB, stimulated BMPR-IB transcription resulting in a significant increase in BMPR-IB mRNA and protein. Moreover, TGF-β1 induced transport of intracellular BMPR-IB to the cell surface. BMP-2 was also found to markedly stimulate the expression of Smurf1 and Smad6, which have previously been shown to cooperatively target cell surface BMPR-IB for proteosomal and lysosomal degradation. Red arrows represent the main findings in the thesis.

⊕ : Induction; -----> : Intracellular transport of cytoplasmic BMPR to the cell surface.

been reported for BMP regulation of the ALP gene in rat compared with human marrow stromal cells (Osyczka *et al.*, 2004). In addition, a substantial proportion of the AB cells isolated in the present study appear to be cells of osteoblastic lineage, and they express BMPR-IA, -IB and -II at the cell surface (Chapter 4) and can evidently function as an osteogenic target of the BMP (Chapter 8).

Although the BMPR have been shown to be present in many tissues and cell types (Mehler *et al.*, 1997; Reddi, 1997; Reddi, 2000; Nakashima and Reddi, 2003), the present study using FCM is the first to show direct evidence of the expression of cell surface BMPR proteins (Chapter 4). Unexpectedly, only a small proportion of the total cellular level of one BMPR (*i.e.*, the BMPR-IB protein) was found to be localised at the cell surface of the AB cells in standard culture medium, with even BMPR-IA and -II showing readily measurable levels of intracellular protein. However, the relatively low level of surface BMPR-IB nevertheless appeared to be highly regulated by cytokines and growth factors. Thus, as shown in Chapter 6, inflammatory cytokines induced the shedding of a soluble form of BMPR-IB and thereby further reduced the low level of BMPR-IB surface expression, although the surface expression of BMPR-IA and -II was unaffected. The functional importance of the cytokine-induced loss of surface BMPR-IB expression by the AB cells was demonstrated by a marked suppression of the osteoblastic response to BMP-2 (*i.e.*, Smad1/5/8 phosphorylation, ALP activity and OC expression). However, in contrast to the cytokines, osteogenic growth factors up-regulated surface BMPR-IB expression, again the expression of cell surface BMPR-IA and -II being unaffected. Moreover, the growth factor-induced increase in surface BMPR-IB markedly enhanced BMP-2 responses by the AB cells *in vitro* (Chapter 7), which could be crucial to the initiation of bone regenerative processes *in vivo*.

Much of the information thus far available about the biosynthesis, post-translational processing and intracellular transport of the BMPR is based mainly on data obtained from studies of the TGFβR, which are closely related to the BMPR within the TGFβR superfamily (Massague *et al.*, 1994; Massague, 1998). Studies of cell surface expression of the TGFβR-I have shown that not all TGFβR-I molecules reach the plasma membrane (Koli and Arteaga, 1997; Wells *et al.*, 1997), with as much as 50% of newly synthesised receptors remaining in the ER (Wells *et al.*, 1997). In addition, the intracellular

localisation of TGFβR-I has been shown to be associated with growth arrest in the cell cycle and an increase in nuclear translocation and reduction of surface expression of the TGFβR-I (Zwaagstra *et al.*, 2000). In the present study, it was found that a major proportion of BMPR-IB had an intracellular localisation in the AB cells, being identified in both the cytoplasm and nucleus. These results thus suggest possible roles for BMPR-IB in these compartments, and future studies of putative BMPR-IB binding protein(s) may provide additional insight into BMPR-IB function in bone cells.

The present study has also demonstrated that the BMPR can be shed from the cell surface of bone cells. Although the specific molecular mechanisms of BMPR shedding are not yet known, important differences were observed between the effect of PMA, a well known synthetic PKC agonist, and the naturally-occurring cytokines IL-1β and TNF-α (Chapter 5 and 6). Thus, PMA was able to generate soluble forms of BMPR-IA, -IB and -II, indicating that shedding of all three BMPR can occur via the PKC pathway. However, previous studies suggest that the shedding process can be mediated not only by activation of 'proteases' via a PKC-dependent signal transduction pathway, but also by PKC-independent pathways (Reiland *et al.*, 1996; Vecchi *et al.*, 1996; Vecchi and Carpenter, 1997; Izumi *et al.*, 1998; Vecchi *et al.*, 1998; Rizoli *et al.*, 1999; Umata *et al.*, 2001; Dello and Rovida, 2002). The latter may be involved in the BMPR-IB shedding induced by IL-1β- and TNF-α, as reported in the present study (Chapter 6), which, unlike PMA, prompted the shedding of BMPR-IB only, and not BMPR-IA and -II. This demonstrates that cytokine-induced signalling pathways also mediated the formation of a soluble form of this receptor specifically, as has been reported for the IL-1β- and TNF-α-enhanced shedding of IL-6R via the activation of a distinct metalloprotease TACE (Franchimont *et al.*, 2005a), mediated by the MAPK signalling pathway (Diaz-Rodriguez *et al.*, 2002; Fan *et al.*, 2003). It is therefore likely that different signal transduction pathways are involved in the PMA- and in the cytokine-induced shedding of the BMPR in the AB cells. Identification of the specific signalling pathways and molecules responsible for the cytokine-induced shedding of BMPR-IB in the AB cells could enable antagonists to be developed which inhibit shedding of this receptor and thus enhance the sensitivity and response of bone cells to the BMP.

In addition to shedding, internalisation by endocytosis has also been shown to regulate the expression and signalling of transmembrane growth factor receptors (Braulke, 1999; Zwaagstra *et al.*, 1999; Citores *et al.*, 2001; Zwaagstra *et al.*, 2001; Belleudi *et al.*, 2002; Wang *et al.*, 2002; Wang *et al.*, 2003). Although none of the cytokines and growth factors studied here was found to be involved in internalisation of the BMPR in the AB cells, these receptors have previously been shown to undergo endocytosis following BMP-2 stimulation in rat skeletal myoblasts (Jortikka *et al.*, 1997). However, whether such processes play an important role in modulating BMPR expression and signalling in bone remains to be examined by techniques which can localise the BMPR directly rather than indirectly by using the BMP ligand, as previously reported (Jortikka *et al.*, 1997).

Because of their potent biological activities, the BMPs are considered to be potentially valuable for the treatment of various orthopaedic and craniofacial disorders, including spinal fusion, bone fracture and periodontal diseases (Boden *et al.*, 2002; Govender *et al.*, 2002; Nakashima and Reddi, 2003). Thus, rhBMP have recently been used both in experimental models and clinically to promote wound healing and regenerative processes in human bone and periodontal tissues (Bessho *et al.*, 1999; Cochran *et al.*, 2000; Cheng *et al.*, 2003; Park *et al.*, 2003; Osyczka *et al.*, 2004), having the potential for regeneration of AB, alveolar ridge augmentation and oral implant therapy (Reddi, 1998; Nakashima and Reddi, 2003). However, very high concentrations of exogenously applied rhBMP appear to be required to produce an osteogenic effect in orthopaedic and periodontal clinical trials (Boyne *et al.*, 1997; Howell *et al.*, 1997) as well as in spinal fusion applications (McKay and Sandhu, 2002). Such high doses may elicit adverse effects such as heterotopic ossification and excessively large increases in bone turnover and immunological reactions (Ripamonti *et al.*, 1996; Lin *et al.*, 2005), suggesting that augmenting BMP action could be of important clinical significance. A number of studies have therefore focused on this area (Chan *et al.*, 2003; Li *et al.*, 2003b; Kugimiya *et al.*, 2005b; Lin *et al.*, 2005; Toyoda *et al.*, 2005). For example, a synthetic peptide B2A2 (Lin *et al.*, 2005) and a PTH-related peptide (Chan *et al.*, 2003) have been reported to enhance BMP-2-induced osteoblast differentiation of pluripotent mesenchymal cells. An alternative approach, used in the present study, has focused on the enhancement of BMP action by up-regulating the expression of functionally active BMPR-IB and thus the response of the target cells to the BMP.

The importance of the BMPR-IB signalling pathway in osteoblast differentiation has been supported by several lines of evidence in the present study. First, diminished BMPR-IB expression caused by cytokine-induced shedding resulted in a marked reduction in osteogenic responses of the AB cells to BMP-2 (Chapter 6). Secondly, in Chapter 7, up-regulation of BMPR-IB by osteogenesis-associated growth factors was found to significantly promote BMP-2-induced osteoblast differentiation of the AB cells. Finally, the pivotal role of BMPR-IB expression was further demonstrated by knock-down of this receptor using siRNA-mediated RNAi in the AB cell cultures. This was found to strongly inhibit the expression of a number of key genes associated with osteoblast differentiation (Chapter 8). These observations demonstrate conclusively that promoting BMPR-IB-mediated signalling can stimulate osteoblast differentiation of primary human AB cells, as has been shown in a pre-adipocyte cell line (Skillington *et al.*, 2002), in adipose-derived stromal cells (Wan *et al.*, 2006), in a bone-derived mesenchymal cell line (Chen *et al.*, 1998) and in osteoprogenitor-like cells (Nishitoh *et al.*, 1996) *in vitro*. *In vivo,* BMPR-IB also appears to play a key role in transmitting signals involved in skeletal development and bone formation, since mice lacking BMPR-IB display severe skeletal defects (Kawakami *et al.*, 1996; Zou *et al.*, 1997; Yi *et al.*, 2000; Zhao *et al.*, 2002).

It is noteworthy, however, that stimulation of osteoblast differentiation by BMP-2 is not necessarily wholly dependent on the expression of BMPR-IB (Namiki *et al.*, 1997; Hay *et al.*, 2004; Kaps *et al.*, 2004). This may be due to cell-specific differences in signalling molecules and/or transcription factors activated in different cell types after the formation of BMPR-IA/BMPR-IB and BMPR-II complexes in these cells. Thus, while the present study used primary human AB cells, previous studies employed an osteosarcoma OHS4 cell line, a myoblast C2C12 cell line and an undifferentiated mesenchymal C3H10T1/2 cell line (Namiki *et al.*, 1997; Hay *et al.*, 2004; Kaps *et al.*, 2004). However, the C2C12 and C3H10T1/2 cell lines do not express BMPR-IB (Namiki *et al.*, 1997; Kaps *et al.*, 2004) and, even after forced overexpression of these cells with wild-type BMPR-IB, the expression of functional BMPR-IB at the cell surface was still very low (Namiki *et al.*, 1997). This relatively low level of surface BMPR-IB was apparently not sufficient to initiate subsequent intracellular signalling, thus resulting in the lack of BMP-2 induced osteoblast differentiation (Namiki *et al.*, 1997; Kaps *et al.*, 2004).

The present finding of TGF-β1 modulation of BMPR-IB expression and function is of particular interest since this osteogenic growth factor positively regulates BMPR-IB at multiple levels in the AB cells. The results have shown that after TGF-β1 treatment, surface translocation of functionally active BMPR-IB was immediately, if transiently, induced (Chapter 4). Additionally, TGF-β1 also activated transcription of the BMPR-IB gene, subsequently resulting in the increased expression of both the BMPR-IB transcript and surface protein (Chapter 7). Notably, both of these short and longer-term effects of TGF-β1 appeared to be functionally relevant. Thus, the immediate effect of TGF-β1 might be of value for enhancing the apparent initial and rapid osteogenic effects of BMP when used clinically with a collagen sponge carrier since the half-life of the BMP in a collagen sponge used as a delivering vehicle has been reported to be less than 12 h (Jepsen *et al.*, 1999).

Although only BMP-2 was used here as an activator of BMPR-IB signalling for examining the functional importance of BMPR-IB-mediated osteoblast differentiation, it is likely that BMPR-IB signalling is also regulated by other members of the BMP family, such as BMP-4, GDF-5, BMP-6 and BMP-7. These have previously been shown to bind to BMPR-IB (ten Dijke *et al.*, 1994b; Nishitoh *et al.*, 1996; Erlacher *et al.*, 1998; Ebisawa *et al.*, 1999), and their osteogenic roles have also been established both *in vitro* and *in vivo* (Maliakal *et al.*, 1994; Luo *et al.*, 1995; Thomas *et al.*, 1996; Yamaguchi *et al.*, 1996). Further studies are therefore required to clarify the precise and possibly unique role of each of the BMP in BMPR-IB signalling, in order to maximise the potential use of BMPR-IB as a therapeutic target for enhanced osteogenesis *in vivo* by these different BMP.

The present results have demonstrated the fundamental role played by BMPR-IB in BMP-induced osteoblast differentiation in the AB cells, but the role of this receptor in the contribution of mesenchymal stem cells (MSC) to bone formation and fracture healing is not yet known. While BMPR-IB mRNA expression in MSC is barely detectable *in vitro* (Kaps *et al.*, 2004), it is possible that local growth factors and systemic hormones could up-regulate the expression of BMPR-IB in human MSC *in vivo*. The results of the present study (Chapter 7), together with a recent study demonstrating that TGF-β1 enhances BMP-mediated functions via up-regulation of BMPR-IB in human MSC *in vitro* (Xu *et*

al., 2006), strongly suggest that BMPR-IB is a major component of enhanced bone formation *in vivo*.

9.2. Concluding remarks

This thesis has demonstrated that the expression of the BMPR, especially BMPR-IB, is differentially regulated *in vitro* by a number of biological factors, including inflammatory cytokines, growth factors and hormones. The level of BMPR-IB expression has been shown to be crucial to the responsiveness of AB cells to the BMP and thus to BMP activity, suggesting that BMPR-IB could be a valuable therapeutic target for enhanced bone formation *in vivo*.

9.3. Future work

In addition to the areas of further study which have already been mentioned in previous chapters, some additional studies which could be of particular interest are discussed below.

9.3.1. Shedding of BMPR-IB

This thesis has provided strong evidence that shedding of a soluble form of BMPR-IB is a central regulatory mechanism controlling the sensitivity of AB cells to the BMP. Although both PKC-dependent and -independent pathways have been implicated in this process, the precise mechanisms which regulate BMPR-IB shedding in these cells is not yet known. Future studies to identify the signalling proteins involved in the cytokine-induced shedding of BMPR-IB could enable specific antagonists to be developed which inhibit this process and thus increase BMPR-IB expression, enhancing the potential efficacy of BMP-induced bone healing *in vivo*.

9.3.2. Internalisation of BMPR-IB

A recent report has shown that endocytic internalisation of BMPR has a profound influence on BMP signalling (Hartung *et al.*, 2006). In view of the findings here of the biological importance of BMPR-IB in AB cells, it would be of value to clarify the mechanism of BMPR-IB endocytosis by studying BMPR-IB internalisation directly using an anti-BMPR-IB antibody rather than indirectly using the BMP ligand, as reported

previously (Jortikka *et al.*, 1997). For this purpose, the internalisation and endocytic pathways, including intracellular transport, receptor recycling to the plasma membrane and lysosomal degradation, of BMPR-IB could be determined using a fluorochome-labelled BMPR-IB antibody. Moreover, a number of key endocytic proteins, such as β-arrestin 2 (a G protein-coupled receptor binding protein), Eps15R (a constitutive component of clathrin-coated pits) and caveolin-1 (a structural component of caveolae), have been reported to mediate the endocytic pathway of a number of transmembrane proteins, including TGFβR-III and BMPR-II, via clathrin-coated pits and via caveolae (Chen *et al.*, 2003; Liu and Shapiro, 2003; Raiborg *et al.*, 2003; Hartung *et al.*, 2006). RNAi targeting of these key endocytic molecules to alter specific intracellular vesicular transport could provide an important insight into the regulation of BMPR-IB by these processes in the AB cells.

9.3.3. Regulation of BMPR-IB by T-lymphocyte-AB cell interaction

The results in Chapters 6 and 7 have shown that the BMPR-IB protein is specifically and negatively controlled by the inflammatory cytokines IL-1β and TNF-α, and is positively regulated by the osteogenic growth factors TGF-β1, FGF-2 and PDGF-AB. Since T-lymphocytes are known to be a major source of a wide range of cytokines as well as growth factors during both inflammatory and healing processes in bone (Taubman and Kawai, 2001), it is possible that T-lymphocytes could also play an important role in the regulation of BMPR-IB in bone. This is consistent with the observation that T-lymphocytes frequently infiltrate into the site of bone inflammation and directly adhere to bone cells *in vivo* (Tanaka *et al.*, 1998; Gortz *et al.*, 2004), while previous *in vitro* studies have also revealed that direct cell-to-cell contact of T-lymphocytes with other cell types, including osteoblasts, can mediate a number of fundamental cellular events (Olsen *et al.*, 1988; Tanaka *et al.*, 1995; Saho *et al.*, 2003). In addition, the BMPs have previously been shown to also be indirectly involved in cell adhesion of human osteoblastic cells by stimulating a marked increase in two key cell-to-cell adhesion molecules, N-cadherin and E-cadherin (Cheng *et al.*, 1998), indicating a possible mechanism of cross-talk between BMP/BMPR and cadherin signalling in the AB cells. Thus, the BMPR-IB could be regulated by T-lymphocytes through both soluble mediators and direct cell-to-cell contact, possibly via adhesion molecule signalling pathways. Future experiments to determine the effects of T-lymphocytes on BMPR-IB expression and

function could clarify the mechanisms involved in T-lymphocyte-bone cell interaction. It is noteworthy that in preliminary *in vitro* studies not shown here, T-lymphocytes were found to form stable cell-to-cell contacts with AB cells, resulting in the apparent activation of resting T-lymphocytes into T-lymphoblastic cells, which are known to be much more active in cytokine secretion as well as the expression of cell-associated proteins such as adhesion molecules and other cell surface receptors (Taubman and Kawai, 2001). This phenomenon could be of major importance clinically in chronic bone diseases, such as osteoarthritis and periodontitis, and also in the adverse chronic inflammatory response to bone implant surgery *in vivo*.

9.3.4. Functional roles of BMPR-IB

While the present work has clearly demonstrated the biological significance of surface-associated BMPR-IB in AB cells, the results have also suggested the possibility of other roles since substantial levels of this receptor have been identified in the cytoplasm and nucleus. To investigate any additional and hitherto unknown functions of intracellular BMPR-IB in these cells, it would be of value to identify possible BMPR-IB binding proteins in cytoplasmic and nuclear extracts of the cells using two-dimensional gel electrophoresis and mass spectrometry, as have previously been described (Hassel *et al.*, 2004). Identification of putative intracellular BMPR-IB binding proteins could provide further insight into the interactions of the intracellular BMPR-IB (*i.e.*, cytoplasmic and nuclear receptors) with other regulatory molecules as well as their downstream signalling pathways, and thus the functions of these receptors.

Since the present study has strongly demonstrated a crucial role of BMPR-IB in osteoblast differentiation of the AB cells *in vitro*, in future studies it would be important to examine the role of this receptor *in vivo*. Mice carrying a targeted disruption of the BMPR-IB gene (BMPR-IB$^{-/-}$) have previously been shown to be viable (Yi et al., 2000), and could be used to examine the role of BMPR-IB-mediated signalling in AB formation during embryonic development and particularly in adult healing and regenerative processes *in vivo*. Thus, morphological and histological analysis of growth and development of AB in these BMPR-IB$^{-/-}$ mice at different embryonic and postnatal stages could help to determine the role and requirement of BMPR-IB in different phases during AB formation. Immunohistochemical analysis of AB sections could also be carried out to

examine the expression of osteogenic transcription factors and bone matrix proteins in relation to osteoblast differentiation in these mice. Moreover, examination of osteoblast differentiation of the AB cells obtained from these transgenic mice could further establish the osteogenic role of BMPR-IB observed in the present *in vitro* study. In addition to normal bone formation process, the healing potential of AB in these mutants following fracture injury could also be examined to determine whether the lack of BMPR-IB expression ablates repair and regeneration *in vivo*. Finally, gene therapy (Park *et al.*, 2003), for example by introducing BMPR-IB gene-transfected MSC into artificially generated AB defects, could clarify whether the expression of the BMPR-IB gene enhances bone healing in these transgenic null mice.

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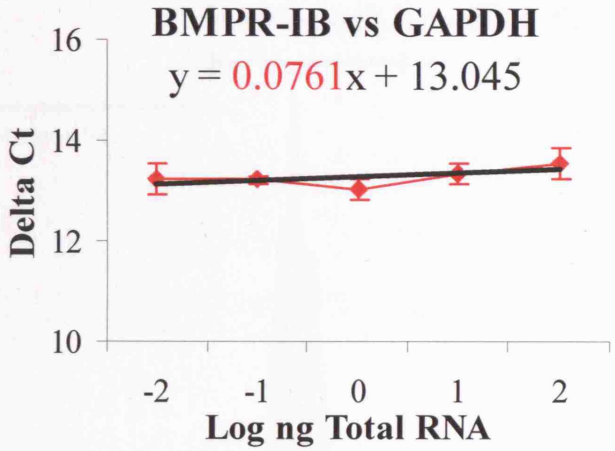
APPENDIX I

SUPPLEMENTAL DATA-CHAPTER 2



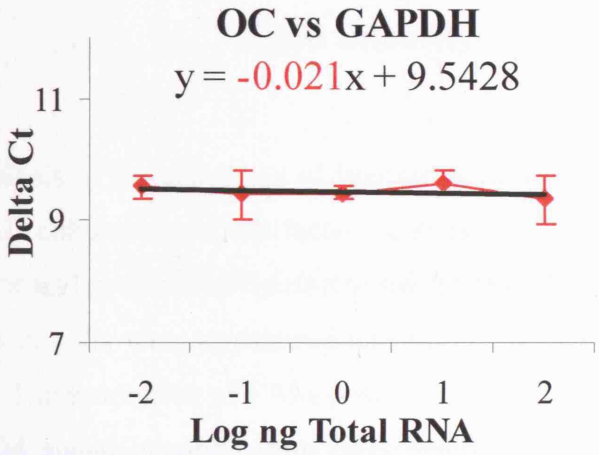


Figure 1. Q-PCR relative efficiency plot of BMPR-IB and OC amplifications versus GAPDH amplification. Note that as a guideline by the manufacturer, the efficiencies of the target gene and GAPDH amplifications are approximately equal when the absolute value (in red) of the slope of Log input amount versus ΔCt is less than 0.1. The expression of the two genes examined can thus be quantitatively analysed by a comparative C_T method.

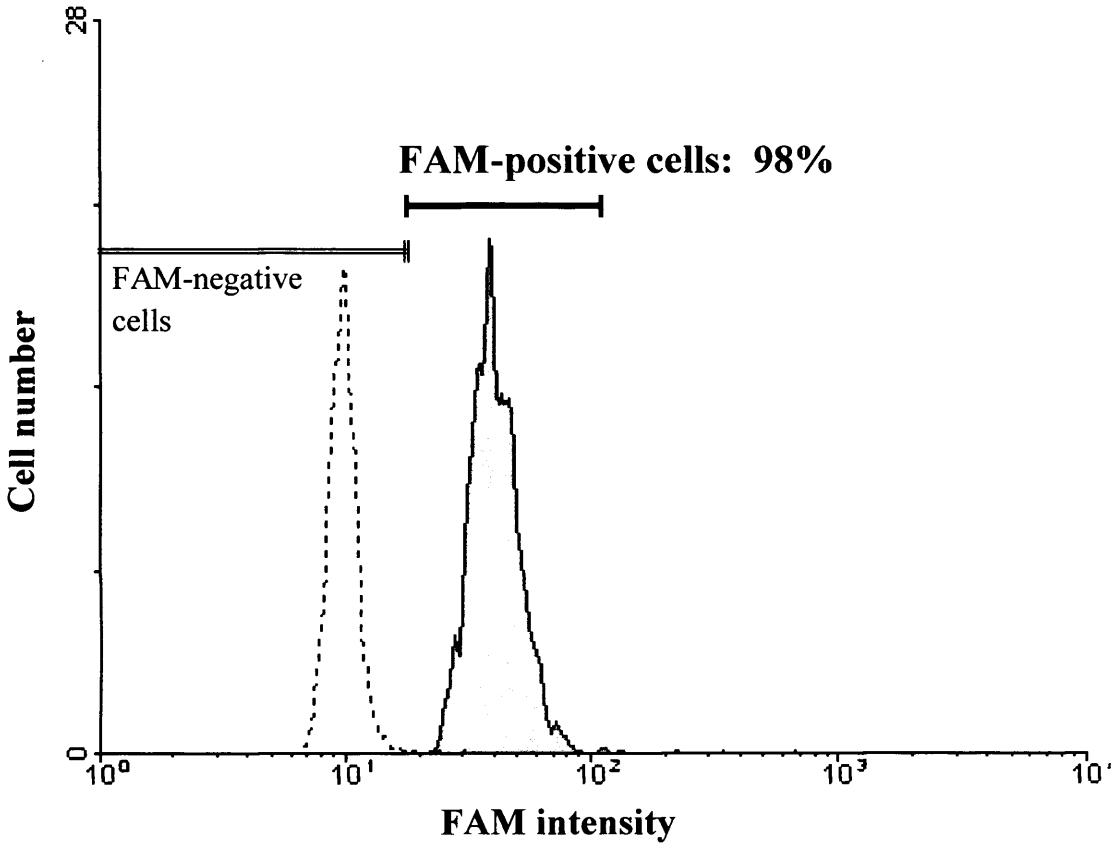


Figure 2. FCM analysis of the efficiency of lipofectamine-mediated siRNA transfection of AB cells. The AB cultures were transfected with carboxyfluorescein (FAM)-labelled siRNA in the absence and presence of lipofectamine, as described in Section 2.11.2. After 24 h post-transfection, cells were trypsinised and the FAM intensity of individual cells measured by FCM. The proportion of FAM-positive cells in cultures was determined by subtracting the FAM signal obtained from cells treated with FAM-conjugated siRNA alone, defined as FAM-negative signal (clear histogram), from the signal derived from cells transfected with lipofectamine-FAM-conjugated siRNA complexes (shaded histogram). Note that in lipofectamine-mediated siRNA-transfected cells, 98% of cells were found to be FAM-positive, suggesting that the efficiency of siRNA transfection of AB cells using lipofectamine was approximately 98%. The similar results were obtained from two separate experiments.

APPENDIX II
SUPPLEMENTAL DATA-CHAPTER 4

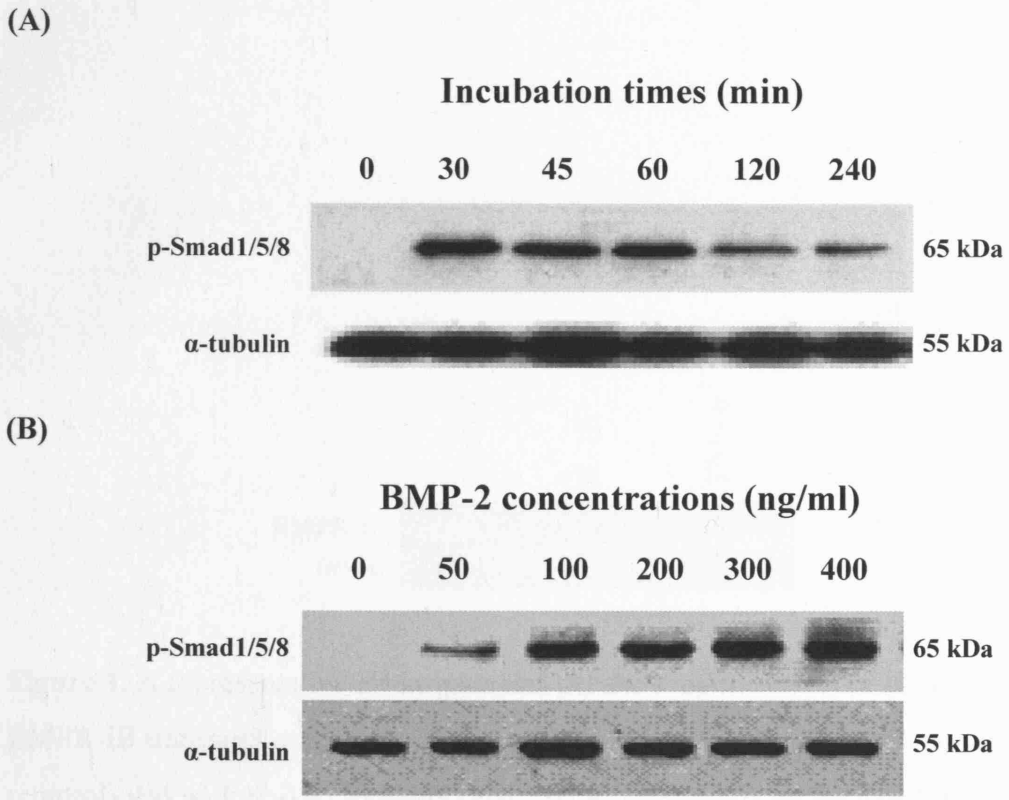


Figure 1. Expression of p-Smad 1/5/8 in response to BMP-2 stimulation by AB cells. In (A), cells were treated with 100 ng/ml of BMP-2 for the indicated times and subjected to WB. In (B), cells were treated with the indicated concentration of BMP-2 for 30 min and subjected to WB. Immunoreactive bands of 65 and 55 kDa molecular weight proteins represent the presence of p-Smad1/5/8 and α-tubulin, respectively. The expression of α-tubulin was used as the internal control.

APPENDIX III
SUPPLEMENTAL DATA-CHAPTER 6

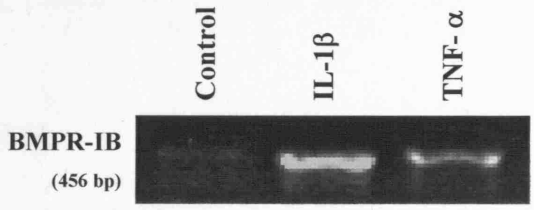
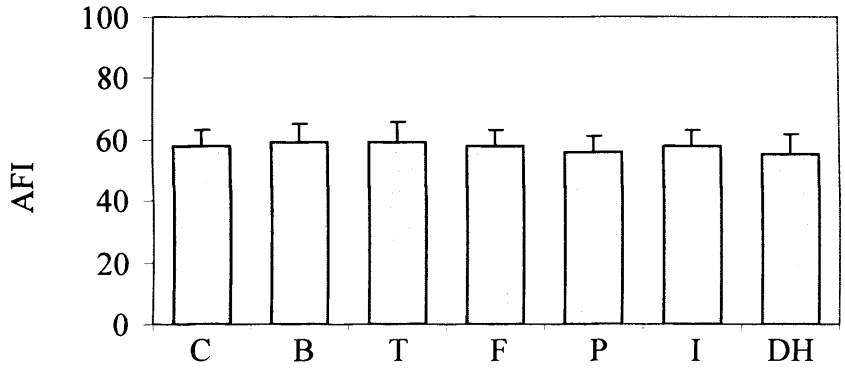


Figure 1. A representative electrophoresis gel showing the effect of IL-1β and TNF-α on BMPR-IB transcript expression. Exponentially growing AB cells were incubated without (control) and with IL-1β (50 ng/ml) and TNF-α (50 ng/ml) for 24 h and the expression of BMPR-IB transcripts examined by RT-PCR using primers that amplify the transcript sequences corresponding to the transmembrane domain and a part of the extracellular region of BMPR-IB, as described in the Materials and Methods. Note that only one form of PCR product corresponding to the BMPR-IB transcript was detected in all three samples.

APPENDIX IV

SUPPLEMTAL DATA-CHAPTER 7

(A) Surface BMPR-IA



(B) Surface BMPR-II

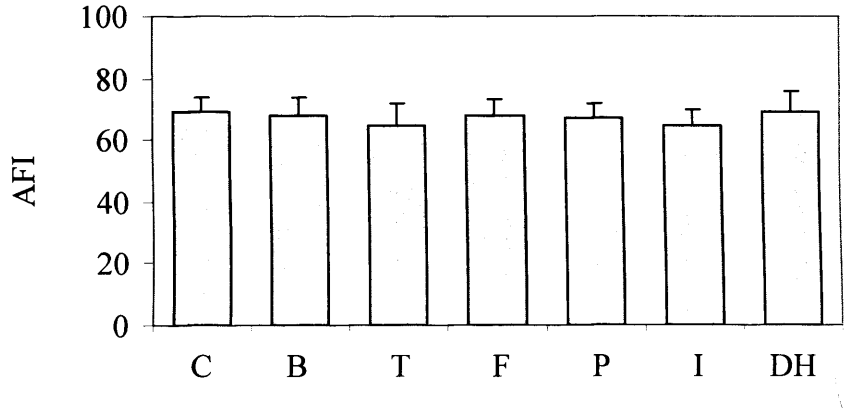


Figure 1. Effects of the ligand BMP-2 and osteogenic growth factors and hormones on surface expression of the BMPR-IA and -II protein. Cells were incubated with BMP-2 (50 ng/ml), TGF-β1 (0.1 ng/ml), FGF-2 (0.1 ng/ml), PDGF-AB (1 ng/ml), IGF-1 (50 ng/ml) and DHT (100 ng/ml) for 24 h and the cell surface expression of BMPR analysed by FCM. The effects of these mediators on the expression of cell surface BMPR-IA and-II protein are summarised in (A) and (B), respectively. Data are presented as the mean AFI (± SE) of untreated and treated cultures of three separate experiments. C: untreated control cells; B: BMP-2; T: TGF-β1; F: FGF-2; P: PDGF-AB; I: IGF-1; DH: DHT.