

**Adenosine receptor expression and function  
during mesenchymal stem cell differentiation  
and osteoblast transdifferentiation.**

A thesis submitted for the degree of

**Doctor of Philosophy**

to the

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by

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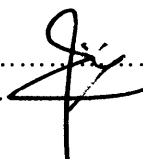
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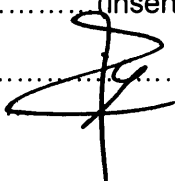
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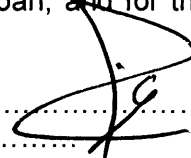
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## Publications and presentations

### Published abstracts

Gharibi, B., Elford, C., Ham, J., and Evans, B.A.J. (2007) Adenosine stimulates mineralisation of rat mesenchymal stem cell *in vitro*. *Journal of Bone and Mineral Research* 22, 1125, P14. Presented as a poster at the Bone Research Society annual meeting 2007, Aberdeen, UK.

Gharibi, B., Lewis, M.B., Elford, C., Ham, J., and Evans, B.A.J. (2008) Adenosine is an important regulator of mesenchymal stem cell differentiation into osteoblasts. *Endocrine Abstracts* 15, P16. Presented as a poster at the British Endocrine Society annual meeting 2008, Harrogate, UK.

Gharibi, B., Elford, C., Lewis, M., Ham, J., and Evans, B.A.J. (2008) Evidence for adenosine receptor regulation of osteogenesis versus adipogenesis in mesenchymal stem cells. *Calcified Tissue International* 83, 9, OP7. Presented as an oral poster at the Joint Meeting of the Bone Research Society and the British Orthopaedic Research Society 2008, Manchester, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Mesenchymal stem cell differentiation to osteoblasts and adipocytes is associated with differential adenosine receptor expression. *Endocrine Abstracts* 19, P12. Presented as a poster at the British Endocrine Society annual meeting 2009, Harrogate, UK.  
**Awarded poster prize.**

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Adenosine receptor expression and function as mesenchymal stem cells differentiate to osteoblasts. *Calcified Tissue International* 85, 76, P045. Presented as a poster at the Joint Meeting of the Bone Research Society and the British Society for Matrix Biology 2009, London, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Modulation of A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> adenosine receptor expression and function as osteoblasts differentiate to adipocytes. *Calcified Tissue International* 85, 76, P046. Presented as a poster at the Joint Meeting of the Bone Research Society and the British Society for Matrix Biology 2009, London, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Adenosine Receptors Regulate Osteoblastogenesis and Adipogenesis, and are Differentially Expressed by these Two Cell types. *Journal of Bone and Mineral Research* 24, (suppl 1). Available at [<http://www.asbmr.org/Meetings/AnnualMeeting/AbstractDetail.aspx?aid=c4309120-01d3-420f-8357-df2956f18d00>]. Presented as a poster at the American Society for Bone and Mineral Research annual meeting 2009, Denver, USA,



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## Other presentations

Gharibi, B., Elford, C., Ham, J., and Evans, B.A.J. (2007) Adenosine: an important regulator of mesenchymal stem cell differentiation. Presented as a poster at the Cardiff University postgraduate research day, Cardiff, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2008) Adenosine is an important regulator of mesenchymal stem cell differentiation into osteoblasts. Presented as a poster at the Welsh Endocrine and Diabetes Society annual meeting, Newport, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2008) A potential role for the adenosine signal pathway in controlling the balance between osteoblastogenesis and adipogenesis. Presented as an oral presentation at the Cardiff University postgraduate research day, Cardiff, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2008) Evidence for adenosine receptor regulation of osteogenesis versus adipogenesis in mesenchymal stem cells. Presented as an oral presentation at the Cardiff Institute of Tissue Engineering and Repair (CITER) annual meeting, Abergavenny, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Mesenchymal stem cell differentiation to osteoblasts and adipocytes is associated with differential adenosine receptor expression. Presented as a poster at the UK MSC meeting, Sheffield, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Can modulation of adenosine receptor signal pathways control the balance of bone and fat in osteoporosis? Presented as a poster at the Welsh Endocrine and Diabetes Society meeting, Newport, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Mesenchymal stem cell differentiation to osteoblasts is accompanied with increases in adenosine A2 receptor expression. Presented as an oral presentation at the UK Purine meeting, Sheffield, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Transdifferentiation of human and mouse osteoblasts to adipocytes is accompanied with changes in adenosine receptor expression. Presented as poster at the UK Purine meeting, Sheffield, UK.

Gharibi, B., Ham, J., and Evans, B.A.J. (2009) Transdifferentiation of human and mouse osteoblasts to adipocytes is accompanied with changes in adenosine receptor expression. Presented as a poster at the Cardiff University postgraduate research day, Cardiff, UK. **Awarded poster prize.**

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## Summary

The mechanisms involved in osteoblast and adipocyte differentiation from the common progenitor, mesenchymal stem cell (MSC) are not fully understood. The nucleoside, adenosine, exists in all cells and is known to be involved in cell growth, proliferation and apoptosis by interacting with four distinct receptors ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$ ). The aims of this study was to investigate the expression and function of adenosine receptors in 1) MSCs and as they differentiated into osteoblasts and adipocytes and in 2) mouse 7F2 and human osteoblasts and during their transdifferentiation to adipocytes.

Rat MSCs and 7F2 osteoblasts expressed all four adenosine receptors. Osteoblast differentiation was associated with increases in  $A_{2A}$  and  $A_{2B}$  receptor expression and their activation stimulated the expression of alkaline phosphatase, core binding factor  $\alpha 1$  and mineralisation. Adenosine also stimulated adipogenesis (lipid accumulation, peroxisome proliferator-activated receptor, CCAAT/enhancer binding protein  $\alpha$  and lipoprotein lipase expression) of MSCs which was accompanied by increased  $A_1$  and  $A_{2A}$  receptor expression.

Transdifferentiation of 7F2 cells to adipocytes was associated with increased  $A_1$ , but decreased  $A_{2A}$  and  $A_{2B}$  receptor expression. Loss of  $A_2$  receptors in adipocytes was supported by reduced cAMP and extracellular signal regulated kinase responses to adenosine. Adenosine also stimulated transdifferentiation of human osteoblasts to adipocytes by inducing lipoprotein lipase and inhibiting alkaline phosphatase and osteocalcin expression. Overexpression of  $A_1$  receptors in 7F2 cells stimulated adipogenesis (lipid accumulation and lipoprotein lipase expression) whereas overexpression of  $A_{2B}$  receptors stimulated alkaline phosphatase expression and inhibited adipogenesis.

These results show that adenosine receptor expression and function is involved in lineage specific differentiation or transdifferentiation;  $A_{2B}$  receptors are associated with MSCs and osteoblasts and  $A_1$  receptors with adipocytes. Targeting adenosine signal pathways may thus be useful as an adjunct therapy for the prevention or treatment of conditions in which there is insufficient bone or excessive adipocyte formation.

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## Abbreviations

$\mu$	micro
$\alpha$ -MEM	minimal essential medium alpha
$\beta$ -GP	$\beta$ -glycerophosphate
$^{\circ}$ C	centigrade
AA	ascorbic acid 2-phosphate
ADA	adenosine deaminase
ADP	adenosine diphosphate
ADRP	adipose differentiation related protein
AGRA	glycyrrhetic acid
AK	adenosine kinase
ALP	alkaline phosphatase
AMP	adenosine monophosphate
ANOVA	analysis of variance
aP2	adipose protein 2
APRT	adenine phosphoribosyltransferase
APS	ammonium persulfate
ARP	acidic ribosomal protein
ATF1	activating transcription factor-1
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factors
BMPs	bone morphogenic proteins
bp	base pairs
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding proteins

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<b>cAMP</b>	cyclic adenosine monophosphate
<b>CATK</b>	cathepsin K
<b>Cbfa1</b>	core binding factor a1
<b>CCPA</b>	2-chloro- <i>N</i> 6-cyclopentyladenosine
<b>CD73</b>	ecto-5'-nucleotidase
<b>CFU-Fs</b>	colony-forming unit fibroblasts
<b>CGS 21680</b>	(2-[ <i>p</i> -(2-carboxyethyl) phenethylamino]adenosine-5'- <i>N</i> -ethyluronamide)
<b>cm</b>	centimetre
<b>CNS</b>	central nervous system
<b>CNTs</b>	concentrative nucleoside transporters
<b>CO<sub>2</sub></b>	carbon dioxide
<b>CPA</b>	<i>N</i> 6-cyclopentyladenosine
<b>CPC</b>	cetylpyridinium chloride
<b>CRE</b>	cAMP response enhancer element
<b>CREB</b>	cAMP responsive element binding protein
<b>CSF</b>	colony stimulating factor
<b>C<sub>t</sub></b>	cycle threshold
<b>DAG</b>	diacylglycerol
<b>DDA</b>	2',5'-dideoxyadenosine
<b>Dex</b>	dexamethasone
<b>dH<sub>2</sub>O</b>	distilled water
<b>DMARDs</b>	disease-modifying antirheumatic drugs
<b>DMSO</b>	dimethyl sulfoxide
<b>DNA</b>	deoxyribonucleic acid

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<b>dNTP</b>	deoxyribonucleotide triphosphate
<b>DPCPX</b>	1,3-dipropyl-8 cyclopentylxanthine
<b>dsDNA</b>	double stranded DNA
<b>ECL</b>	enhanced chemiluminescence
<b>ECM</b>	extracellular matrix
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>E-NTPDase</b>	ecto-nucleoside triphosphate diphosphohydrolase
<b>ENTs</b>	equilibrative nucleoside transporters
<b>ERK</b>	extracellular signal-regulated kinases
<b>FAS</b>	fatty acid synthase
<b>FAT</b>	fatty acid translocase
<b>FCS</b>	fetal calf serum
<b>FGFs</b>	fibroblastic growth factors
<b>Formal saline</b>	formaldehyde in PBS
<b>FSC</b>	forward scatter
<b>FZD</b>	frizzled
<b>g</b>	gram
<b>Gai/o</b>	inhibitory alpha subunit
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase
<b>G<math>\alpha</math>s</b>	stimulatory alpha subunit
<b>GJC</b>	Gap-junctional communication
<b>GOI</b>	gene of interest
<b>Golf</b>	G-olfactory
<b>GPCR</b>	G-protein coupled receptor
<b>GPDH</b>	glycerol-3-phosphate dehydrogenase

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<b>GPI</b>	glycosyl phosphatidylinositol
<b>HCl</b>	hydrochloric acid
<b>Hh</b>	hedgehog
<b>HOB</b>	human osteoblast
<b>HRP</b>	horseradish peroxidase
<b>HSC</b>	haemopoietic stem cells
<b>IB-MECA</b>	<i>N</i> 6-3-Iodobenzyladenosine-5'- <i>N</i> -methyluronamide
<b>IBMX</b>	isobutylmethylxanthine
<b>IGFs</b>	insulin-like growth factors
<b>IL-1<math>\beta</math></b>	interleukin-1 $\beta$
<b>IL-6</b>	interleukin-6
<b>IP<sub>3</sub></b>	inositol 1, 4, 5-triphosphate
<b>ISCT</b>	International Society for Cellular Therapy
<b>kDa</b>	kilodalton
<b>l</b>	litre
<b>LPL</b>	lipoprotein lipase
<b>LRP5</b>	lipoprotein receptor-related protein 5
<b>m</b>	milli
<b>M</b>	molar
<b>mA</b>	milliamps
<b>MAPK</b>	mitogen-activated protein kinase
<b>M-CSF</b>	macrophage colony stimulating factor
<b>MEF2</b>	Myocyte Enhancer Factor 2
<b>MEK</b>	MAPK/ERK kinase
<b>MEPE</b>	matrix extracellular phosphoglycoprotein

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<b>mm</b>	millimetre
<b>M-MLV</b>	Moloney Murine Leukemia Virus
<b>MRFs</b>	myogenic regulatory factors
<b>MRS1706</b>	N-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide
<b>MRS1754</b>	(N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy]acetamide
<b>MSC</b>	mesenchymal stem cell
<b>MTS</b>	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium
<b>MTX</b>	methotrexate
<b>NaCl</b>	sodium chloride
<b>NADH</b>	beta-nicotinamide adenine dinucleotide/reduced
<b>NADPH</b>	beta-nicotinamide adeninedinucleotidephosphate/ reduced
<b>NaHCO<sub>3</sub></b>	sodium hydrogen carbonate
<b>NaOH</b>	sodium hydroxide
<b>NECA</b>	5'-N-ethylcarboxamidoadenosine
<b>NK</b>	natural killer
<b>NSAIDs</b>	non-steroidal anti-inflammatory drugs
<b>NSB</b>	non specific binding
<b>OD</b>	optical density
<b>Oligoprobes</b>	oligonucleotide probes
<b>Osf2</b>	osteoblast specific factor 2
<b>OSX</b>	osterix

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<b>P</b>	purinergic
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PBS</b>	phosphate buffered saline
<b>PCR</b>	polymerase chain reaction
<b>PDEs</b>	phosphodiesterases
<b>PDGF</b>	platelet derived growth factors
<b>PKA</b>	protein kinase A
<b>PKC</b>	protein kinase C
<b>PLC</b>	phospholipase C
<b>PMS</b>	phenazine methosulfate
<b>PMSF</b>	phenylmethylsulfonyl fluoride
<b>p-NPP</b>	p-nitrophenylphosphate
<b>PPAR<math>\gamma</math></b>	peroxisome proliferator-activated receptor $\gamma$
<b>Pref-1</b>	preadipocyte factor-1
<b>PSVT</b>	paroxysmal supraventricular tachycardia
<b>PTH</b>	parathyroid hormone
<b>PTHrP</b>	parathyroid hormone-related peptide
<b>PVDF</b>	polyvinylidene difluoride
<b>q-RTPCR</b>	quantitative-RT-PCR
<b>RA</b>	rheumatoid arthritis
<b>RANK-L</b>	receptor activator of nuclear factor $\kappa$ B ligand
<b>RIA</b>	radioimmunoassay
<b>RIPA</b>	radioimmunoprecipitation assay
<b>RNA</b>	ribonucleic acid
<b>RT</b>	reverse transcription



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<b>Runx2</b>	runt related transcription factors 2
<b>SAH</b>	s-adenosylhomocysteine
<b>SDS</b>	sodium dodecyl sulfate
<b>SLC</b>	solute carrier
<b>SLRPs</b>	small leucine-rich proteoglycans
<b>SPSS</b>	Statistical Package for Social Sciences
<b>SREBP-1c</b>	sterol regulatory element binding protein-1c
<b>SSC</b>	side scatter
<b>TAE</b>	tris-acetate-EDTA
<b>TAZ</b>	transcriptional co-activator with PDZ-binding motif
<b>TBS</b>	tris buffered saline
<b>TBS-T</b>	tris buffered saline with tween 20
<b>TEMED</b>	N,N,N',N'-Tetramethylethylenediamine
<b>TGF-<math>\beta</math></b>	transforming growth factor- $\beta$
<b>T<sub>m</sub></b>	melting temperature
<b>TNF<math>\alpha</math></b>	tumor necrosis factor alpha
<b>TRAP</b>	tartrate-resistant acid phosphatase
<b>U</b>	unit
<b>UCSC</b>	University of California Santa Cruz
<b>UDG</b>	uracil-DNA glycosylase
<b>UDP</b>	uridine diphosphate
<b>UTP</b>	uridine triphosphate
<b>w/v</b>	weight for volume
<b>Wnt</b>	wingless type MMTV integration site

# **Chapter 1**

## **General introduction**

## **1.1 Mesenchymal stem cells (MSCs) and osteogenic and adipogenic differentiation**

### **1.1.1 Stem cells**

Stem cells are non-specialised cells defined by their self-renewal ability and differentiation potential. They exist as undifferentiated or poorly differentiated cells that lack the functional characteristics of their progeny, and they maintain this undifferentiated phenotype until they are exposed to appropriate differentiation signals (Fuchs and Segre, 2000; Reya et al., 2001; Thomson et al., 1998). They are capable of asymmetric cell division and give rise to two daughter cells, one of which remains as a stem cell and the other follows a path of proliferation and differentiation. This unique asymmetry enables stem cells to undergo continuous self-renewal and indefinite proliferation allowing a constant pool of undifferentiated stem cells and subsequent specific lineage differentiation to be maintained (Lin, 2008; Morrison and Kimble, 2006). The differentiation potency however is not always the same and stem cells are classified according to their potential for differentiation, as totipotent, pluripotent and multipotent depending on the number of potential cell types that can arise (Wagers and Weissman, 2004). Mammalian zygotes are considered to be totipotent and these cells have the potential to generate all of the cell types in the body (McKay, 2000). Pluripotent cells are descendants of totipotent stem cells and give rise to cells of the three embryonic germ layers: ectoderm, mesoderm, and endoderm (Thomson et al., 1998). Pluripotent cells undergo further specialisation into more developmentally restricted multipotent cells that give rise to a limited number of cell lineages within a single germ layer.

### 1.1.2 Adult stem cells

Many adult tissues contain multipotent stem cells (adult stem cells) that, as opposed to embryonic stem cells, persist throughout life. Adult stem cells are responsible for maintaining the integrity and function of tissues by replacing the cells that are lost through normal tissue turnover or through apoptosis or necrosis following infection or injury (Mimeault and Batra, 2006). They lack tissue specific characteristics and remain quiescent until activated by appropriate signals to divide and differentiate into specialised cells. These cells may be tissue specific, *e.g.* residing in the brain (neuronal stem cells), skin (epidermal stem cells), eye (retinal stem cells) and gut (intestinal crypt stem cells) or may be located in other tissues that serve as stem cell reservoirs *e.g.* bone marrow which harbour multiple populations of stem cells with different properties (Li and Xie, 2005; Moore and Lemischka, 2006; Spradling et al., 2001). The bone marrow contains both haemopoietic stem cells (HSC) and MSCs. HSC have been used for many years in a therapeutic setting as a basis for bone marrow transplantation (Dupont, 1997) and MSCs contribute to the regeneration of multiple mesodermal tissue types (Pittenger et al., 1999). MSCs have been studied extensively in recent years and have been used in clinical settings (Granero-Molto et al., 2008; Horwitz et al., 2002; Koc et al., 2000; Krampera et al., 2006).

### 1.1.3 MSCs

The existence of cells with multilineage mesenchymal differentiation capacity was first demonstrated by Friedenstein and co-workers, who described the osteogenic potential of small population of cells in the bone marrow

(Friedenstein et al., 1966). Moreover, they demonstrated that *in vitro* cultivation of bone marrow cells at low density in a liquid medium containing serum gave rise to colonies of plastic-adherent cells with a fibroblastic appearance, which they defined as colony forming unit-fibroblasts (CFU-Fs). These cells had high proliferative capacity *in vitro* and when transplanted resulted in the generation of bone (Friedenstein et al., 1970; Friedenstein et al., 1974; Owen and Friedenstein, 1988). As a result of these observations the concept of multipotent stem cells capable of differentiating into mesenchymal lineages including myocytes, chondrocytes, adipocytes, osteoblasts and tendonocytes was proposed by Caplan (1991) who was the first to use the term mesenchymal stem cell (Caplan, 1991). These investigations formed the basis of further studies which demonstrated that bone marrow derived stromal cells were able to form a mixture of osseous and cartilaginous tissue *in vivo* or to differentiate into osteoblasts, chondrocytes and adipocytes *in vitro* (Awad et al., 1999; Bruder et al., 1998; Kadiyala et al., 1997; Krampera et al., 2006; Pittenger et al., 1999). Recent studies, however, questioned the ability of indefinite self-renewal and the multipotent properties of MSCs, and for this reason, The International Society for Cellular Therapy (ISCT) proposed that MSCs should be called multipotent stromal cells (Horwitz et al., 2005).

#### **1.1.3.1 Source of MSCs**

MSCs represent a very small percentage (0.0001 - 0.01%) of nucleated cells in the bone marrow, but can be expanded using standard cell culture techniques (Pittenger et al., 1999; Sakaguchi et al., 2005). Human MSCs are often obtained from aspirates of bone marrow harvested from the superior

iliac crest of the pelvis (Pittenger et al., 1999; Risbud et al., 2006). They have also been isolated from the tibial and femoral marrow compartments and thoracic and lumbar spine (D'Ippolito et al., 2002; Murphy et al., 2002). Apart from human MSCs, other well characterised MSC models include those of rat and mouse origin. MSCs from cow, pig, horse and sheep have also been investigated, particularly in possible therapeutic applications (Baddoo et al., 2003; Bosnakovski et al., 2005; Moscoso et al., 2005; Santa María et al., 2004). In rodents, bone marrow is typically obtained from the mid-diaphysis of the tibia or femur, whereas in large animals it is often harvested from the superior iliac crest of the pelvis. In addition to bone marrow, MSCs or MSC-like cells have been isolated from a number of tissues including; adult skeletal muscle, adipose tissue, lung, dental pulp, amniotic fluid and fetal lung and liver (Anker et al., 2003; Campagnoli et al., 2001; Gronthos et al., 2000; Williams et al., 1999; Zuk et al., 2001; Zvaifler et al., 2000).

### **1.1.3.2 Isolation and characterisation of MSCs**

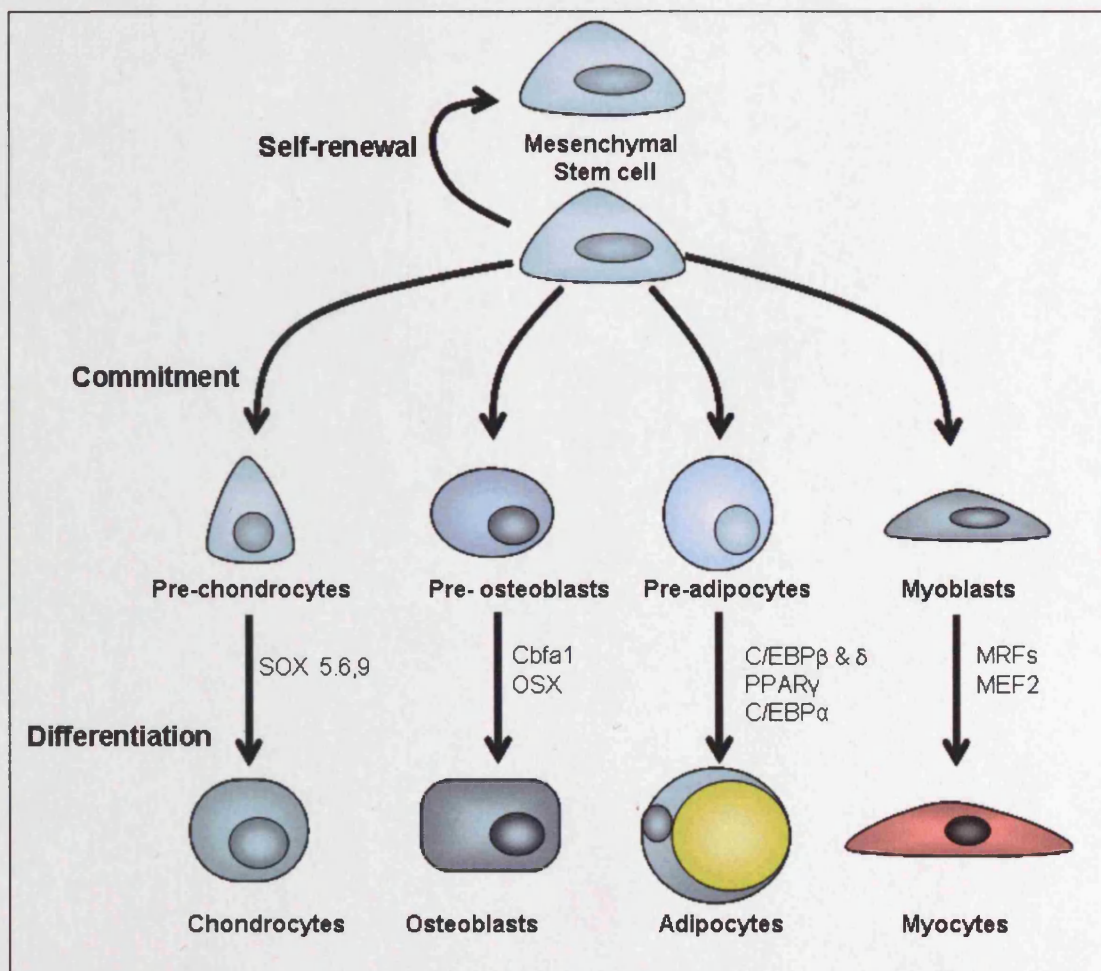
Traditionally, MSCs have been isolated utilising their physical property of selective adherence (compared to haemopoietic cells) to plastic surfaces (Luria et al., 1971). Whilst this eliminates many types of cells, macrophages, endothelial cells, lymphocytes, and smooth muscle cells may also adhere to plastic (Deans and Moseley, 2000). Further purification can be obtained by serial passaging, the resulting cultures are, however, still morphologically and functionally heterogeneous and commonly contain undifferentiated stem cells and lineage restricted precursors with varying differentiation capacities (Colter et al., 2000; Javazon et al., 2001). Considerable efforts have been made to

isolate a more homogenous MSC population using centrifugation over a Percoll gradient which allows the enrichment of nucleated cells and subsequent selection using MSC specific cell surface markers which are absent from haemopoietic cells (Pittenger et al., 1999; Simmons and Torok-Storb, 1991). However, such attempts are hampered by the lack of MSC specific markers. To date, there is no established phenotypic definition for MSCs; they do express a number of cell surface molecules, such as CD44, CD105, CD106, CD166, CD29, CD73, CD90, CD117, STRO-1 and Sca-1 none of which individually or in combination has been shown to be specific for MSCs (Bobis et al., 2006; Tare et al., 2008). On the other hand, MSCs do not express markers typical for haemopoietic cell lineages including, CD11b, CD31, and CD45 (Bobis et al., 2006; Tare et al., 2008). MSCs are thus best characterised using a combination of phenotypic and functional studies and, as suggested by the International Society for Cellular Therapy (ISCT), they should be defined by their property of plastic adherence, their phenotype and their capacity to differentiate into osteoblasts, adipocytes and chondrocytes (Dominici et al., 2006).

### **1.1.3.3 MSCs - commitment and differentiation**

The commitment of MSCs to differentiate into different cell lineages is mediated by a complex interaction of extracellular mediators. The mechanisms are not fully understood, but a number of growth factors and hormones such as bone morphogenic proteins (BMPs), wingless type MMTV integration site (Wnt) proteins, hedgehog (Hh) proteins, fibroblastic growth factors (FGFs), insulin, insulin-like growth factors (IGFs) appear to be involved

(see section 1.3). These factors are thought to regulate the changes in the expression and activation of lineage specific transcription factors that act as master switches to drive the differentiation of uncommitted precursors down a specific lineage (figure 1.1). For example Sox 5, 6 and 9 regulate chondrocytic differentiation and myogenic regulatory factors (MRFs) (including MyoD, myogenin, myogenic factor 5 and MRF 4) and myocyte enhancer factor 2 (MEF2) regulate myogenic differentiation (Blais et al., 2005; Frith and Genever, 2008). Factors that regulate MSC differentiation into adipocytes and osteoblasts will be discussed in section 1.1.3.4 and 5.



**Figure 1.1** Differentiation potential of MSCs. Modified from Harada and Rodan (2003).



#### 1.1.3.4 Adipocyte differentiation

The nuclear receptor peroxisome proliferators activated receptor (PPAR $\gamma$ ) is the key transcription factor involved in adipogenesis (Gimble et al., 1996). PPAR $\gamma$  is expressed as two isoforms PPAR $\gamma$ 1 and PPAR $\gamma$ 2 that are formed as a result of alternative splicing; PPAR $\gamma$ 2 is the main isoform in adipocytes (Tontonoz et al., 1994). PPAR $\gamma$ 2 is expressed early in the adipocyte differentiation process and at high levels in fat tissue (Kersten et al., 2000; Rosen and Spiegelman, 2001) and its requirement in adipogenesis has been demonstrated by both gain and loss of function studies. Activation of PPAR $\gamma$ 2 is sufficient to induce adipocyte differentiation of many cell types but its absence results in complete lack of adipogenesis *in vitro*, indicating an absolute requirement of PPAR $\gamma$ 2 for adipocyte formation (Ren et al., 2002; Tontonoz et al., 1994). The roles of PPAR $\gamma$  in regulation of adipogenesis have also been demonstrated by gene knockout strategies in several mouse models (Barak et al., 1999; Mueller et al., 2002; Rosen et al., 1999; Zhang et al., 2004b). These studies suggest that both PPAR $\gamma$ 1 and PPAR $\gamma$ 2 are involved in adipogenic differentiation, but PPAR $\gamma$ 2 is the most potent isoform.

CCAAT/enhancer binding proteins (C/EBP) are also important transcription factors involved in adipocyte differentiation. Amongst the C/EBP family, C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$  are known to promote adipogenesis while C/EBP $\gamma$  is found to inhibit adipogenesis (Darlington et al., 1998). C/EBP $\beta$  and C/EBP $\delta$  are early markers of adipogenesis and are able to activate expression of PPAR $\gamma$  and C/EBP $\alpha$  (Clarke et al., 1997; Yeh et al., 1995). When activated,

C/EBP $\alpha$  induces expression of several adipogenic genes that are involved in the maturation of adipocytes and is also necessary for insulin dependent glucose uptake. C/EBPs are therefore important contributors at both the commitment and differentiation phases of adipogenesis (Lefterova and Lazar, 2009; Rosen and MacDougald, 2006).

### 1.1.3.5 Osteoblast differentiation

The essential regulators of osteoblast lineage cells are the core binding protein from the runt family of transcription factors (Runx2) and osterix (OSX). Runx2 is also known as osteoblast specific factor 2 (Osf2) or core binding factor a1 (Cbfa1). Cbfa1 directs mesenchymal cells to differentiate into osteoblasts and inhibits their differentiation into adipocytes (Toshihisa, 2006). The role of Cbfa1 in osteoblast differentiation has been demonstrated in null-mutation mice which show a complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation (Komori et al., 1997; Otto et al., 1997). Although cartilage is present in Cbfa1 null mice, these animals show a defect in chondrocyte maturation and a lack of hypertrophic chondrocytes (Inada et al., 1999). On the other hand overexpression of Cbfa1 induces osteoblast differentiation, bone formation and the expression of bone associated markers and matrix mineralisation both *in vitro* and *in vivo* (Ducy et al., 1997; Zheng et al., 2004).

Mice lacking the OSX gene also show a complete lack of osteoblastic differentiation and mineral bone matrix formation. However, unlike Cbfa1-null mice, MSCs in OSX-null mice maintain their full potential to differentiate into

chondrocytes (Day et al., 2005; Hu et al., 2005; Nakashima et al., 2002). OSX may thus be important for directing precursor cells away from the chondrocyte lineage and towards the osteoblast lineage. Furthermore, OSX is not expressed in Cbfa1-null mice, whereas the expression of Cbfa1 is normal in OSX-null mice, indicating that OSX is expressed downstream of Cbfa1 (Day et al., 2005; Hu et al., 2005; Nakashima et al., 2002). Other transcription factors involved in differentiation of osteoblasts include those of the Dlx and Msx homeodomain families that are homologues of the *Drosophila* Distal-less and muscle specific homeobox genes, respectively (Ichida et al., 2004; Kim et al., 2004; Shirakabe et al., 2001; Tadic et al., 2002).

## **1.2 Inter-relationships between osteoblasts and adipocytes**

### **1.2.1 Transcription factors**

Recent studies have shown that transcription factors such as PPAR $\gamma$  may have a dual role in the conversion of MSCs into osteoblasts or adipocytes. These studies suggest that a large degree of plasticity exists between osteoblasts and adipocytes and that they can form a reciprocal relationship. *In vitro* studies have shown that several PPAR $\gamma$  agonists, *i.e.* rosiglitazone, thiazolidinedione, not only induce the differentiation of murine and human bone marrow MSCs into adipocytes, but also negatively regulate osteoblast differentiation by repressing Cbfa1 (Ali et al., 2005; Lazarenko et al., 2006; Rzonca et al., 2004). Overexpression of PPAR $\gamma$  in MSCs has been shown to preferentially induce adipogenesis rather than osteoblastogenesis. When expressed in osteoblasts, PPAR $\gamma$  suppresses the mature osteoblast phenotype and induces expression of genes that are

associated with an adipocyte-like phenotype, such as adipose protein 2 (aP2), fatty acid synthase (FAS), and lipoprotein lipase (LPL) (Lecka-Czernik, 1999).

*In vivo* studies showed that PPAR $\gamma$  function was respectively stimulated or inhibited in animal models of bone loss and bone formation. Treatment of mice with rosiglitazone increased the volume of bone marrow adipose tissue while reducing bone mineral content, bone formation rates, and trabecular bone area (Ali et al., 2005; Rzonca et al., 2004). Similar results were observed when ovariectomised rats received rosiglitazone (Sottile et al., 2004). A less potent ligand, troglitazone, increased bone marrow adipose volume without significantly reducing bone mass and trabecular volume (Tornvig et al., 2001). Furthermore, mice with congenital mutations in PPAR $\gamma$  displayed enhanced bone formation (Cock et al., 2004). This increased bone mass was associated with a 2 fold increase in osteoblast formation and *in vitro* studies showed there was a similar decrease in adipocyte formation from bone marrow derived MSCs (Akune et al., 2004). Consistent with these findings was the observation that primary cultured marrow cells lacking PPAR $\gamma$  expressed several osteoblast markers and key molecules for osteoblast differentiation, including Cbfa1 and OSX (Kawaguchi et al., 2005). On the other hand, Cbfa1 deficient calvarial cells spontaneously differentiate into adipocytes indicating that Cbfa1 has an inhibitory effect on adipocyte differentiation (Kobayashi et al., 2000). Together these data suggests that specific expression of lineage specific transcription factors has a dual role, favouring the differentiation of MSCs toward one cell type and inhibiting differentiation to other cell types.

### **1.2.2 Osteoblasts and adipocytes in health and disease**

A large body of clinical evidence supports the notion that there is an inverse relationship between adipocytes and osteoblasts. For example, ageing which has negative effects on bone mass and osteoblast production is also associated with an increase in marrow adiposity (Duque, 2008; Gimble J M et al., 2006). With advancing age, the number of adipocytes in the bone marrow increases, resulting in the appearance of fatty marrow. This is most pronounced in the long bones, where up to 90% of the marrow cavity is occupied by adipocytes. Marrow adiposity is recognised to be a significant risk factor for the long-term capability of maintaining mechanical strength in the skeleton (Byers et al., 2001).

In contrast to the elderly, healthy teenagers and young adults have high levels of bone in the axial and appendicular skeleton but low levels of adipocytes in the marrow. However even in healthy young individuals, the amount of bone in the axial and appendicular skeleton have been shown to be inversely related to marrow adiposity (Di Iorgi et al., 2008). Reduced bone mass is thus likely to be partly due to an imbalance in the production of bone-forming and fat-forming cells in the marrow. Evidence for this was shown many years ago when histomorphometric and radiological examination of bone indicated that changes in bone marrow stromal cell dynamics with age could result in osteoporosis as a result of replacing the osteoprogenitor cell population with adipose tissue in the marrow (Meunier et al., 1971; Moore and Dawson, 1990). More recently, it has been shown that ageing is associated with changes in the intrinsic differentiation potential and the specific marrow

microenvironment necessary for differentiation of MSCs. MSCs derived from aged rats have reduced expression of Cbfa1, Dlx5, collagen type I and osteocalcin and show increased adipogenesis in response to rosiglitazone when compared with MSCs isolated from adult animals. Similarly, conditioned media from bone marrow cultures of aged animals induced a greater degree of adipogenesis (Moerman et al., 2004). Sera from aged individuals have also been shown to inhibit the expression of Cbfa1, alkaline phosphatase (ALP), collagen type I and osteocalcin (Abdallah et al., 2006).

Adipocyte recruitment at the expense of a reduction in osteoblast number may also contribute to bone loss in postmenopausal women. After the menopause, the rate of bone remodelling increases due to sex steroid deficiency (Manolagas, 2000) which is accompanied by an increase in the number of early osteoclast and osteoblast progenitors (Jilka et al., 1998). Reduction in the number of osteoblast progenitors due to adipocyte recruitment could therefore result in an imbalance between formation and resorption and leads to progressive bone loss. In fact, several groups have reported an inverse relationship between trabecular bone loss and increased marrow adiposity in postmenopausal osteoporosis (Griffith et al., 2006; Shen et al., 2007; Verma et al., 2002; Yeung et al., 2005). Similar findings have been seen in glucocorticoid treatment, increased cortisol production and immobilisation (Ahdjoudj et al., 2002; Valentin et al., 2007; Wang et al., 1977).

Together, these observations strengthen the premise that there is a reciprocal relationship between adipocyte and osteoblast differentiation. The bone loss

seen in osteoporosis could therefore be an outcome of preferential differentiation of mesenchymal cells into the adipocyte at the expense of osteoblasts. Inhibiting marrow adipogenesis with a parallel increase in osteoblastogenesis could be a useful therapeutic strategy to either prevent or treat conditions where there is insufficient bone formation or excessive marrow adipogenesis.

### **1.3 Factors involved in osteoblast and adipocyte differentiation and function**

Many compounds, including growth factors and hormones have been shown to be involved in the differentiation of MSCs to osteoblasts and adipocytes, some of the more important are described below. In addition I have focused on adenosine signal pathways in the context of differentiation as this is largely an unexplored area and the subject of my thesis.

#### **1.3.1 Growth factors**

Osteoblasts produce a range of growth factors including the IGFs, platelet derived growth factors (PDGF), basic fibroblast growth factors (bFGF), transforming growth factor- $\beta$  (TGF- $\beta$ ) and BMPs (Nakamura, 2007). These factors can act in an autocrine and paracrine fashion and are involved in the different stages of the osteoblast differentiation process.

Amongst these factors, BMPs are capable of initiating osteoblastogenesis from uncommitted progenitor cells (Katagiri et al., 1994) by stimulating the transcription of genes that encode Cbfa1 and Dlx5 (Abe et al., 2000; Leboy, 2006; Newberry et al., 1998; Termaat et al., 2005). BMPs are essential for

osteoblasts to achieve a mature phenotype, characterised by expression of ALP, collagen type I, and osteocalcin and the formation of collagen-based extracellular matrix and mineral deposition. Inhibition of the BMP signalling pathway by noggin (a natural antagonist) has been shown to suppress the osteoblast phenotype by inhibiting mineralisation *in vitro* and *in vivo* (Abe et al., 2000; Devlin et al., 2003).

TGF- $\beta$ , an abundant molecule in the bone matrix, plays a stage-dependent role in osteoblast differentiation. It stimulates bone formation by recruiting osteoblast progenitors and stimulating their proliferation, whilst inhibiting osteoblast maturation, differentiation and mineralisation. Blocking the expression of phenotype-specific genes, such as osteocalcin and ALP increases the population of committed osteoblasts (Banerjee et al., 2001; Lee et al., 2000). TGF- $\beta$  and BMPs also influence adipogenic differentiation. TGF- $\beta$  inhibits adipocyte differentiation by blocking the induction of the adipogenic transcription factor C/EBP $\alpha$  via interaction with Smad3, Smad6 and Smad7 proteins (Choy and Derynck, 2003; Choy et al., 2000). In contrast, BMPs, under selected culture conditions, stimulate adipocyte differentiation (Hata et al., 2003; Sottile and Seuwen, 2000).

Other factors such PDGF, IGFs, and the FGF family can all stimulate osteoblast differentiation (Varghese et al., 2000; Zhao et al., 2000). However, similar to TGF- $\beta$ , these factors are only able to influence the replication and differentiation of committed osteoblast progenitors towards the osteoblastic



lineage and are unable to induce osteogenesis in uncommitted mesenchymal pluripotent cells.

### **1.3.2 Leptin**

Leptin, an adipocyte-derived hormone that regulates appetite and energy expenditure, is also found to have complex effects on bone. The actions of leptin on bone can be explained by its ability to act positively and directly, or negatively, via stimulation of growth hormone secretion and its effects on the hypothalamic pituitary axis (Thomas, 2004). The direct effects of leptin on bone appear to be mediated primarily via effects on MSCs (Thomas et al., 1999). Leptin has been shown to stimulate the proliferation of bone marrow MSCs (a murine cell line) and osteoblasts isolated from fetal rats (Steppan et al., 2000; Takahashi et al., 1997). It also increased osteoblastic differentiation of a conditionally immortalised stromal cell line from human bone marrow, rat calvarium cells, and human osteoblasts in long-term cell cultures (Takahashi et al., 1997). At the same time, leptin decreases adipocyte differentiation of stromal precursors (Hamrick et al., 2005). These findings suggest that leptin may regulate the reciprocal differentiation of MSCs along the osteoblast and adipocyte lineages. On the other hand some studies have shown that high concentrations of leptin induce apoptosis of bone marrow MSCs, decrease bone formation, and increase bone resorption (Kim et al., 2003; Martin et al., 2007). An excess of marrow adipogenesis could therefore result in a very high local concentration of leptin in the bone marrow microenvironment and contribute to bone loss.

### 1.3.3 Wnt signalling

Wnts are a large family of secreted glycoproteins that activate the family of 7-membrane-spanning frizzled (FZD) receptors to regulate cell growth, differentiation, function, and death in many organs and tissues, including bone and fat. Wnt signalling can be subclassified as either canonical or non-canonical, depending on whether  $\beta$ -catenin is involved (Clevers, 2006). The canonical Wnt/ $\beta$ -catenin pathway appears to be important in bone development. It is thought to favour differentiation of progenitor cells into osteoblasts as opposed to adipocytes, stimulate osteoblast proliferation and induce osteoblastogenesis (Baron and Rawadi, 2007; Muruganandan et al., 2009). In humans, mutations in the Wnt co-receptor low-density lipoprotein receptor related protein 5 (LRP5) are shown to be associated with alterations in bone mass. Whereas loss of function mutations in the LRP5 gene resulted in low bone mass and skeletal fractures, gain of function mutations of LRP5 have been found to be associated with increased bone density (Gong et al., 2001; Hofbauer et al., 2002; Little et al., 2002). Similar findings were observed by knocking out LRP5 or introducing gain of function mutations in mice (Babij et al., 2003; Clement-Lacroix et al., 2005; Kato et al., 2002). In these animals, bone resorption was unaffected suggesting Wnt signalling pathways only targeted the osteoblasts.

Furthermore, a defect in the  $\beta$ -catenin gene in mesenchymal precursor cells was associated with a lack of skeletal bone (Hu et al., 2005). Similarly, a spontaneous conversion of various cell types to adipocytes was observed following inhibition of  $\beta$ -catenin signalling (Bennett et al., 2002). Activation of

Wnt/ $\beta$ -catenin signalling pathways in mesenchymal precursors has also shown to repress adipogenesis and stimulate osteoblastogenesis. For example, overexpression of Wnt10b increases bone mass and strength, whilst inhibiting adipogenesis and blocking the induction of key adipogenic transcription factors C/EBP $\alpha$  and PPAR $\gamma$  (Bain et al., 2003; Bennett et al., 2002; Rawadi et al., 2003; Ross et al., 2000).

#### **1.3.4 Transcriptional co-activator with PDZ-binding motif (TAZ)**

TAZ is a transcriptional modulator that regulates the functional interaction of specific transcription factors with chromatin (Hong et al., 2005; Kanai et al., 2000). Recently, TAZ has been implicated in the regulation of differentiation of MSCs into osteoblasts and adipocytes. It is believed that TAZ directly interacts with the regulatory regions of Cbfa1 and PPAR $\gamma$  and promote osteoblastic differentiation by co-activating Cbfa1, whilst inhibiting adipogenesis by repressing PPAR $\gamma$ . Binding of TAZ to Cbfa1 is found to increase the expression of osteoblast related genes during terminal osteoblast differentiation. In contrast, binding to PPAR $\gamma$  markedly inhibits its ability to induce the expression of adipocyte genes such as aP2. In addition, siRNA inhibition of TAZ in preadipocytes and MSCs is shown to induce adipogenesis at the expense of osteoblastogenesis (Hong et al., 2005). TAZ expression appears to increase during osteoblast differentiation and decrease during adipocyte differentiation (Hong and Yaffe, 2006).

### 1.3.5 Hh signalling

Hh signalling is another pathway that is thought to be involved in the regulation of osteoblastogenesis vs adipogenesis. In MSCs activation of Hh signalling appears to promote osteoblast rather than adipocyte differentiation. Overexpression of Hh signalling is shown to inhibit the expression of the adipocyte markers aP2, leptin, C/EBP $\alpha$  and PPAR $\gamma$  whilst inducing the osteoblast markers osteocalcin and Cbfa1 in C3H10T1/2 cells (Spinella-Jaegle et al., 2001). Furthermore, disruption of the Hh signalling pathway resulted in failure of cells to undergo osteoblast differentiation (Long et al., 2004). In preadipocytes, Hh signalling induced an almost complete inhibition of adipocyte differentiation as shown by Oil Red O staining and adipocyte marker gene expression (Suh et al., 2006). Hh signalling has also shown to inhibit maturation of human MSCs via the targeting of C/EBP $\alpha$  and PPAR $\gamma$  genes (Fontaine et al., 2008). On the other hand, in preosteoblasts, recombinant human sonic Hh potently increases osteoblastic differentiation as demonstrated by increased ALP activity, mineralisation and expression of osteoblast marker genes (Geertje van der et al., 2003).

### 1.3.6 Cyclic adenosine monophosphate (cAMP) signalling

cAMP is a secondary messenger in intracellular signal cascades that is activated by a wide variety of cellular actions in response to a large number of extracellular agents. In the context of bone, parathyroid hormone (PTH) up regulates cAMP whereas adenosine can both stimulate and inhibit cAMP via different receptor systems. cAMP is produced by adenylate cyclases from 5'-AMP in the adenosine triphosphate (ATP) metabolic pathway and is rapidly

degraded by cyclic nucleotide phosphodiesterases (PDEs) that hydrolyse the 3'-phosphoester bond to the inactive noncyclic nucleotide 5'-AMP (Fimia and Sassone-Corsi, 2001). Changes in cAMP levels are translated into intracellular responses through activation of effector proteins, the most important of them being cAMP-dependent protein kinase or protein kinase A (PKA). PKA is a heterotetrameric holoenzyme consisting of two catalytic subunits and one regulatory subunit. cAMP binds to and causes the release of the free active catalytic subunits which subsequently migrate into the nucleus where they phosphorylate and activate transcriptional activators including the cAMP responsive element binding protein (CREB). Subsequently CREB binds the cAMP response enhancer element (CRE) typically found in the promoter of many cAMP responsive genes to activate transcription (Daniel et al., 1998; Sands and Palmer, 2008). Activation of this signalling pathway is involved in many cell activities including growth and differentiation.

The importance of the cAMP/PKA/CREB pathway in the induction of adipocyte differentiation and maturation has been known for many years. Indeed, agents that increase cAMP, such as isobutylmethylxanthine (IBMX) have been used, *in vitro*, as a component of cell culture medium to initiate adipogenic differentiation (Farmer, 2006). Activation of the cAMP pathway stimulates adipogenesis in murine preadipocytes (3T3-L1) and in human MSCs by increasing the expression of many adipocytic genes (Petersen et al., 2008; Reusch et al., 2000; Watanabe et al., 2003; Yang et al., 2008). A remarkable observation is the finding that adipocyte differentiation in mouse embryonic fibroblasts from CREB<sup>-/-</sup> mice is impaired (Zhang et al., 2004a) and

siRNA mediated depletion of CREB and the closely related activating transcription factor-1 (ATF1) in 3T3-L1 cells results in loss of adipogenic differentiation (Fox et al., 2006). Overexpression of constitutively active CREB is sufficient for initiation of adipogenesis as determined by triacylglycerol storage, cell morphology, and the expression of adipocyte marker genes, whereas expression of a dominant-negative form of CREB blocks adipogenesis (Reusch et al., 2000). Furthermore, the cAMP/CREB pathway appears to be associated with a number of events during adipocyte differentiation. Activation of cAMP in preadipocytes suppresses Wnt10b and Sp1, both of which are negative regulators of adipogenesis (Bennett et al., 2002; Tang et al., 1999). On the other hand, cAMP/CREB is thought to induce C/EBP $\beta$  and PPAR $\gamma$  expression and the production of endogenous PPAR $\gamma$  ligands (Bennett et al., 2002; Petersen et al., 2008; Tzameli et al., 2004; Yang et al., 2008; Zhang et al., 2004a).

The cAMP pathway also has a positive effect on osteoblastogenesis although some of the findings are conflicting. Activation of cAMP pathway through PTH has been shown to regulate a number of osteoblastic differentiation genes such as osteopontin, collagen type I, bone sialoprotein and osteocalcin (Boguslawski et al., 2000; Ogata et al., 2000; Yang and Gerstenfeld, 1996). PTH has also shown to enhance BMP signalling through cAMP and promote osteoblastic function and anabolic actions of new bone formation (Nakao et al., 2009). PKA appears to participate synergistically in BMP-2-induced osteoblastic differentiation (Sugama et al., 2006; Zhao et al., 2006) and inhibiting PKA in murine premyoblast C2C12 cells been shown to repress

OSX and collagen type I expression and ALP enzyme activity. On the other hand, stimulation of cAMP with 8-Br-cAMP, dibutyryl-cAMP or forskolin accelerates BMP-2 induced ALP activity and osteocalcin expression (Tsutsumimoto et al., 2002; Zhao et al., 2006) and increases osteoblast differentiation *in vitro* and bone formation *in vivo* (Siddappa et al., 2008).

The cAMP pathway has also been shown to be involved in the induction of *in vitro* vascular calcification by enhancing osteoblast-like differentiation of calcifying vascular cells (Tintut et al., 1998). Moreover, PDE inhibitors that cause elevation of the intracellular cAMP have been reported to induce osteoblastic differentiation and inhibit osteoclastic differentiation *in vitro* (Tsutsumimoto et al., 2002; Waki et al., 1999). PDE silencing also induced osteoblastogenesis as shown by upregulation of several osteogenic genes and increased mineralisation (Pekkinen et al., 2008). These data are supported by *in vivo* studies, in which PDE inhibitors have been shown to increase bone mass in mice by mainly promoting bone formation (Kinoshita et al., 2000). In addition, CRE binding sites have been reported in promoter regions of osteoblast associated genes (Huang et al., 2005; Pearman et al., 1996) and PKA is shown to phosphorylate Cbfa1 via a PKA phosphorylation site (Selvamurugan et al., 2000).

In contrast to a stimulatory role for cAMP in osteoblastogenesis, other researchers have suggested a negative action of cAMP on bone formation. For example, in the osteoblastic cell line, MC3T3-E1, treatment with cAMP elevating agents resulted in degradation of Cbfa1 and inhibition of

osteoblastic differentiation (Tintut et al., 1999). Parathyroid hormone-related peptide (PTHrP) also inhibits Cbfa1 expression through the PKA pathway (Li et al., 2004). In human MSCs, cAMP reduces the expression of Cbfa1 and osteopontin (Yang et al., 2008) whereas in rodent cells, it inhibits ALP, osteocalcin, and collagen type I (Siddappa et al., 2009).

### 1.3.7 Nucleotides

Nucleotides, such as ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP) are important extracellular signalling molecules that act through cell surface receptors, designated purinergic (P)2 receptors. The P2 receptors are grouped into the P2X ligand-gated ion channels and P2Y G-protein coupled receptors (GPCRs). Currently, there are seven known P2X subtypes (P2X<sub>1-7</sub>) and eight P2Y subtypes P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>. Each of these receptors has been cloned, characterised and display distinct tissue expression and pharmacology (Burnstock, 2007; Ralevic and Burnstock, 1998). Multiple P2 receptors have been localised to bone, where they elicit a range of responses including osteoblast proliferation, bone formation activities and osteoclast formation and resorptive capacity (Gallagher, 2004). At least eight different P2 receptors (P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, P2X<sub>7</sub>, P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub>) are present in human osteoblasts and osteoblastic cell lines, but the functional roles for these have not been completely clarified (Alqallaf et al., 2009; Gallagher and Buckley, 2002; Hoebertz et al., 2003; Hoebertz et al., 2000).



The P2X<sub>5</sub> receptor has been implicated in the stimulation of DNA synthesis and thought to be involved in the regulation of osteoblast proliferation (Nakamura et al., 2000). The P2X<sub>7</sub> receptor is involved in skeletal responses to mechanical loading and in cell membrane blebbing in response to high concentrations of ATP (Li et al., 2005; Panupinthu et al., 2007). In addition studies of mice lacking the P2X<sub>7</sub> showed increased bone resorption by osteoclasts and decreased bone formation by osteoblasts (Ke et al., 2003). Activation of P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors has been shown to modulate the actions of systemic factors such as PTH (Bowler et al., 1999; Buckley et al., 2001). Activation of P2Y<sub>2</sub> or P2Y<sub>4</sub> with UTP and ATP strongly inhibits mineralisation (Orriss et al., 2006; Orriss et al., 2007).

In contrast to the reports of P2 receptor signalling in bone, little is known about its receptors in MSCs and adipocytes. A recent study by Coppi et al (2007) suggest that ATP is released by undifferentiated human MSCs, affecting their proliferation and cell fate (Coppi et al., 2007). ATP and its receptors have also been shown to mediate lipogenesis, which regulate fat stores independently from insulin stimulated glucose transport (Schodel et al., 2004). ATP has also been shown to stimulate aP2 expression, without having effect on differentiation in 3T3-L1 preadipocyte cells (Omatsu-kanbe et al., 2006). Expression of the P2Y<sub>11</sub> receptor in adipocytes on the other hand appears to be involved in the inhibition of insulin-stimulated leptin production and stimulation of lipolysis (Lee et al., 2005). Many of the effects of ATP are however caused by its breakdown product, adenosine, which has been known for a long time to be involved in the activities of adipocytes (see section 1.4.9).

## **1.4 Adenosine signalling pathway**

### **1.4.1 Adenosine**

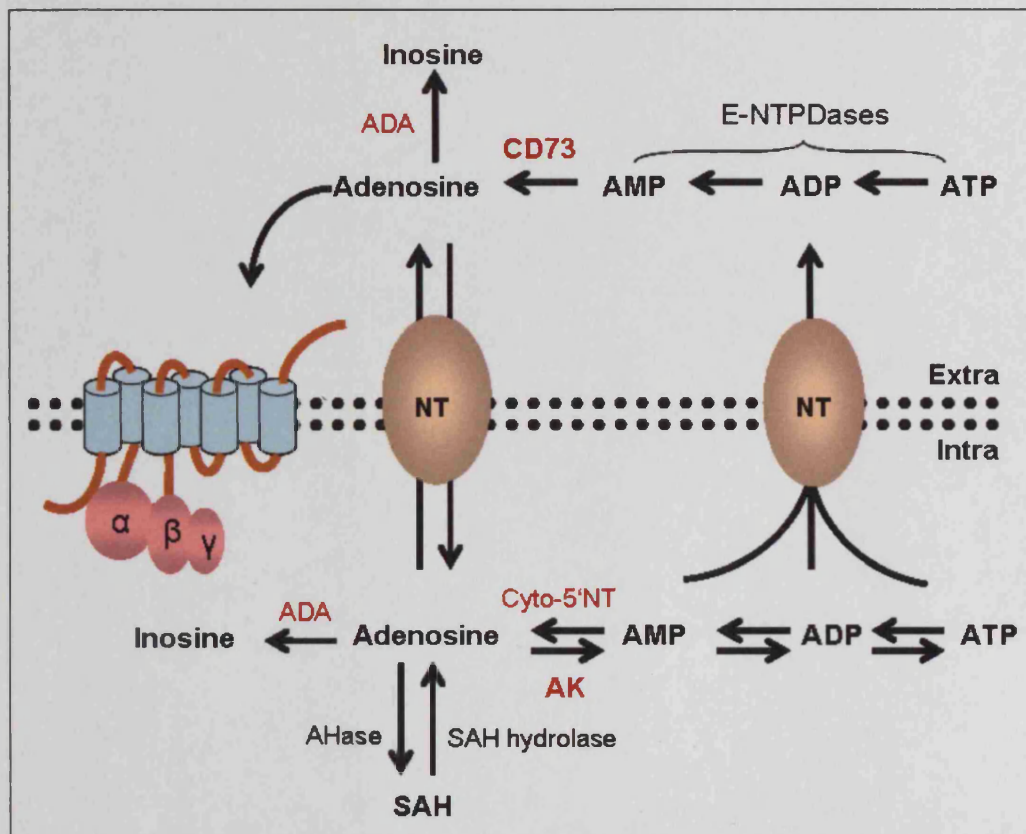
Adenosine is an endogenous purine ribonucleoside consisting of adenine (the purine base) in glycosidic linkage with the sugar, ribose. Besides its role as a structural element in building nucleic acids and in energy metabolism, adenosine also modulates a variety of physiological responses by interacting with specific cell surface receptors. Adenosine was first documented as a physiological regulator in the cardiovascular system by Drury and Szent-Gyorgyi (1929); later Sattin and Rall (1970) postulated that the regulatory effects of adenosine are due to their interaction with specific receptors on the cell surface. It is now established that there are four different adenosine receptor subtypes;  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$ . These are also known as P1 receptors and have been cloned and pharmacologically characterised from several species including human, rat and mouse (Fredholm et al., 2001; Linden, 2001). Adenosine activates adenosine receptors with different affinities; the high affinity  $A_1$  (3 - 30 nM) and  $A_{2A}$  (1 - 20 nM) receptors are activated by physiological concentrations and the low affinity  $A_{2B}$  (5000 - 20000 nM) and  $A_3$  (>1000 nM) receptors require higher concentrations of adenosine. High concentrations of adenosine are found to occur in extreme or pathological conditions (Latini and Pedata, 2001; Yuzlenko and Kiec-Kononowicz, 2006).

### **1.4.2 Adenosine metabolism**

Adenosine is normally present in both intracellular and extracellular spaces although extracellular levels under cellular resting conditions are low. It is established that adenosine is produced by two different pathways that involve

different substrates, *i.e.* adenosine monophosphate (AMP) and S-adenosylhomocysteine (SAH) (Schrader et al., 1981; Zimmermann, 2000) (Figure 1.2). Under basal conditions, intracellular adenosine is mainly formed from the sequential dephosphorylation of ATP by a cascade of phosphatases (Zimmermann, 2000). The other possible intracellular source of adenosine involves the hydrolysis of SAH by SAH hydrolase. This pathway is responsible for about one-third of adenosine production under normoxic conditions but does not appear to be important in hypoxic conditions (Latini and Pedata, 2001). Adenosine can also be formed in the extracellular space by breakdown of ATP, ADP and AMP that have been transported out of the cell. Under basal conditions, adenosine levels in cells and tissue fluids are low (30 - 300 nM). When cells become stressed such as during hypoxic events, inflammation and tissue injury, increased metabolic activity due to high cellular energy demands and low oxygen supply, induces a rise in the dephosphorylation of ATP to adenosine (Latini and Pedata, 2001; Pastor-Anglada et al., 2001). In addition there is marked efflux of ATP, ADP and AMP into the extracellular space. These processes bring about a marked increase in cytoplasmic adenosine concentrations and an accumulation of adenosine in the extracellular space (Hyde et al., 2001; Pastor-Anglada et al., 2001). Such conditions may lead to several hundred fold increases in extracellular adenosine and reach concentrations of up to 30  $\mu$ M (Latini and Pedata, 2001; Pastor-Anglada et al., 2001). Increased adenosine re-stabilises the ratio of energy supply and demand, stimulates vasodilatation and exerts protective effects on the heart and brain (Fredholm et al., 2001; Jacobson and Gao, 2006). The half-life of adenosine is however short and extracellular adenosine is rapidly converted

into inosine by adenosine deaminase (ADA) and/or removed by nucleoside transporters from the outside to the inside of the cells where it is phosphorylated back to AMP by adenosine kinase (AK).



**Figure 1.2 Pathways of adenosine synthesis and metabolism.** Modified from Blackburn (2003). Adenosine is produced intracellularly from the dephosphorylation of AMP by cytosolic-5' nucleotidases (cyto-5'NT) or by hydrolysis of S adenosylhomocysteine (SAH) by SAH hydrolase. Adenosine can also form extracellularly by breakdown of ATP, ADP and AMP that have been transported out of the cell. Extracellular ATP and ADP are catabolized by a cascade of ectonucleoside triphosphate diphosphohydrolase (E-NTPDases) to AMP and ecto-5'-nucleotidase (CD73) in turn, rapidly dephosphorylates AMP to adenosine. Extracellular adenosine can signal via adenosine receptors or it can be deaminated to inosine by adenosine deaminase (ADA) or be transported into cells via nucleoside transporters (NT). Intracellular adenosine can be secreted or converted back to AMP via adenosine kinase (AK). Alternatively it can be deaminated to inosine by ADA.

#### 1.4.2.1 Enzymes involved in metabolism of adenosine

A variety of surface-located enzymes are involved in the extracellular hydrolysis of ATP; these, include the ecto-nucleoside triphosphate diphosphohydrolase family (E-NTPDase) 1, 2, 3 and 8 that break down ATP

to ADP and AMP. E-NTPDase1 (CD39) hydrolyses ATP to ADP at the same rate as ADP to AMP, whereas NTPDase2 (CD39L1), NTPDase3 (CD39L3) and NTPDase8 (ecto-ATPase) show a preference for ATP over ADP as substrate and hydrolyse ATP up to thirty times faster than ADP (Kukulski et al., 2005; Robson et al., 2006; Zimmermann, 2001).

The rate limiting step in extracellular ATP hydrolysis is the conversion of AMP to adenosine which is mediated by ecto-5'-nucleotidase (CD73), a glycosyl phosphatidylinositol (GPI)-linked, membrane-bound glycoprotein (Dunwiddie et al., 1997; Sträter, 2006). It is interesting to mention that CD73 is expressed and is used as one of the positive markers for selection of MSCs (Bobis et al., 2006; Tare et al., 2008). The activity of CD73 controls the levels of ATP/ADP *versus* adenosine by altering the balance of expression and whether P2 or P1 receptors become activated. Similar enzymes are also involved in the intracellular hydrolysis of ATP to adenosine, a notable difference however is that CD73 is replaced by structurally unrelated cytosolic-5'-nucleotidase (Sträter, 2006).

The important enzymes that catabolise adenosine are, as mentioned above, ADA and AK; ADA is a high capacity and high  $K_m$  (70  $\mu\text{M}$ ) enzyme that acts mainly on extracellular adenosine whereas AK is a low capacity and low  $K_m$  (40 nM) enzyme and acts mainly intracellularly (Lloyd and Fredholm, 1995; Sychala, 2000; Sychala et al., 1996). AK is fully active during resting conditions but is inhibited when adenosine concentrations rise (Tabrizchi and Bedi, 2001). Under steady state conditions endogenous adenosine

concentrations are in the nanomolar range and the dominant pathway for adenosine metabolism is phosphorylation back to AMP. The activity of ADA becomes more relevant at higher adenosine concentrations, such as that seen under ischaemic conditions (Lloyd and Fredholm, 1995).

#### **1.4.2.2 Nucleoside transporters**

Adenosine generated intracellularly is transported across cell membranes via bi-directional nucleoside transporters, which are important in regulation of extracellular adenosine concentrations (Noji et al., 2004). These transport proteins have been cloned and characterised according to their functional characteristics and molecular structure. Two main categories have been identified: equilibrative nucleoside transporters (ENTs; solute carrier (SLC) 29A1-29A2) that balances the intra- and extracellular levels of adenosine through facilitated diffusion and concentrative nucleoside transporters (CNTs; SLC 28A1–28A3) that mediate the influx of nucleosides into the cell under the force of a transmembrane sodium gradient (Baldwin et al., 1999; Cabrita et al., 2002; Mangravite et al., 2003). The role of these transporters in regulation of adenosine concentration has been studied utilising various adenosine transport blockers (for example dipyridamole), which decrease and increase extracellular adenosine concentrations (Latini and Pedata, 2001).

#### **1.4.3 Adenosine receptor classification**

The original classification of adenosine receptors was based on the order of potency of ligands, differing responses to ligands, and antagonism by methylxanthines. Adenosine receptors were first divided into A<sub>1</sub> and A<sub>2</sub>

receptors in studies showing that activation of these receptors by adenosine and other adenosine receptor antagonists inhibited or stimulated adenylate cyclase activity in cultured mouse brain cells. The order of potency of adenosine analogues was different for the two receptors and the effects were antagonised by methylxanthines (van Calker et al., 1979).  $A_2$  receptors were later subdivided into types  $A_{2A}$  and  $A_{2B}$ . The proposal that  $A_2$  receptors could be divided into two classes was based on the discovery of high-affinity  $A_2$  receptors in rat striatum and low-affinity  $A_2$  receptors throughout the brain, both of which were able to activate adenylate cyclase (Daly et al., 1983). This classification was confirmed by ligand-binding assays and differential affinity towards adenosine derivatives, and later by molecular biological evidence (Fredholm et al., 2001). The  $A_3$  adenosine receptor is the most recent subtype identified, as it had remained pharmacologically obscure until Meyerhof et al cloned its gene in 1991 (Zhou et al., 1992). Molecular cloning and expression studies have provided clear evidence for the existence of all four adenosine receptors.

#### **1.4.4 Structure of adenosine receptors**

##### **1.4.4.1 Gene structure**

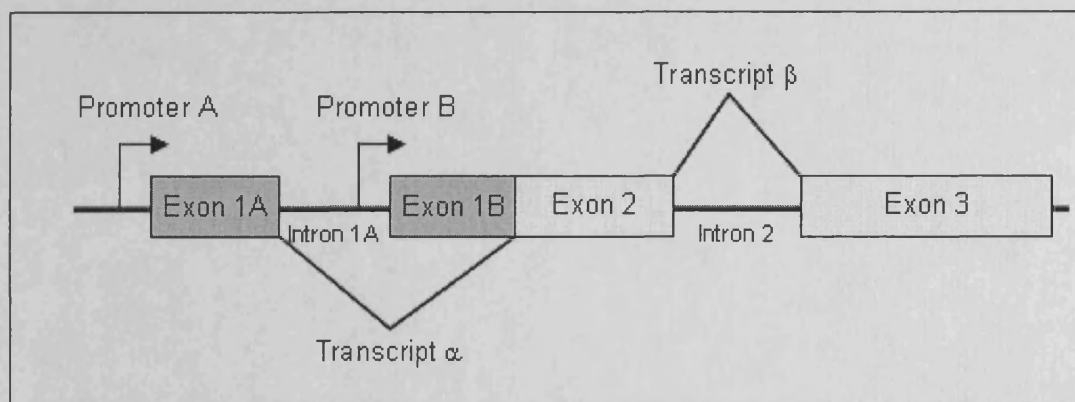
The four adenosine receptors have been cloned and their gene sequences reveal that they all belong to the superfamily of GPCRs (Fredholm et al., 2001). The  $A_1$  receptor gene has been cloned in man and animals (rat, mouse, dog, cow, rabbit, guinea pig, and chick) and show high sequence homology amongst the different species (Yaar, 2005). Although differences in the gene sequence between mammalian species for the  $A_1$  receptor is 10% at

most, this is adequate to cause differences in ligand binding and desensitisation. For instance, the affinity of the non-selective agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) for A<sub>1</sub> is shown to be 9-fold higher in rat compared to human cortical membranes and inhibition of adenylate cyclase by the selective agonist N6-cyclopentyladenosine (CPA) is significantly greater in guinea pig atria and ventricles when compared with rat (Kapicka et al., 2003; Maemoto et al., 1997).

The human A<sub>1</sub> receptor gene has been mapped to chromosome 1q32.1. Similar to other adenosine receptors, the structure of the A<sub>1</sub> receptor contains two exons interrupted by a single intron which is in the coding region corresponding to the second intracellular protein loop (Fredholm et al., 2000; Olah and Stiles, 2000). Genes of all adenosine receptors include part of the coding sequence and the entire 3'-untranslated region as the last exon.

There are, however, variations in the 5'-untranslated sequence of adenosine receptors. The A<sub>1</sub> receptor contains two exons interrupted by an intron at this region. This creates two types of transcripts for the human A<sub>1</sub> receptor containing either exons 1B, 2, and 3 (transcript  $\alpha$ ) or 1A, 2, and 3 (transcript  $\beta$ ) (figure 1.3). Two separate promoters, which control the relative abundance of these transcripts, have been reported as promoter A and promoter B. Promoter B and exon 1B are part of intron 1A when promoter A is active (Ren and Stiles, 1994; Ren and Stiles, 1995).





**Figure 1.3 Schematic diagram illustrating the structure of the  $A_1$  receptor gene.** Adapted from Fredholm (2000).

The  $A_{2A}$  receptor has been cloned from a number of species including human, rat, mouse, dog and guinea pig with about 90% sequence homology. The human  $A_{2A}$  receptor gene has been mapped to chromosome 22q11.23 (Moreau and Huber, 1999). Two alternative transcripts exist for the  $A_{2A}$  receptor gene in rat, which are controlled by two independent promoters (Chu et al., 1996)

Adenosine  $A_{2B}$  receptors have been cloned from human, mouse and rat and localised to either chromosome 11 or chromosome 17 (for the human gene). The human  $A_{2B}$  receptor shows 86 to 87% homology with the rat and mouse sequences (Feoktistov and Biaggioni, 1997). A human  $A_{2B}$  pseudogene with 79% homology has also been identified and localised to chromosome 1q32. The pseudogene contains multiple deletions, point mutations, and frame shifts and is therefore unable to code for a functional receptor (Jacobson et al., 1995).

Initially isolated as an orphan receptor, the  $A_3$  receptor has now been cloned in human, rat, dog, rabbit and sheep and localised to human chromosome

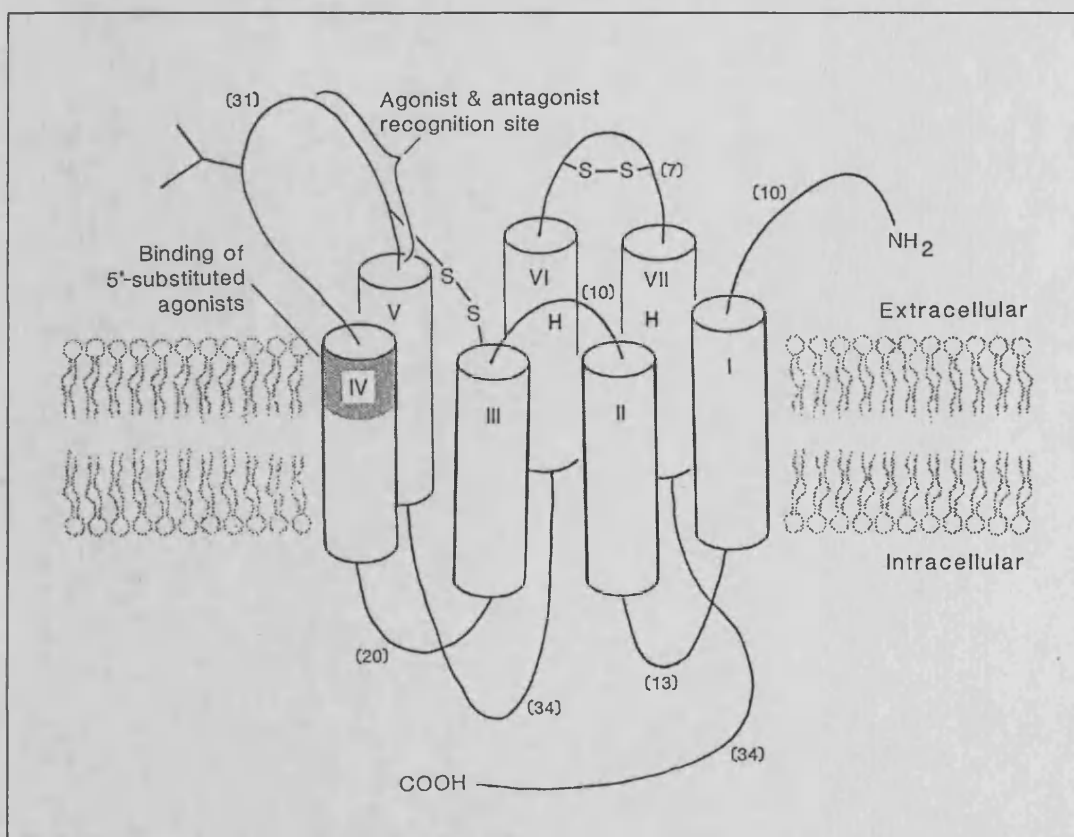
1p21-p13 (Meyerhof et al., 1991). This receptor has the highest interspecies sequence variation with a 30% difference identified in the human and rabbit genes; this reflects the observed differences in potencies of agonist and antagonist compounds such as methylxanthines (Fredholm et al., 2001). The human A<sub>3</sub> receptor has two transcripts of 2 and 5 kilobases, with the latter being less abundant (Atkinson et al., 1997).

#### 1.4.4.2 Protein structure

In regard to protein structure, adenosine receptors are typical of GPCRs, with seven  $\alpha$ -helical transmembrane spanning domains connected by three extracellular and three intracellular loops with the amino-terminus located in the extracellular space and the C-terminus on the cytoplasmic side (figure 1.4). Each of the seven transmembrane domains is composed of approximately 21 - 28 hydrophobic amino acids. The extracellular loops, in particular loop 2 and the transmembrane domains are important for ligand binding (Olah et al., 1994; Rivkees et al., 1995). The histidine residues (His250 and His278) in transmembrane 6 and transmembrane 7 are, for example, particularly important for ligand binding to the A<sub>2A</sub> receptor (Moreau and Huber, 1999). The 2nd extracellular loop contains N-linked glycosylation sites (Moreau and Huber, 1999); glycosylation does not appear to affect ligand binding but may be involved in the inhibition of receptor dimerisation and protection from proteolytic digestion (Linden, 2001). The 3rd intracellular loop and the C-terminus appear to be important for G-protein coupling (Olah, 1997; Palmer and Stiles, 1995; Tucker et al., 2000). In the absence of

glycosylation, adenosine receptors such as the  $A_{2A}$  and  $A_3$  subtypes are susceptible to phosphorylation and desensitisation (Palmer and Stiles, 1997).

The molecular mass of  $A_1$ ,  $A_{2B}$ , and  $A_3$  receptors are very similar: 36.7, 36.4 and 36.6 kilodalton (kDa) respectively, whereas  $A_{2A}$  receptors, as a result of a longer C-terminus have a larger predicted size (45 kDa) (Palmer and Stiles, 1995). There is considerable amino acid sequence homology between the same adenosine receptors in different species but sequences between different adenosine receptors are quite different. For example the  $A_1$  and  $A_3$  receptors and the  $A_{2A}$  and  $A_{2B}$  receptors display 49% and 45% sequence homology respectively (Olah and Stiles, 2000; Stefano Moro, 2006).



**Figure 1.4 Schematic diagram illustrating the structure of  $A_1$  receptor.** From Ralevic and Burnstock (1998).

### 1.4.5 Adenosine receptor signalling pathways

Adenosine receptors are either associated with a stimulatory alpha subunit ( $G_{\alpha s}$ ) or an inhibitory alpha subunit ( $G_{\alpha i/o}$ ) (Palmer and Stiles, 1995). Classically, this leads to the stimulation or inhibition of adenylate cyclase and changes in cAMP production. Adenylate cyclase can exist as nine different isotypes (I-IX) and comprise a family of transmembrane proteins, that catalyse the cyclisation of AMP (Fimia and Sassone-Corsi, 2001). Adenosine receptors are also now known to activate other pathways including the phospholipase C (PLC), protein kinase C (PKC) pathway and mitogen-activated protein kinases (MAPKs) (Gao et al., 1999; Rogel et al., 2005; Schulte and Fredholm, 2003b). Adenosine receptors have also shown to regulate  $K^+$ ,  $K_{ATP}$ , N, P and Q type  $Ca^{2+}$  channels (Fredholm et al., 2001; Ralevic and Burnstock, 1998) (figure 1.5).

The  $A_1$  receptor is coupled to pertussis toxin-sensitive  $Gi_{1,2,3}$  and  $G_o$  proteins and the inhibition of adenylate cyclase (Londos et al., 1980; van Calker et al., 1979). Activation of  $A_1$  receptors via PLC leads to the production of inositol 1, 4, 5-triphosphate ( $IP_3$ ), diacylglycerol (DAG) and  $Ca^{2+}$  mobilisation and activation of  $K^+$  channels (Rogel et al., 2005) and  $K_{ATP}$  channels whilst inhibiting Q, P and N type  $Ca^{2+}$  channels (Fredholm et al., 2001). These have been shown in cardiac muscle, neurons, ventricular myocytes and coronary arteries (Yaar, 2005).

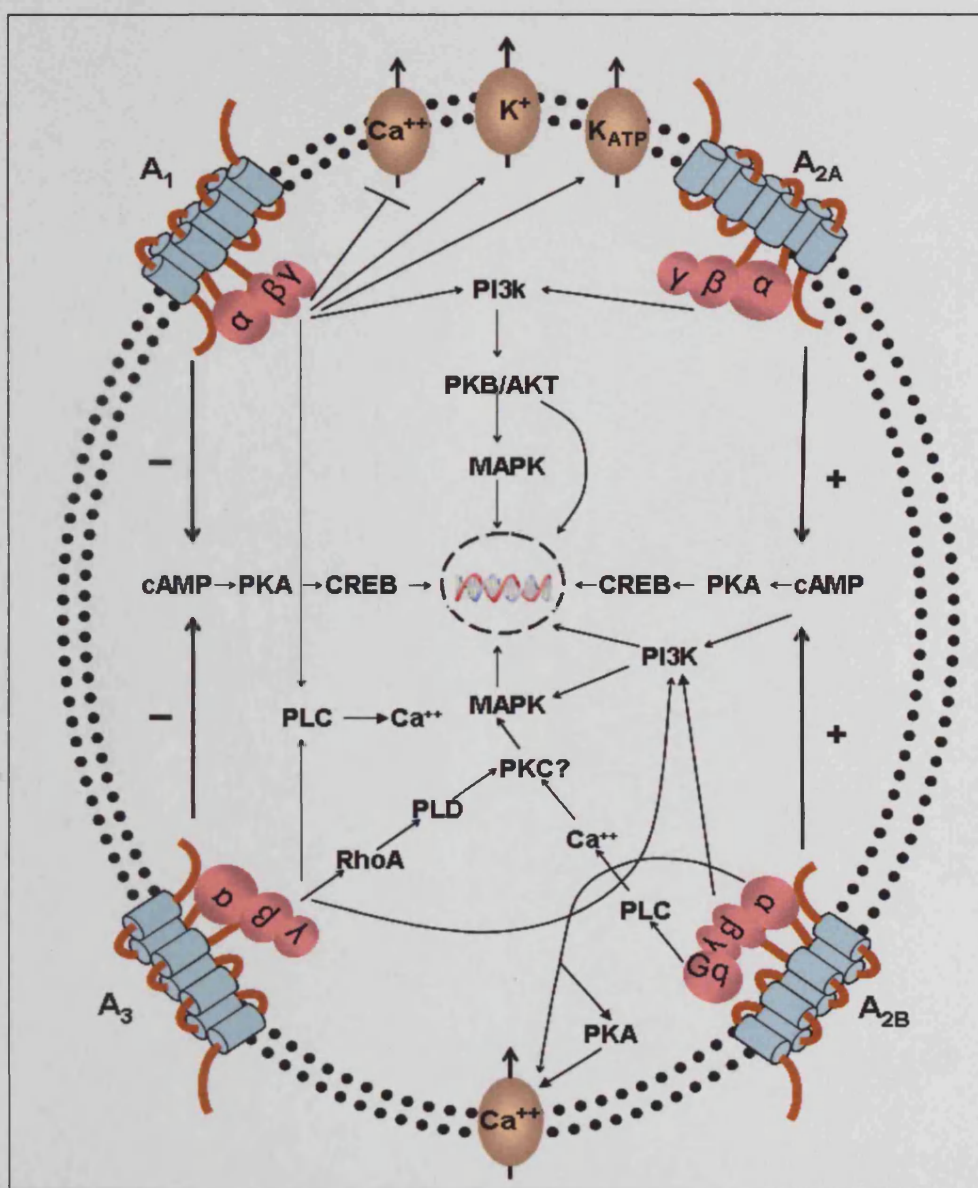
The  $A_{2A}$  receptor is positively coupled to adenylate cyclase via activation of cholera toxin-sensitive Gs-proteins (Palmer and Stiles, 1995). Gs-coupling

seems to be the main route in  $A_{2A}$  signalling in the periphery but not in striatum, where  $A_{2A}$  receptor concentrations are highest. The striatal  $A_{2A}$  receptors act largely through activation of G-olfactory (Golf) which is abundant in the striatum, and is similar to  $G_s$  in its ability to activate adenylate cyclase (Kull et al., 2000). In COS-7 cells,  $A_{2A}$  receptors appears to mediate activation of  $G\alpha_{15}$  and  $G\alpha_{16}$  proteins and the induction of inositol phosphates to raise intracellular calcium and activate PKC (Offermanns and Simon, 1995).

The  $A_{2B}$  receptor, like the  $A_{2A}$  receptor, is also coupled to  $G_s$  proteins and stimulates adenylate cyclase. Some of the actions of the  $A_{2B}$  receptor are mediated through the activation of PLC and  $Gq/11$  proteins (Feoktistov and Biaggioni, 1997; Linden et al., 1999) and the stimulation of inositol phosphate formation has been observed in the human mast cell line, HMC-1. Activation of  $A_{2B}$  receptors also appears to increase intracellular calcium, either from intracellular stores by PLC coupling or from the extracellular space by promoting calcium influx directly and activation of PKA (Feoktistov and Biaggioni, 1997; Gao et al., 1999). This effect can be antagonised by the  $A_{2B}$  receptor selective antagonist, enprofylline (Feoktistov and Biaggioni, 1997).

Similar to the  $A_1$  receptor, the main transduction system that is associated with the  $A_3$  receptor is inhibition of adenylate cyclase via activation of pertussis toxin-sensitive  $Gi_{2,3}$ -proteins (Palmer and Stiles, 1995). Activation of the  $A_3$  receptor has also been shown to stimulate phospholipases C and D via activation of  $Gq/11$  (Abbracchio et al., 1995). In HL-60 promyeloid leukaemia cells  $A_3$  receptor activation results in the influx of calcium and its

release from intracellular space. The  $A_3$  receptor mediates cardiac protection through activation of the  $K_{ATP}$  channel and RhoA-phospholipase D1 signalling and suppression of melanoma cell function via the Wnt signalling pathway (Tracey et al., 1998). In common with other adenosine receptors, the  $A_3$  receptor also couples to MAPK, which is involved in cell growth, survival, death and differentiation (Mozzicato et al., 2003; Schulte and Fredholm, 2002; Schulte and Fredholm, 2003b).



**Figure 1.5 Possible signalling pathways via  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  receptor stimulation.** Adapted from Feoktistov and Biaggioni (1998); Jacobson and Gao (2006); Schulte and Fredholm (2003b).

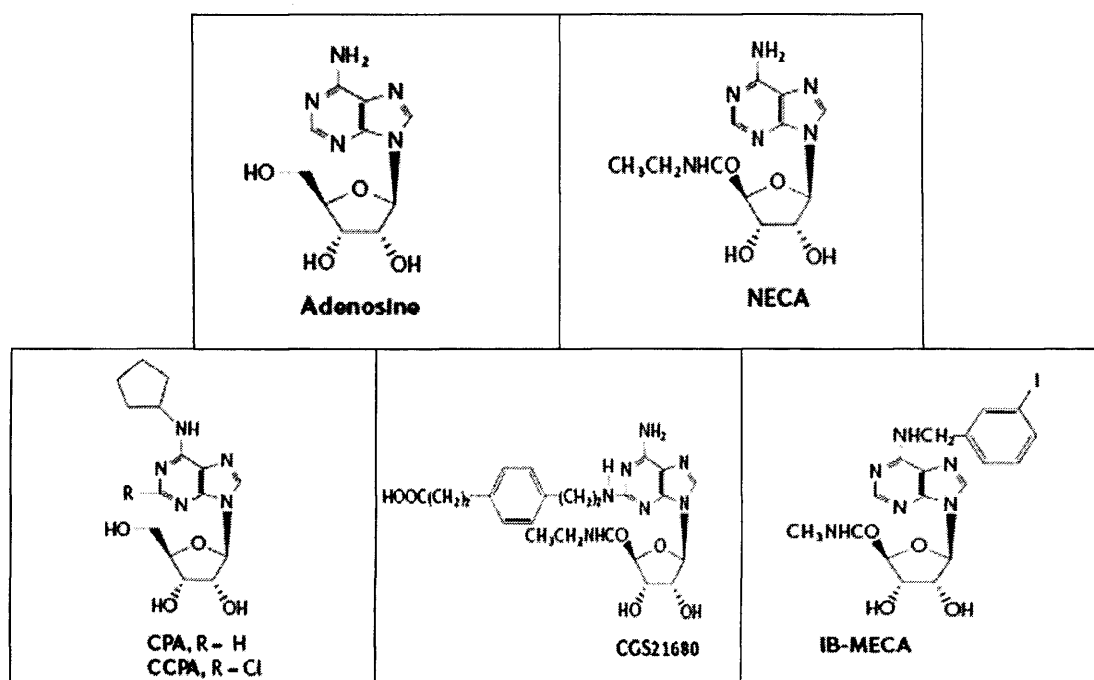
### 1.4.6 Adenosine receptor agonists

Adenosine has a limited use for the investigation of adenosine receptor function due to its short half life. A large number of adenosine receptor agonists with greater stability than adenosine have been produced, mainly by modification of the adenosine molecule itself (figure 1.6). Several positions in adenosine molecular structure have been modified to increase affinity to specific receptor subtypes without disturbing the agonistic activity: the N and C2 positions of the adenine ring and the 3-, 4- or 5- position of the ribose moiety of adenosine (Jacobson and Gao, 2006). Table 1.1 shows some of common adenosine receptor agonist and their  $K_i$  value.

Modification of adenosine at both N<sup>6</sup> and C2-positions led to the development of 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA) as a potent and selective A<sub>1</sub> receptor ligand (Klotz et al., 1997). Substitution with small alkyl amide groups at the 5-position of adenosine gave rise to a non-selective agonist NECA. Further modification of NECA at the 2-position led to the development of CGS 21680 (2-[*p*-(2-carboxyethyl) phenethylamino]adenosine-5'-N-ethyluronamide) a selective agonist for A<sub>2A</sub> receptor (Hutchison et al., 1989; Klotz, 2000).

Although agonists with an apparent high affinity and selectivity for A<sub>2B</sub> receptors have been recently developed *i.e.* LUF5835 (EC<sub>50</sub> = 10 nM) and BAY 60-6583 (Beukers et al., 2004; Chen et al., 2009), these compounds are not well characterised or commercially available. Action at A<sub>2B</sub> receptors is often investigated by comparing responses to NECA and CGS 21680; a strong response to NECA and a weak response to CGS 21680 are indicative

of  $A_{2B}$  receptor activation whereas a dominant response to CGS 21680 indicates  $A_{2A}$  receptor activity. NECA itself strongly binds the  $A_1$ ,  $A_{2A}$  and  $A_3$  receptors ( $K_i$  approx 10 nM) but only weakly ( $K_i$  2.4  $\mu$ M) to the  $A_{2B}$  receptor (Feoktistov and Biaggioni, 1998). Stimulation of adenylate cyclase activity has been used to demonstrate activation of both  $A_{2A}$  and  $A_{2B}$  receptors; an agonist profile  $NECA > N^6$ -3-Iodobenzyladenosine-5'-*N*-methyluronamide (IB-MECA)  $>$  CGS 21680 is indicative of  $A_{2B}$  receptors (Feoktistov and Biaggioni, 1997). Agonists that are selective for  $A_3$  receptors are compounds that contain an  $N^6$ -benzyl substitution as well as a 5'-modification (Muller, 2000). IB-MECA (or its chlorinated derivative) is one of these agonists that is widely used for studying the  $A_3$  receptor (Adachi et al., 2007).



**Figure 1.6 Structure of adenosine receptor agonists.** From Jacobson and Gao (2006)

#### 1.4.7 Adenosine receptor antagonists

The structural core for the development of adenosine receptor antagonists has been modification of naturally occurring xanthines, theophylline and



caffeine (Moro et al., 2006). As is the case for agonists, potent and selective xanthine antagonists stem from multiple substitutions of the parent heterocycle. Table 1.1 shows some of the common adenosine receptor antagonists and their  $K_i$  value.

The main sites of the xanthine structure that allows for the variation of the pharmacological profile is the C8 residue (Klotz, 2000). Among  $A_1$  receptor antagonists, 8-cycloalkyl xanthine derivatives are well known potent ligands. One such derivative is DPCPX (1,3-dipropyl-8 cyclopentylxanthine), with a 1000 fold higher potency at the rat  $A_1$  receptor ( $K_i$ -value 0.3 nM) compared to the rat  $A_{2A}$  receptor (Redman and Silinsky, 1993). Several  $A_{2A}$  receptor xanthine antagonists (8-styrylxanthines) have been synthesised, However they suffer from sensitivity to photoisomerisation (Moro et al., 2006; Yuzlenko and Kiec-Kononowicz, 2006) Non-xanthine antagonists such SCH 58261 and SCH 442416 with a high affinity ( $K_i$  0.5 and 0.048 nM respectively) and selectivity for  $A_{2A}$  receptors have therefore been developed and found useful in discriminating  $A_{2B}$  and  $A_{2A}$  receptor function (Feoktistov and Biaggioni, 1998; Fredholm et al., 2003). Xanthines that have been developed as selective antagonists of the  $A_{2B}$  receptor bear a C8-substitution combined with N1- and N3- (and sometimes N7) substitutions. For example 8-phenyl xanthine substitution such as the *p*-cyanoanilide derivative MRS 1754 (*N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide, with a  $K_i$ -value of 2 nM, is found to be 204-, 255-, and 289 fold more selective when compared with human  $A_1$ ,  $A_{2A}$  and  $A_3$  receptors respectively (Giovanni Baraldi et al., 2006). Other  $A_{2B}$  receptor highly selective

antagonists, include MRS 1706, [3H] OSIP3991 and MRE2029-F20 (Giovanni Baraldi et al., 2006; Jacobson and Gao, 2006). In contrast to  $A_1$  and  $A_2$  receptors the  $A_3$  receptor is insensitive to xanthine molecules (Muller, 2001) with binding affinities of around 100  $\mu$ M. Therefore the majority of  $A_3$  antagonists have a non-xanthine structure; MRE 3008F20 is the most selective antagonist (KD 0.8 nM) for the human  $A_3$  receptor (Jacobson and Gao, 2006; Muller, 2001).

Compound	$A_1$	$A_{2A}$	$A_{2B}$	$A_3$
<b>Agonists</b>				
Adenosine	3-30	1-20	5000-20000	>1000
NECA	14	20	2400	6.2
CCPA	0.8	2300	18800	42
CGS 21680	289	27	888000	67
IB-MECA	3.7	2500	11000	1.2
<b>Antagonists</b>				
PSB 36	0.12	552	187	2300
SCH 442416	1110	0.048	>10000	>10000
MRS 1706	157	112	1.39	230
MRS 1754	403	503	2	570

**Table 1.1 Binding affinity for selective agonists and antagonists for adenosine receptors ( $K_i$  values in nM).** Adapted from Jacobson and Gao (2006).

#### 1.4.8 Adenosine receptor distribution and function

All four subtypes are widely distributed throughout the body and their spectra of physiological and pathological functions have been described in many tissues and organs, such as in the cardiovascular system, the central nervous system (CNS), immune system and the respiratory system. Adenosine  $A_1$  receptors are ubiquitously expressed within the CNS with particularly high expression in the cerebral cortex, hippocampus, cerebellum, thalamus, brain

stem, and spinal cord. In this setting, A<sub>1</sub> receptors have a neuroprotective role by blocking neurotransmitter release (Ribeiro et al., 2002). Additionally, A<sub>1</sub> receptors have been reported to be potentially useful in the treatment of dementia and anxiety disorders, and mice lacking the receptor show increased anxiety-related behaviour and a loss of the analgesic effects of adenosine (Johansson et al., 2001). In support of these findings, A<sub>1</sub> receptor agonists were shown to improve memory defects and reduced anxiety in rats (Maemoto et al., 2004).

The A<sub>1</sub> receptors are also highly expressed in the cardiovascular system, where they have been shown to reduce heart rate and atrial contractility, and the attenuation of stimulatory actions of catecholamines (Fraser et al., 2003; Zablocki et al., 2004). In patients with paroxysmal supraventricular tachycardia (PSVT), normal heart rhythm is restored by intravenous infusion of adenosine and activation of A<sub>1</sub> receptors (Jacobson and Gao, 2006). Overexpression or activation of A<sub>1</sub> receptors produced a protective response in the cardiac tissues of mice subjected to ischemia/reperfusion, whilst mice lacking the receptor showed reduced heart recovery and systolic blood pressure after an ischemic event (Regan et al., 2003; Reichelt et al., 2005). A<sub>1</sub> receptors are also abundant in testis, white adipose tissue, spleen, pituitary, liver, eye, and kidney (Fredholm et al., 2001).

Like the A<sub>1</sub> receptor, the A<sub>2A</sub> receptor also plays important roles in CNS and the cardiovascular system. Studies of mice lacking the A<sub>2A</sub> receptor showed several disturbances in CNS activity including, decreased exploratory activity,

increased aggressiveness and hypoalgesia, attenuated psychostimulant responses and reduced alcohol sensitivity (Chen et al., 2003; Ledent et al., 1997; Naassila et al., 2002). Antagonising the  $A_{2A}$  receptor is potentially a treatment for Parkinson's (Xu et al., 2005). In the cardiovascular system, activation of  $A_{2A}$  is involved in vasodilation in the aorta and coronary artery (Fredholm et al., 2001).  $A_{2A}$  receptors also have anti-inflammatory effects and their activation in immune cells has been shown to inhibit T-cell activation, proliferation and production of inflammatory cytokines whilst enhancing the production of anti-inflammatory cytokines.  $A_{2A}$  receptor knockout animals are more susceptible to inflammation and the production of proinflammatory cytokines (Hasko and Pacher, 2008; Sitkovsky et al., 2004).

$A_{2B}$  receptors are abundantly expressed in the cecum, colon, bladder, kidney, lung, and pituitary (Fredholm et al., 2001). The physiological role of the  $A_{2B}$  receptor is less studied in comparison to other adenosine receptors, probably due to the lack of potent selective agonists and until recently, the  $A_{2B}$  knockout mice had not been created (Jacobson and Gao, 2006). Nonetheless, the  $A_{2B}$  receptor is shown to be involved in the control of cardiovascular tone, cardiac myocyte contractility, regulation of inflammation, modulation of neurosecretion and neurotransmission, regulation of intestinal tone and secretion and regulation of mast cell function (Dubey et al., 2002; Dubey et al., 2001; Eckle et al., 2007; Feoktistov and Biaggioni, 1997; Giovanni Baraldi et al., 2006; Hasko et al., 2009; Hua et al., 2007).

The A<sub>3</sub> receptor is highly expressed in lung and liver and has also been detected in the testis, lung, kidney, placenta, heart, brain, spleen, liver, uterus, bladder, aorta, proximal colon and eye (Fredholm et al., 2001). Expression of the A<sub>3</sub> receptor appears to mainly involve pathological conditions such as ischemia, inflammation and cancer. The receptor also seems to have cardioprotective, proinflammatory, anti-inflammatory, neuroprotective and neurodegenerative effects in different pathophysiological situations (Gessi et al., 2008). The A<sub>3</sub> receptor is also highly expressed in tumour cells such as melanoma, lymphoma, pineal gland, colon, and prostate carcinomas (Gessi et al., 2004; Madi et al., 2003; Madi et al., 2004). Within cancer cells A<sub>3</sub> receptors appear to have anti-tumour effects such as inducing apoptosis and inhibiting tumour cell growth both *in vitro* and *in vivo* (Fishman et al., 2002a; Fishman et al., 2002b).

#### **1.4.9 Adenosine receptor signalling pathways in adipocytes and adipogenesis**

Adenosine is known for many years to be involved in the regulation of several aspects of adipose tissue functions including lipolysis and lipogenesis. The first evidence was reported about 50 years ago by Dole showing that adenosine decreased the lipolytic effect of adrenaline in rat adipose tissue (Dole, 1961). Later, adenosine was shown to be released by adipocytes *in vitro* and *in vivo* and this was sufficient to inhibit cAMP accumulation and lipolysis (Lonroth et al., 1989; Schwabe et al., 1973; Schwabe et al., 1974). Adenosine has been shown to be involved in both lipolysis (break down of fat) and lipogenesis (formation of fat) in various mammalian cell culture systems

including human, rat, mouse and dog (Johansson et al., 2008; Lonroth et al., 1989; Sollevi and Fredholm, 1981; Szkudelski et al., 2008; Vannucci et al., 1989).

The pharmacological evidence and molecular studies suggest that the action of adenosine on adipocytes is mainly through activation of  $A_1$  receptors (Fatholahi et al., 2006; Johansson et al., 2008; Schoelch et al., 2004; Vannucci et al., 1989). Many  $A_1$  adenosine receptor agonists have been shown to induce antilipolytic effects (Dhalla et al., 2007a; Dhalla et al., 2003; Fatholahi et al., 2006). Furthermore, overexpression of  $A_1$  receptors and subsequent inhibition of lipolysis in adipocytes have been suggested as a potential cause of a lower and slower rate of weight loss in obese African-American women (Barakat et al., 2006). The related observation of reduced adenosine sensitivity and decreased adenosine receptor number in adipocytes isolated from obese men also highlight the importance of adenosine in the activities of adipocytes (Kaartinen et al., 1991).

Adenosine, acting through the  $A_1$  receptor, regulates glucose and lipid metabolism and enhances the antilipolytic action of insulin (Schwabe et al., 1974). Overexpression of the adenosine  $A_1$  receptor in adipose tissue is shown to be associated with reduced insulin resistance in mice and rats on a high fat diet (Dhalla et al., 2007a; Dong et al., 2001) by lowering free fatty acids and triglyceride concentrations (Dhalla et al., 2007b). Adenosine appears to regulate glucose transport and facilitate insulin regulation of glucose metabolism (Schwabe et al., 1974; Smith et al., 1984), whereas

removal of adenosine or antagonism of adenosine receptors is found to reduce insulin-stimulated glucose transport (Steinfelder and Pethö-Schramm, 1990). Adenosine also has similar stimulatory action on leptin secretion. Activation of the A<sub>1</sub> receptor has been shown to increase circulating leptin levels, whilst, targeted disruption of adenosine action was associated with a reduction in secretion of leptin (Cheng et al., 2000; Rice et al., 2000).

As described above, there have been several studies on the role of adenosine receptors on adipocyte function; there is however little information on how expression of these receptors may change during adipogenic differentiation. There is some evidence to support this hypothesis; Vassuex and colleagues (1993) reported the presence of A<sub>2</sub> receptors in preadipocytes and the A<sub>1</sub> receptor in mature adipocytes in the stromal-vascular fraction of the epididymal fat pad. Similar findings were reported in human primary preadipocytes and in a mouse preadipocyte cell line, Ob1771 (Børglum et al 1996). A<sub>2</sub> receptors were markedly reduced during adipocyte differentiation of Ob1771 cells and the A<sub>1</sub> receptor was only present in fully differentiated cells. Induced expression of the A<sub>1</sub> receptors also appeared to induce adipogenesis (Tatsis-Kotsidis and Erlanger, 1999). In addition, universal adenosine receptor agonists were shown to enhance corticosterone-induced adipocyte differentiation by 47% (Børglum et al., 1996). Together these data suggest a role for adenosine and A<sub>1</sub> receptors in adipocyte differentiation and function.

#### **1.4.10 Adenosine signalling pathways in osteoblasts and osteoblastogenesis**

There is little information on the expression and function of adenosine receptors in osteoblasts or during their differentiation from precursor stem cells. Recent studies in our department described the expression of adenosine receptors, in particular the  $A_2$  subtypes in human primary osteoprogenitor cells and the immortalised human cell line HCC-1 (Evans et al., 2006). Subsequently adenosine receptor expression was demonstrated in mouse MSCs (Katebi et al., 2009) and in the human osteoblastic cell line MG-63 (Russell et al., 2007). These cells also have the capacity to produce adenosine as they express high levels of CD73 (Evans et al., 2006; Katebi et al., 2009). In these studies, adenosine acting via  $A_{2B}$  receptors stimulated interleukin-6 (IL-6) secretion and inhibited osteoprotegerin but not the receptor activator of nuclear factor  $\kappa$ B ligand (RANK-L) expression (Evans et al., 2006). Activation of  $A_{2A}$  receptors in osteoblasts was shown to inhibit LPS-induced IL-6 release (Russell et al., 2007). These data suggest that adenosine receptors may play a role in the control of inflammation as well as in osteoclastogenesis and bone resorption.

In patients with rheumatoid arthritis (RA), adenosine has been shown to reduce the levels of proinflammatory cytokines such as tumor necrosis factor alpha ( $TNF_{\alpha}$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) (Forrest et al., 2005). Additionally, methotrexate (MTX) which is one of the most widely used drugs for the treatment of RA is thought to exert its effects by increasing the level of adenosine and activation of  $A_{2A}$  and  $A_3$  receptors (Cronstein, 2005). Studies in



$A_{2A}$  and  $A_3$  receptor knockout mice support this finding, as MTX treatment reduces leukocyte and  $TNF\alpha$  accumulation in wild-type mice, but not in receptor null mice (Montesinos et al., 2003). Furthermore, adenosine receptor antagonists reversed the anti-rheumatic effects of MTX in adjuvant-induced arthritis in rats (Montesinos et al., 2000) and  $A_3$  receptor agonism has been shown to prevent bone resorption in adjuvant-induced arthritis (Rath-Wolfson et al., 2006).

$A_{2A}$  receptors have also been reported to play a role in promoting proliferation, development and protein expression in mouse bone marrow MSCs. Disruption of  $A_{2A}$  receptor signalling using antagonists or in null animals was associated with a reduction in CFU-Fs and in procollagen type I expression (Katebi et al., 2009). Adenosine was also shown to be mitogenic for MC3T3 osteoblasts (Shimegi, 1998) and activation of  $A_1$  and  $A_{2A}$  receptors have been shown to prevent oxidative stress induced cell death in MC3T3 (Fatokun et al., 2006). In contrast to this, other researchers have reported a lack effect of adenosine on osteoblast function, *i.e.* mineralisation and production of intracellular  $Ca^{2+}$  (Hoebertz et al., 2002; Orriss et al., 2006). ATP, on the other hand, strongly inhibits mineralised bone nodule formation and stimulates  $Ca^{2+}$  production.

To date, data suggest that adenosine receptors have a role in adipocyte and osteoblast function but there is little information on how these receptors are modulated during adipocyte and osteoblast lineage specific differentiation. There is also little information on whether adenosine can induce the process of adipogenesis or osteoblastogenesis.

### **1.5 Aims of the study**

The preferential differentiation of MSCs into adipocytes rather than osteoblasts is thought to contribute to bone loss and diseases such as osteoporosis. Currently PTH (Blick et al., 2008; Cranney et al., 2006) is the only compound in clinical use, that induces bone formation. The understanding of the mechanisms involved in adipocyte and osteoblast differentiation could be considered as targets for therapeutic intervention. Our previous findings and those of others suggest that adenosine is likely to have a role in adipocyte and osteoblast function and differentiation.

I have, thus, explored how adenosine receptors are modulated as MSCs are induced to differentiate into osteoblasts and adipocytes and how these processes may be influenced by adenosine. I have also investigated whether adenosine is involved in the transdifferentiation of osteoblasts to adipocytes. Specific aims and objectives are described in the appropriate chapters.

Specifically the aims were:

- To investigate the expression and function of adenosine receptors in MSCs and during their differentiation to osteoblasts and adipocytes.
- To investigate the role of adenosine signalling pathways in the regulation of osteoblastic and adipogenic differentiation of MSCs.

- To investigate the expression and function of adenosine receptors in 7F2 and HOB cells and as they are induced to transdifferentiate to adipocytes.
- To investigate the role of exogenous adenosine in the regulation of adipocyte differentiation of 7F2 and HOB cells.

## **Chapter 2**

### **Materials and methods**

## 2.1 Materials

### 2.1.1 Reagents

General laboratory chemicals and reagents were obtained from Sigma Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK) unless otherwise stated. All cell signalling reagents were obtained from TOCRIS Biosciences (Bristol, UK) (see table 2.1 for details).

Compound	Dissolved in	Description
Adenosine	PBS	Adenosine receptors natural ligand
NECA	DMSO	Adenosine receptors universal agonist
CCPA	DMSO	A <sub>1</sub> receptor agonist
CGS 21680	DMSO	A <sub>2A</sub> receptor agonist
IB-MECA	DMSO	A <sub>3</sub> receptor agonist
PSB 36	DMSO	A <sub>1</sub> receptor antagonist
SCH 442416	DMSO	A <sub>2A</sub> receptor antagonist
MRS 1754	DMSO	A <sub>2B</sub> receptor antagonist
MRS 1706	DMSO	A <sub>2B</sub> receptor antagonist
Forskolin	DMSO	Adenylate cyclase activator
PMA	DMSO	PKC activator
RO 20-1724	DMSO	PDE inhibitor
RO-32-0432	DMSO	PKC inhibitor
U-73122	DMSO	PLC inhibitor
PD 98059	DMSO	MAPK inhibitor
Wortmannin	DMSO	PI3K inhibitor
2',5'-dideoxyadenosine (DDA)	DMSO	Adenylate cyclase inhibitor

**Table 2.1 Details of cell signalling reagents.**

### 2.1.2 Cell culture consumables and reagents

Tissue culture plastic dishes and flasks were all purchased from Fisher Scientific (Loughborough, UK). Cell culture reagents and materials were purchased from Invitrogen (Paisley, Scotland), Sigma Aldrich (Poole, UK) and Lonza (Berkshire, UK) unless otherwise stated.

## **2.2 Cell culture**

### **2.2.1 Culture medium**

Cells were cultured in minimal essential medium alpha ( $\alpha$ -MEM) (without ribonucleosides or deoxyribonucleosides). A stock solution of  $\alpha$ -MEM was prepared by dissolving 10.08 g of  $\alpha$ -MEM and 2.2 g sodium hydrogen carbonate ( $\text{NaHCO}_3$ ) in one litre distilled water ( $\text{dH}_2\text{O}$ ). The medium was sterilised by passing through a 0.2  $\mu\text{m}$  bottle top rac filter (VacuCap 90) and 500 ml aliquots were transferred into sterile glass bottles. The medium was stored at 4°C and L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50  $\mu\text{g}/\text{ml}$ ) and 10% fetal calf serum (FCS) were added prior to use. This medium will be referred in this thesis as normal growth medium. FCS was first tested for its ability to support cell growth and the same batch was used throughout the project.

### **2.2.2 Isolation and expansion of MSCs**

8 - 10 week old male Wistar rats were sacrificed by carbon dioxide inhalation according to UK Home Office regulation. The femurs and tibiae were dissected away from attached muscle, cleaned of all connective tissue and the proximal end of the femur and distal end of tibia were excised to expose the marrow. The bones were put into bottomless 0.5 ml centrifuge tubes and inserted into 1.5 ml centrifuge tubes; the marrow was collected after centrifugation for 1 minute at 6,500 x g. The pellet was resuspended in 1 ml of normal growth medium and dispersed by passage through a 16-gauge needle. From the cell suspension 4  $\mu\text{l}$  was resuspended in 796  $\mu\text{l}$  of medium (1:200), mixed well and 10  $\mu\text{l}$  of this suspension was mixed with 10  $\mu\text{l}$  of 2%

acetic acid (to disrupt the red blood cells). Cells were counted using a haematocytometer and plated at a density of  $2 \times 10^6/\text{cm}^2$  in  $75 \text{ cm}^2$  tissue culture flasks. The flasks were incubated at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$ :95% air atmosphere for three days. The medium was then changed to remove the non-adherent cells, and every 3 - 4 days thereafter. The cells were expanded and used at passage 0 - 3.

### **2.2.3 7F2 cell line**

The 7F2 mouse osteoblast cell line was kindly provided by Dr Colin Farquharson (University of Edinburgh, UK) and used at passage 8 - 20. The cells are derived from the bone marrow of  $\text{p53}^{-/-}$  mice and represent mature osteoblasts as they express ALP, secrete type I collagen and mineralise extensively (Thompson et al., 1998). The cells were grown in normal growth medium in  $25 \text{ cm}^2$  tissue culture flasks and maintained at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$ :95% air atmosphere. The cells were passaged by trypsinisation on reaching confluency (split 1:5) as described in section 2.2.5 and the culture medium was routinely changed every 3 - 4 days.

### **2.2.4 Human osteoblast (HOB) cells**

Primary human osteoblast cells were purchased from Lonza (Berkshire, UK). The cells were grown in normal growth medium in  $25 \text{ cm}^2$  tissue culture flasks and maintained at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$ :95% air atmosphere. Upon reaching confluency cells were passaged by trypsinisation as described in section 2.2.5 and the culture medium was routinely changed every 3 - 4 days. The cells were expanded and used at passage 2 - 6.

### 2.2.5 Cell culture maintenance

On reaching confluence cells were detached by trypsinisation. The medium was removed and the cells were washed with sterile phosphate buffered saline (PBS) and dislodged by incubation with 0.5 - 1 ml (per 25 cm<sup>2</sup> and 75 cm<sup>2</sup> tissue culture flasks respectively) of 0.25% trypsin/ 0.25% EDTA (ethylenediaminetetraacetic acid) (Worthington Biochemical Corp. distributed by Lorne Laboratories, Ltd. Reading, Berkshire UK) for 3 - 5 minutes at room temperature. Cell detachment was monitored by light microscopy and aided by gentle intermittent taping on the side of flask. The trypsin was inactivated by the addition of 3 ml of normal growth medium. The cells were then either reseeded in a number of new flasks or were set up for an experimental procedure. For the latter, the cell suspension was centrifuged for 3 minutes at 500 x *g* at room temperature. The supernatant was discarded and the cells were resuspended in a known volume of normal growth medium, counted with a haemocytometer (section 2.2.6) and seeded at a known density into 6, 12, 24 or 96 well plates.

### 2.2.6 Cell counting

Cell suspensions were diluted appropriately (to give 20 - 100 cells per square of haemocytometer chambers) in a known volume of normal growth medium and 7 µl was transferred to each chamber of a haemocytometer. Cells were counted using x100 magnification. Counts from 10 squares of both haemocytometer chambers were averaged and the number of cells was calculated as follows:

Cell number per ml = the average count x dilution factor x 10<sup>4</sup>



### **2.2.7 Cryopreservation of cells**

For long-term storage in liquid nitrogen, 7F2 cells were grown in 75 cm<sup>2</sup> tissue culture flasks until reaching 90% confluency. The cells were then detached by trypsinisation and suspended in 3 ml of freezing medium (50%  $\alpha$ -MEM, 40% FCS, 10% dimethyl sulfoxide (DMSO)). A uniform cell suspension was prepared by repeated pipetting and 1 ml aliquots were dispensed into cryotubes (Nunc, Thermo Fisher Scientific, Loughborough, UK), transferred into cryocontainers which contained isopropanol at room temperature and placed in a -80°C freezer. On the following day, the vials were transferred to liquid nitrogen for storage. To resurrect cells from liquid nitrogen, they were thawed rapidly by plunging the vial into a 37°C water bath. The thawed cells were transferred to a 25 cm<sup>2</sup> flask containing 10 ml of warm normal growth medium and incubated overnight at 37°C in a 5% CO<sub>2</sub>:95% air atmosphere. The medium was then replaced to eliminate the remaining DMSO in the culture.

## **2.3 Analysis of mRNA expression**

### **2.3.1 RNA extraction**

Total cellular RNA was extracted using TRIzol® reagent (Invitrogen, Paisley, Scotland), a monophasic solution of phenol and guanidine isothiocyanate that solubilises cell components while maintaining the integrity of the RNA. Cells were directly lysed with appropriate amount of TRIzol® reagent based on the area of the culture dish, and transferred to an RNase/DNase free 1.5 ml tube. Cell lysates were homogenised by passing several times through a pipette tip and incubated for 5 minutes at room temperature for complete dissociation of

nucleoprotein complexes. Following the incubation, 200  $\mu$ l of chloroform was added and the mixture shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. Samples were centrifuged at 12,000  $\times g$  for 15 minutes at 4°C to separate the mixture into a lower red, phenol-chloroform phase (containing the DNA), an interphase, and a colourless upper aqueous phase (containing the RNA). The aqueous phase was transferred to a fresh RNase/DNase free 1.5 ml tube and RNA was precipitated by mixing with 500  $\mu$ l of isopropanol. Samples were incubated at room temperature for 10 minutes and the precipitated RNA pelleted by centrifugation at 12,000  $\times g$  for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol by vortexing and centrifugation at 7,500  $\times g$  for 5 minutes at 4°C. The ethanol supernatant was removed and the RNA air-dried and re-suspended in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 minutes at 55°C.

### **2.3.2 DNase treatment of RNA**

Contaminating DNA was removed by DNase I treatment using the TURBO DNA-free kit (Ambion, Warrington, UK). 0.1 volume of 10x TURBO DNase Buffer and 1  $\mu$ l of TURBO DNase were added to each RNA sample, mixed gently and incubated for 20 - 30 minutes at 37°C. The digestions were stopped by treatment with 5  $\mu$ l DNase Inactivation reagent for 2 minutes at room temperature. Following centrifugation at 10,000  $\times g$  for 1.5 minutes, the supernatant, which contained the RNA, was transferred into a fresh tube.

### 2.3.3 RNA quantification

Total RNA was quantified using a GeneQuant spectrophotometer (GE Healthcare UK Ltd., Buckinghamshire, UK) by measuring optical density at 260 nm wavelength and the purity was confirmed by checking the ratio at a wavelength of 260/280 nm (RNA is detected at 260 nm, whereas protein at 280 nm). RNA with a ratio of 1.8 to 2 was considered to be sufficiently pure.

### 2.3.4 Reverse transcription (RT)

cDNA was synthesised from 1 µg of total RNA in a final volume of 20 µl reaction mixture containing Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase with its supplied buffer, Rnasin ribonucleotide inhibitor, oligo dT<sub>15</sub> primer and deoxyribonucleotide triphosphate (dNTP) mix (all from Promega, Southampton, UK) as described in table 2.2. Initially RNA samples were heated for 10 minutes at 65°C to denature the secondary structure and quickly cooled on ice to stop them reforming. The RNA was then added to the reaction mix and the RT reaction carried out for 60 minutes at 37°C. For any one sample there was a no-RT control to check for residual DNA. The cDNA was stored at -20 °C until required.

Reagents	Concentration/reaction	Volume/reaction
M-MLV RT	100 U	0.5 µl
M-MLV RT Buffer	5x	4 µl
dNTP mix	10 mM	1 µl
Oligo dT primer	0.1 µg	1 µl
Rnasin RI	8 U	0.2 µl
RNA	1 µg	-
H <sub>2</sub> O	-	to 20 µl

**Table 2.2 Concentrations and volumes of reagents used for RT.**

### 2.3.5 Primer design

Sequences for the genes of interest (GOI) were retrieved from University of California Santa Cruz (UCSC) genome browser program (<http://genome.ucsc.edu/>). In order to distinguish amplification of mRNA from any possible contamination by genomic DNA; where possible forward and reverse primers were designed in different exons. Primers were designed with Primer3 (<http://frodo.wi.mit.edu/>) software and obtained from Invitrogen (Paisley, Scotland). The desired amplicon length was chosen to be less than 250 base pairs (bp) to increase the efficiency of the polymerase chain reaction (PCR) reaction. The primers were designed to have a melting temperature ( $T_m$ ) close to 65°C and minimal self-dimerisation. All primer sequences were tested via BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to eliminate those with significant homologies to other eukaryotic sequences. Each primer set was tested to ensure a single PCR product of expected size and a single melting peak on quantitative-RT-PCR (q-RT-PCR). The identity of PCR products was then checked by sequencing. The amplification efficiency for the primers was calculated in q-RT-PCR analysis by measuring the slope of a standard curve created from serially diluted templates using MX 3000 PRO software (Stratagene, La Jolla, CA). The primer sequences (all 5' - 3') and amplicon size are indicated in tables 2.3, 2.4 and 2.5.

Primers	Forward	Reverse	Size (bp)
A <sub>1</sub>	GTCCTCATCCTCACCCAGAG	CAGATTGTTCCAGCCAAACA	189
A <sub>2A</sub>	TCTTCAGTCTCCTGGCCATC	TCCAACCTAGCATGGGAGTC	156
A <sub>2B</sub>	CTCCATCTTCAGCCTTCTGG	GGAATGGAGTCAATCCGATG	152
APRT*	GCTGCGTGCTCATCCGAAAG	CCTTAAGCGAGGTCAGCTCC	247
ALP	GGACATGCAGTACGAGCTGA	CCACCAAATGTGAAGACGTG	282
Osteocalcin	GGCAGCGAGGTAGTGAAGAG	CTGGAGAGGAGCAGAACTGG	230
PPAR $\gamma$	CAGTGGGGATGTCTCATAA	CTTTTGGCATACTCTGTGAT	390
LPL	GAGATTTCTCTGTATGGACC	CTGCAAATGAGACACTTTCTC	275

**Table 2.3 Human primer sequences.**

\*APRT: adenine phosphoribosyltransferase

Primers	Forward	Reverse	Size (bp)
A <sub>1</sub> *	ATTGCTGTGGATCGATAC C	GAATCCAGCAGCCAGCTAT	100
A <sub>2A</sub>	CTTCGCTATCACCATCAGCA	AAATTGCAATGATGCCCTTC	200
A <sub>2B</sub> *	TCCATCTTTAGCCTCTTG	TCCTCTTGCTCGTGTC	100
A <sub>3</sub>	TTCTTGTTTGCCTTGCTG	AGGGTTCATCATGGAGTTCG	129
CD73	GGACTGATTGATCCCCTCCT	TTGTCCCTGGATTTGAGAGG	192
ADA	AAGGAGACCTCACCCCTGAT	GTCTCATCCCCAGCCAAGT	198
AK	CTATGCTGCCGAGAACAACA	TGCCTCTTCGAGTTCACCTT	225
Cbfa1	GGCCCTGGTGTAAATGGT	AGGCTGTTTGACGCCATAGT	178
Collagen type I	AGCTGGTGCTAAGGGTGAAG	GCAATACCAGGAGCACCATT	174
ALP	CTGCAAGGACATCGCCTATC	CATCAGTTCTGTTCTTGGGGTA	101
Osteocalcin	CCTTCATGTCCAAGCAGGAG	GTCCGCTAGCTCGTCACAAT	152
PPAR $\gamma$	TTCAGAAGTGCCTTGCTGTG	CCAACAGCTTCTCCTTCTCG	84
C/EBP $\alpha$	GCCAAGAAGTCGGTGGATAA	AACACCTTCTGCTGCGTCTC	125
LPL	CTTCAACCACAGCAGCAAAA	GGCCCGATACAACCAGTCTA	148
$\beta$ -actin*	TGTCACCAACTGGGACGATA	GGGGTGTGAAGGTCTCAA	165

**Table 2.4 Rat primer sequences.**

\* A<sub>1</sub> and A<sub>2B</sub> adenosine receptor sequences were obtained from (Novak et al., 2008) and  $\beta$ -actin was obtained from (Bonfeld et al., 2008).

Primers	Forward	Reverse	Size (bp)
A <sub>1</sub>	GATCGGTACCTCCGAGTCAA	TTGGCTATCCAGGCTTGTTTC	160
A <sub>2A</sub>	GGCTATTGCCATCGACAGAT	ATGGGTACCACGTCCTCAAA	228
A <sub>2B</sub>	TGCTCACACAGAGCTCCATC	AGTCAATCCAATGCCAAAGG	158
A <sub>3</sub>	TCCCTGATTACCACGGACTC	TCCTTCTGTTCCCCACATTC	151
CD73	CCTCTCAATCCAGGGACAA	TTTGAAGGTGGATTTCTG	220
ADA	GCGTGGTCTATGTGGAAGT	GACCTTGATGCCAAATGCTT	165
AK	CTATGCTGCCGAGAACAACA	TGCCTCTTCGAGTTCACCTT	225
C/EBP $\beta$	CAAGCTGAGCGACGAGTACA	CAGCTGCTCCACCTTCTTCT	157
PPAR $\gamma$	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT	220
GPDH*	ATGCTCGCCACAGAATCCACAC	AACCGGCAGCCCTTGACTTG	124
LPL	GCGTAGCAGGAAGTCTGACC	CATCAGGAGAAAGGCGACTG	108
Osteocalcin	AGACTCCGGCGCTACCTT	TAGGCGGTCTTCAAGCCATA	104
ARP*	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGTTGC	72

**Table 2.5 Mouse primer sequences.**

\*ARP: acidic ribosomal protein

\*GPDH: glycerol-3-phosphate dehydrogenase

### 2.3.6 PCR

#### i. Principle

PCR is a technique for amplification of linear fragments of DNA within two specific short DNA sequences (primers). The starting material for nucleic acid amplification by PCR are a DNA template, which contains the segment of nucleic acid that is to be amplified, a pair of DNA primers, a thermostable DNA polymerase (*Taq* DNA polymerase) and a set of nucleotides (dNTPs), dATP, dCTP, dGTP, and dTTP. The basic PCR procedure includes repeated cycles of amplifying target nucleic acid sequences. Each cycle consists of three steps: initially, a double strand of DNA is separated into two single

strands by heat at 90 - 95°C (denaturation). The reaction is then cooled to 55 - 65°C to allow the primers to bind to their target sequence on the single strand (annealing). Finally, the reaction is warmed to 72°C to allow *Taq* polymerase to create new DNA fragments complementary to the target DNA (extension).

## ii. Method

For gene detection PCR was performed in a TC-512 thermocycler (Techne, Cambridge, UK) using 1 µl of the cDNA with reagents from Promega (Southampton, UK) in a 50 µl reaction as shown in table 2.6. The thermal profile for PCR was 95°C for 1 minute (denaturation), followed by 40 cycles, each of which is comprised of 30 seconds at 95°C, 1 minute at 60°C and 1 minute at 72°C. The extension step was 10 minutes at 72°C. No template controls (negative controls) were included for each set of primers used in PCR amplification.

Reagent	Concentration/reaction	Volume/reaction
cDNA template		1 µl
dNTP mix	10 mM	1 µl
Taq polymerase	2 U	0.4 µl
10x Taq buffer MgCl <sub>2</sub> KCl Tris-Hcl	1.5 mM 50 mM 10 mM	5 µl
Primer mix	25 µM for each primer	1 µl
H <sub>2</sub> O		to 50 µl

**Table 2.6 Concentrations and volumes of reagents used for PCR.**

### **2.3.7 Agarose gel electrophoresis**

The sizes of PCR products were obtained using 2% agarose gel containing ethidium bromide (final concentration 1:20,000). Briefly, gels were prepared by adding agarose (Melford Laboratories Ltd, Ipswich, UK) to tris-acetate-EDTA (TAE) buffer consisting of 40 mM tris base/ glacial acetic acid and 1 mM EDTA and heating in a microwave oven until dissolved. The solution was allowed to cool for few minutes and then poured into a gel tray and allowed to set for 30 minutes at room temperature. PCR products, 100bp DNA ladder (Promega, Southampton, UK) and negative and positive controls were prepared for electrophoresis by mixing with an appropriate volume of gel loading buffer (Promega, Southampton, UK) and applied to the gel. The gel was run at 100 volts using the Mupid-21 mini gel migration system (Cosmo Bio Ltd, Japan) for approximately 30 minutes or until the tracking dye migrated two-thirds down the gel. The gel was visualised and photographed under UV transillumination using a Multi Image™ light cabinet and Alphamager™ documentation and analysis system (Alpha Innotech Corporation, San Leandro, USA).

### **2.3.8 DNA Sequencing**

The identity of PCR products were confirmed by direct sequencing. PCR products were gel extracted and purified using the Wizard® SV Gel and PCR Clean-Up System based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts (Promega, Southampton, UK). The band of interest was excised with a minimal volume of agarose with a clean scalpel and weighed. The guanidine isothiocyanate (Membrane Binding Solution) was



added to the sample at a ratio of 100  $\mu$ l of solution per 10 mg of gel slice, mixed by vortexing and incubated at 65°C for 10 minutes or until the gel slice was completely dissolved. To isolate the DNA, the sample was applied to a SV mini-column (containing silica), incubated for 1 minute at room temperature and centrifuged at 16,000  $\times$  *g* for 1 minute. The flow-through was discarded and the DNA fragment was washed twice in Membrane Wash Solution (700  $\mu$ l and 500  $\mu$ l respectively) and centrifuged at 16,000  $\times$  *g*. The flow-through was discarded and the SV column was centrifuged for an additional 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol.

Extracted cDNA was collected into a clean 1.5 ml microcentrifuge tube by the addition of 50  $\mu$ l of nuclease-free water directly to the centre of the column, incubation at room temperature for 1 minute and a final centrifugation for 1 minute at 16,000  $\times$  *g*. To quantify the amount of purified cDNA, 5  $\mu$ l of samples were run on an agarose gel and compared to DNA fragments from a 100bp ladder (Promega, Southampton, UK).

Extracted DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Warrington, UK). The sequencing reactions were set up as described in table 2.7 and subjected to cycling conditions as described in table 2.8 using a TC-512 thermocycler. Following the reaction, the cDNA samples were precipitated by addition of 2  $\mu$ l of 3 M sodium acetate (pH 4.6) and 50  $\mu$ l of 95% ethanol and incubation at room temperature for 15 minutes. The precipitated DNA was then centrifuged for 20

minutes at  $16,000 \times g$  and the DNA pellets were washed by addition of  $250 \mu\text{l}$  of 70% ethanol and centrifugation at  $16,000 \times g$  for 5 minutes. The pellets were dried by placing the tubes with the lids open in a heat block at  $90^\circ\text{C}$  for 1 minute. The sequencing was performed on an ABI 377 sequencer (Applied Biosystems) by Central Biotechnology Services, Cardiff University School of Medicine.

Reagents	Concentration	Amount
Ready Reaction Premix	2.5x	4 $\mu\text{l}$
BigDye Sequencing Buffer	5x	2 $\mu\text{l}$
Primer		3.2 pmol
Template		2 - 4 ng
H <sub>2</sub> O		to 20 $\mu\text{l}$
Final Volume	1x	20 $\mu\text{l}$
H <sub>2</sub> O	-	to 20 $\mu\text{l}$

**Table 2.7 Concentrations and volumes of reagents used for sequencing reaction.**

25 cycles	
Denaturation	96°C for 30 seconds
Annealing	50°C for 15 seconds
Extension	60°C for 4 minutes

**Table 2.8 Cycling conditions used for sequencing reaction.**

### 2.3.9 qPCR

#### i. Principle

Real time or qPCR is the state of the art technique based on conventional PCR chemistry, to quantify nucleic acid expression in real time by the labelling of primers, oligonucleotide probes (oligoprobes) or amplicons with reporter

molecules capable of fluorescing. The fluorescent reporter molecules fall into two groups: 1) DNA-binding dyes (e.g. SYBR green) and 2) Dye-labelled probes such as hydrolysis probes (e.g. TaqMan probes). The former of these has been used in this investigation *i.e.* SYBR Green.

### **A. SYBR Green**

SYBR Green is the most commonly used double stranded DNA (dsDNA) binding dye. In solution (*i.e.* not bound to DNA), it displays relatively low fluorescence; however on binding to dsDNA, the level of fluorescence emitted by the molecule increases by 1000 fold (Wittwer et al., 1997). Therefore, as the amount of dsDNA in the PCR increases, the amount of fluorescence increases proportionally. One potential disadvantage of assays based on DNA-binding dye chemistry is the inherent non-specificity. SYBR Green will bind to all dsDNA molecules, including primer dimers or genomic DNA. However it is possible to achieve additional specificity and to verify the identity of the product in each amplified sample by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. Each dsDNA molecule has a specific  $T_m$  based on its nucleotide composition at which 50% of the DNA becomes single stranded. The presence of two or more peaks suggests that more than one amplified sequence was obtained, and the amplification was not specific for a single DNA target (Ririe et al., 1997).

### **B. Analysis**

The amount of template in a sample can be described either relatively or absolutely. Both relative and absolute quantitation of gene expression utilises

the cycle threshold ( $C_t$ ) value to quantitate cDNA and thereby determine gene expression. The  $C_t$  is defined as the PCR cycle in which fluorescence intensity is greater than background fluorescence. The  $C_t$  value is directly correlated to the starting target concentration of the sample. The greater the amount of initial DNA template in the sample, the lower the  $C_t$  value for that sample. Relative quantification describes the change in expression of a target sequence in a test sample relative to the same sequence in an external standard or a reference sample, also known as a calibrator.

Typically the calibrator is an untreated sample or a zero-time point. The differences in  $C_t$  values between an unknown sample and calibrator are expressed as fold changes (*i.e.* upregulated or downregulated) relative to the calibrator sample. Using this approach, it is necessary to normalise the results with a normalising reference or housekeeping gene (*e.g.* cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH),  $\beta$ -actin, 18S rRNA, etc.) that is not expected to change under the experimental conditions. This normalisation controls for possible differences in the amount of RNA used and in the efficiency of the reverse transcription reaction from sample to sample and experiment to experiment. This produces a corrected relative value for the target specific mRNA product, which can be compared between samples and allows an estimate of the relative expression of the GOI in those samples. In addition amplification efficiencies of the target and the reference must be determined and be incorporated into calculations because they directly affect the accuracy of any expression results (Livak and Schmittgen, 2001).

**ii. Method**

Real-time PCR was performed using an MX3000p thermal cycler system (Stratagene, Cambridge, UK) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Paisley, Scotland). All reactions were set up in a total volume of 25  $\mu$ l containing 12.5  $\mu$ l of SYBR green Master Mix, 1  $\mu$ l of cDNA template, 1  $\mu$ l of the forward and reverse primers, and 10.5  $\mu$ l of nuclease free water. The mixtures were prepared in 96 well PCR plates, centrifuged for 1 minute at  $1000 \times g$  and amplified using the following cycling parameters: 2 minutes at 50°C (uracil-DNA glycosylase (UDG) incubation), 2 minutes at 95°C (activation of Platinum DNA polymerase), followed by 45 cycles consisting of denaturation at 90°C for 15 seconds and annealing at 60°C for 30 seconds (amplification of the specific target sequence).

Amplification was confirmed by monitoring the dissociation curve (at the end of the PCR, samples were subjected to a melting curve analysis: 60 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 95°C) (see an example of thermal profile in figure 2.1). All reactions were performed in at least duplicate. Template negative controls were also included in all amplification experiments.

The relative quantitative expression of GOI was analysed using the MxPro software (Stratagene). The relative quantitation value for each target gene compared with the calibrator for that target is first calculated separately for each assay, following normalisation of GOI assays to assigned normalisers.

To do this, the following equation was used:

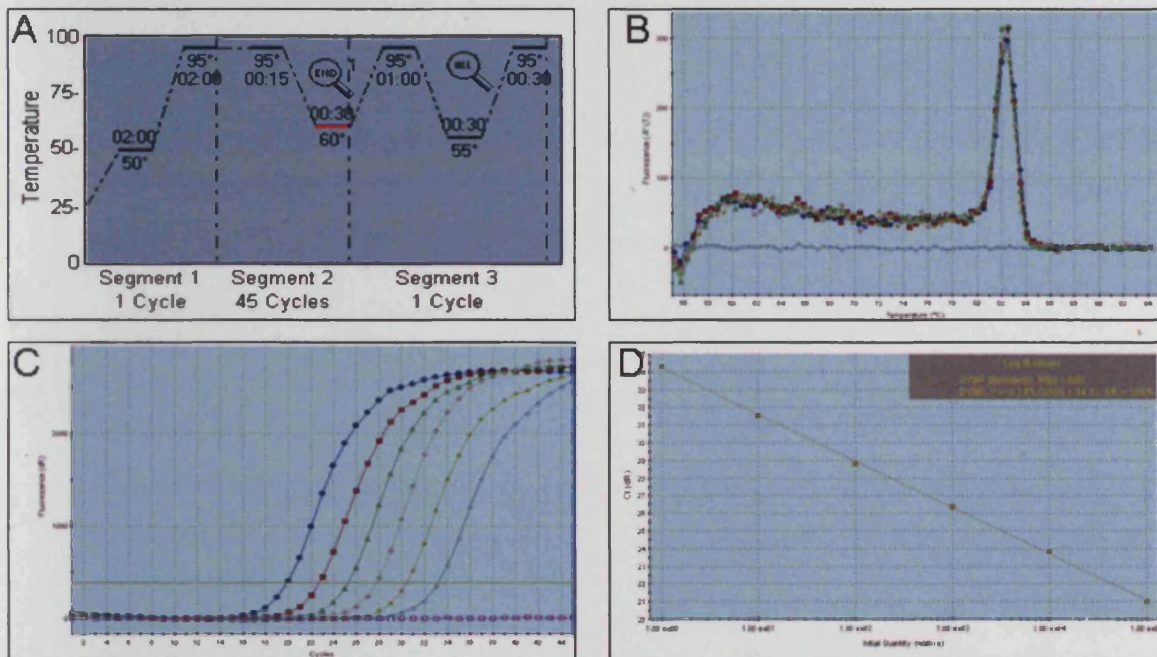
$$\text{Relative quantity to the calibrator} = \frac{(1 + E_{GOI})^{\Delta C_t_{GOI}}}{(1 + E_{norm})^{\Delta C_t_{norm}}}$$

Where

$E_{GOI}$  = efficiency of the target assay

$E_{norm}$  = efficiency of the normaliser assay

$$\Delta C_t = (C_{t_{calibrator}} - C_{t_{unknown}})$$



**Figure 2.1** Example of q-RT-PCR readout. (A) Thermal profile, (B) dissociation curve, (C) amplification curve and (D) efficiency curve of a 10 fold dilution series using rat ALP primers.

## 2.4 Analysis of protein expression

### 2.4.1 Cell lysis

Cells were washed twice with PBS and lysed (250  $\mu$ l per well of 6 well plate) in radioimmunoprecipitation assay (RIPA) buffer (50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloric acid (HCl), pH 7.5, 150

mM sodium chloride (NaCl), 1% Nonidet P-40, 0.1% Sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate) containing a protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin all at 10 µg/ml), 1 mM sodium orthovanadate and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF). Plates were stored overnight at -80°C and lysates were then transferred into 1.5 ml centrifuge tubes. Lysates were clarified by centrifugation (3 minutes at 6,500  $\times$  g), and protein levels determined with the use of the Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Hertfordshire, UK) as described in section 2.6. Aliquots of cell lysates (15 - 30 µg of total cellular protein) and the pre-stained protein marker (Cell Signalling Technology, Hertfordshire, UK) were mixed with an equal volume of loading buffer (4% SDS, 2%  $\beta$ -mercaptoethanol, 20% glycerol, 100 mM Tris-HCl, pH 6.8, 0.004% pyronin Y), boiled for 5 minutes, centrifuged to remove the insoluble material, and then separated on SDS-polyacrylamide gel electrophoresis (PAGE) as described in section 2.4.3.

#### **2.4.2 Phosphorylation assay**

For detection of signal transduction molecules (extracellular signal-regulated kinases (ERK); CREB) cells were serum-starved for about 17 hours. Briefly, cells were washed twice with PBS and incubated in serum free medium overnight at 37°C in a 5% CO<sub>2</sub>:95% air atmosphere. Medium was changed and cells were incubated for an additional two hours prior to stimulation with the test compounds as described in the results. The reaction was terminated by two quick washes in ice-cold PBS containing 1 mM sodium orthovanadate and lysed in ice-cold RIPA buffer with inhibitors as described above and subjected to Western blot analysis (section 2.4.3).

### **2.4.3 Western blotting**

#### **i. Principle**

SDS-PAGE and Western blotting were used for identification and comparative quantification of proteins of interest. SDS-PAGE relies on the migration of charged molecules in a gel matrix and facilitates the separation and resolution of a mixture of proteins according to molecular weight. SDS is an anionic detergent that denatures proteins, providing them with the same conformational properties and also with a similar net negative charge. SDS coated proteins are therefore subjected to the same field strength and migrate in an electric field with equal charge toward the anode. SDS-PAGE also involves linearisation of proteins by dissociation of disulphide bonds with reducing agents such as 2-mercaptoethanol. SDS-PAGE separation is, therefore, determined not by intrinsic electrical charge of the polypeptide, but by their molecular weight through the sieving action of the acrylamide gel matrix. SDS-PAGE separated proteins are then blotted to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane for immuno-blotting analysis (Bolt and Mahoney, 1997; Towbin et al., 1979).

#### **ii. Methods**

##### **A. SDS-PAGE**

The separation of proteins was carried out using the discontinuous system and denaturing conditions originally described by Laemmli (Laemmli, 1970). The discontinuous system utilises different buffer ions and pH in the gel and in the electrode reservoirs. It is composed of a slightly acidic (pH 6.8), non-restrictive large pore stacking gel which overlays a smaller pore resolving gel.



High resolution of protein separation is achieved, as proteins concentrate into narrow zones during migration through the large-pore stacking gel, before entering the separation gel, where higher pH (8.8) and acrylamide concentration causes proteins to be resolved according to size.

The percentage of acrylamide used in the separation gel is based on the size of the protein of interest; low molecular weight proteins are resolved on higher percentage gels while proteins with higher molecular weight are resolved on lower percentage gels. 10% gel was routinely used for separating proteins in the range of 30 - 80 kDa. Proteins of greater than 80 kDa should be run on an 8% gel and those of less than 30 kDa on a 12% gel. The recipes for the stacking and resolving gels can be found in tables 2.9 and 2.10. Once prepared the resolving gel mixture was mixed by swirling and 7 ml of the mixture was immediately transferred into a 1.5 mm gel casting system (Bio-Rad, Hertfordshire, UK). The gel was overlaid with 500  $\mu$ l of N-butanol and allowed to set for 45 minutes at room temperature. Once the resolving gel has polymerised, the gel surface was washed twice with 1 ml of dH<sub>2</sub>O. The stacking gel was poured on top of the resolving gel and a 10 well comb was placed between the glass plates. The stacking gel was allowed to set for 30 - 45 minutes at room temperature and the comb was removed. Samples with known concentration of protein (15 - 30  $\mu$ g protein) were loaded onto each well and subjected to SDS-PAGE using a mini protean II system (Bio-Rad, Hertfordshire, UK). Electrophoresis was then performed at a constant current of 200 volts for about 30 - 45 minutes in running buffer.

Component	10% (w/v) Gel
30% Acrylamide*	3.3 ml
dH <sub>2</sub> O	2.92 ml
1M Tris (pH 8.8)	3.75 ml
10% SDS	100 µl
10% APS**	100 µl
TEMED***	5 µl

**Table 2.9 Resolving gel components.**

Component	4% (w/v) Gel
30% Acrylamide*	1.3 ml
dH <sub>2</sub> O	6.1 ml
0.5M Tris (pH 6.8)	2.5 ml
10% SDS	100 µl
10% APS**	100 µl
TEMED***	10 µl

**Table 2.10 Stacking gel components.**

\* Made up with acrylamide:bis-acrylamide ratio of 29:1

\*\* APS: Ammonium Persulfate (Freshly prepared in H<sub>2</sub>O)

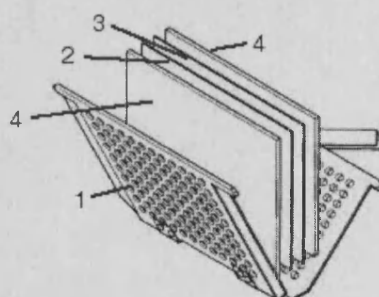
\*\*\* TEMED: N,N,N',N'-Tetramethylethylenediamine (Added immediately prior to pouring the gel)

## B. Western blotting

Proteins from SDS-PAGE gels were transferred to a PVDF membrane (Hybond-P, GE Healthcare, Buckinghamshire, UK) using a mini protean II system (Bio-Rad, Hertfordshire, UK). Following electrophoresis the resolving gel was soaked in blotting buffer along with PVDF membrane and filter paper that were all cut to the same size as the gel. The Western blot transfer cassette was assembled according to manufacturer's instructions. The membrane was carefully located on top of the gel and these were sandwiched between the filter papers. A Pasteur pipette was drawn across the surface of the assembled sandwich to remove air bubbles that might have formed

between the layers as they can disrupt the transfer of proteins from gels to membrane. The assembled sandwich was placed between sponge pads within the cassette as shown in figure 2.2. The cassette was then loaded along with a freezing block into the blotting tank with the membrane located between the gel and the positive electrode. The proteins were transferred onto the membrane by electrophoresis in continuously stirred chilled blotting buffer for 1 hour at 350 mA using a POWER PAC 1000 (Bio-Rad, Hertfordshire, UK).

- 1 Cassette
- 2 Gel
- 3 Membrane
- 4 Filter paper



**Figure 2.2 Western blot transfer cassette.**

### **C. Immunoprobing of Western blots**

The PVDF membrane was removed from the tank and cassette and washed with Tris buffered saline (TBS) (0.137 M NaCl, 0.02 M Tris-HCl, pH 7.6) and 0.1% Tween 20 (TBS-T) for 5 minutes. All the washing and staining steps were performed with continuous mechanical agitation. To prevent non-specific antibody binding, the membrane was blocked for a minimum of 1 hour with 5% milk powder in TBS-T at room temperature. After blocking, the membrane was probed with a primary antibody specific to the protein of interest in blocking buffer by overnight incubation at 4°C or at room temperature for one hour. Table 2.11 gives details of antibodies used in these studies, along with

their corresponding dilutions. Following incubation with primary antibody the blot was washed with TBS-T (3 × 5 minutes) and probed with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (at a dilution of 1:5000) for 1 hour in blocking buffer at room temperature. The blot was extensively washed in TBS-T (2 × for 30 seconds; 1 × for 15 minutes; 3 × for 5 minutes) before visualisation by chemiluminescence.

#### **D. Detection of immuno-labelled protein**

Specific antibody binding was visualised with enhanced chemiluminescence (ECL) plus Western blotting detection reagents (GE Healthcare, Amersham, UK). The ECL Plus contains lumigen PS-3 acridan substrate, which is catalysed by HRP to produce an acridinium ester intermediate that reacts with peroxide in alkaline conditions and emits light. This light is captured on autoradiography film. The ECL reagent consisted of two solutions (A and B) that were mixed in 40:1 ratio according to manufacturer's instructions and the protein side of the blot was overlaid with a sufficient volume (~ 2.5 ml for a 5 × 8 cm blot) for 5 minutes. The excess ECL solution was then removed and the blot covered with saran wrap. The blot was transferred to a photographic cassette with the protein side facing up and exposed to an autoradiography film (Hyperfilm™ ECL; GE Healthcare, Amersham, UK) for periods ranging from 30 seconds to 60 minutes depending on signal strength. The film was developed and the bands obtained were analysed using an Alphamager™ documentation and analysis system (Alpha Innotech Corporation, San Leandro, USA).

Antibody	Ab Host/type	Species Reactivity	Dilution	MW (kDa)	Supplier	Catalog #
Anti-A <sub>1</sub>	Rabbit/polyclonal	H, R	1:1000	36	Abcam®	ab3460
Anti-A <sub>2A</sub>	Rabbit/polyclonal	H, D	1:2000	45	Abcam®	ab3461
Anti-A <sub>2B</sub>	Rabbit/polyclonal	H, M, R	1:1000	36	Alpha diagnostics	A2BR23-A
Anti-A <sub>3</sub>	Rabbit/polyclonal	H	1:1000	36	Abcam®	ab13160
Anti-CD73	Goat/polyclonal	H, M, R	1:500	71	Santa Cruz Biotechnology	sc-14684
Anti-β-actin	Goat/polyclonal	H, M, R	1:1000	43	Santa Cruz Biotechnology	sc-1616
Anti-ERK1	Rabbit/polyclonal	H, M, R	1:2000	44	Santa Cruz Biotechnology	sc-94
Anti-ERK2	Rabbit/polyclonal	H, M, R	1:2000	42	Santa Cruz Biotechnology	sc-153
Anti-p-ERK1/2	Mouse/monoclonal	H, M, R	1:2000	42, 44	Cell Signalling Technology	9106
Anti-CREB	Rabbit/polyclonal	H, M, R	1:1000	44	Santa Cruz Biotechnology	SC-186
Anti-P-CREB	Rabbit/monoclonal	H, M, R	1:1000	43	Cell Signalling Technology	9198
Anti-Phospho-Threonine	Mouse/monoclonal	H, M, R	1:1000		Cell Signalling Technology	9386
Anti-goat IgG-HRP			1:5000		Santa Cruz Biotechnology	SC-2354
Anti-rabbit IgG-HRP			1:5000	-	GE Healthcare	NA934V
Anti-mouse IgG-HRP			1:5000		GE Healthcare	NA931V

**Table 2.11 Summary of antibodies.** Reactivity Key: H=Human; M=Mouse; R=Rat; D=Dog

#### 2.4.4 Stripping and re-probing PVDF blots

To check sample loading, the blot was stripped and re-probed with a housekeeping antibody (anti-β-actin). The blot was incubated with stripping buffer (6.25 ml, 0.5 M Tris (pH 6.8), 10 ml 10% SDS, 0.35 ml mercaptoethanol and 33.5 ml of dH<sub>2</sub>O) in a hybridisation oven with gentle mixing for 30 minutes at 60°C. The blot was then washed (3 × for 10 minutes) with a large volume of TBS-T, blocked and re-probed with appropriate housekeeping antibody as described in section 2.4.3 Western blotting.

### **2.4.5 Pro-Q Diamond**

#### **i. Principle**

Pro-Q Diamond dye is a fluorescence based detection method for the selective and sensitive analysis of phosphorylated proteins. The dye binds noncovalently to phosphoproteins and allows direct detection of phosphoserine, phosphothreonine and phosphotyrosine containing proteins.

#### **ii. Method**

Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel, and blotted to a PVDF membrane. After blotting, the membrane was allowed to dry completely. Dried PVDF membranes were re-wetted by dipping into 100% methanol for a few seconds and were stained for phosphoproteins using Pro-Q Diamond dye (Invitrogen, Paisley, Scotland). Briefly, the transblotted proteins were fixed to the membrane by immersing the membrane in 7% acetic acid/10% methanol solution (25 ml) for 10 minutes. The membrane was then washed four times in 25 ml of dH<sub>2</sub>O for 5 minutes per wash and incubated in 25 ml of Pro-Q Diamond stain for 15 minutes. The membrane was destained with 25 ml of a solution of 50 mM sodium acetate and 20% acetonitrile (pH 4.0) for 15 minutes. This wash was repeated twice for a total of three washes. The membrane was then air-dried, visualised and photographed under UV transillumination using the Multi Image™ light cabinet and Alphamager™ documentation and analysis system (Alpha Innotech Corporation).

## **2.5 Analysis of cAMP production**

### **2.5.1 Sample preparation**

Cells in 24 well plates were washed with serum free medium containing the experimental treatments used for that particular experiment. Cells were incubated with varying concentrations ( $10^{-8}$  to  $10^{-4}$  M) of adenosine, NECA, or CGS 21680 in the presence of  $10^{-4}$  M RO 20-1724 (cAMP specific PDE inhibitor) for 15 minutes at 37°C. The reaction was stopped by rapid aspiration of medium and degradation of cyclic nucleotides was prevented by the addition of 200  $\mu$ l of 0.1 M HCl. Acid extracts were dried by overnight vacuum centrifugation. The dried samples were dissolved in 1 ml of assay buffer (50 mM sodium acetate with 0.25% bovine serum albumin (BSA), pH 5.2). This extract was stored at -20°C and used directly in the immunoassay system.

### **2.5.2 cAMP radioimmunoassay**

#### **i. Principle**

Intracellular cAMP contents were measured by an in-house acetylation radioimmunoassay (RIA) (Brooker et al., 1979; Harper and Brooker, 1975). This is a sensitive and specific method, which allows measurement of pmolar concentrations of the nucleotide. RIAs are based upon competition of substrate with a radiolabelled substrate for binding sites on antibody molecules. With a constant and limiting amount of antibody, the amount of radioactive label bound by the antibody will be inversely proportional to the concentration of substrate in the sample. Free and antibody-bound antigens are separated by precipitation of the antibody-bound fraction with either a second antibody or polyethylene glycol. The radioactivity in the antigen-

antibody complex is then counted and the concentration of substrate in samples is determined by interpolation from a standard curve of known substrate concentrations.

## ii. Method

Standards were prepared in assay buffer from a cAMP stock (20 pmol) by 2 fold serial dilution over the range of 4 - 0.039 pmole/tube. Samples were prepared by diluting in assay buffer in a final volume of 400  $\mu$ l based on the results of the prior test assays. Also included were assay blanks *i.e.* for non-specific binding (NSB) and for background subtraction ( $C_0$ ) which received assay buffer only. Standards, blanks and appropriately diluted samples were acetylated by adding 10  $\mu$ l of triethylamine and immediate vortexing, followed by addition of 5  $\mu$ l acetic anhydride. The mixture was again mixed by vortexing for few seconds and 100  $\mu$ l of diluted [ $^{125}$ I]-cAMP label (NEN Life Sciences Products, UK) (~ 10,000 cpm) was then added along with 100  $\mu$ l of affinity purified rabbit antisuccinyl cAMP (in house) in a final dilution of 1:10000. Non-specific binding was determined in the absence of antiserum (NSB tube). The tubes were incubated overnight at 4°C and the antibody-bound [ $^{125}$ I]-cAMP was then separated from the free form by precipitation of the antibody-bound fraction by adding 100  $\mu$ l of bovine gamma globulins (2% in PBS) and 750  $\mu$ l of polyethylene glycol (20% in PBS). Table 2.12 shows the components added for NSB, total binding ( $C_0$ ) and samples and standards (C). Tubes were mixed to homogeneity and centrifuged at 3000 x *g* at 4°C for 20 minutes. The supernatants were aspirated and the amount of [ $^{125}$ I]-



cAMP radioactivity in the pellets was determined by counting for 50 seconds in a mini-assay type 6-20 gamma counter (Thermo Scientific, Cheshire, UK).

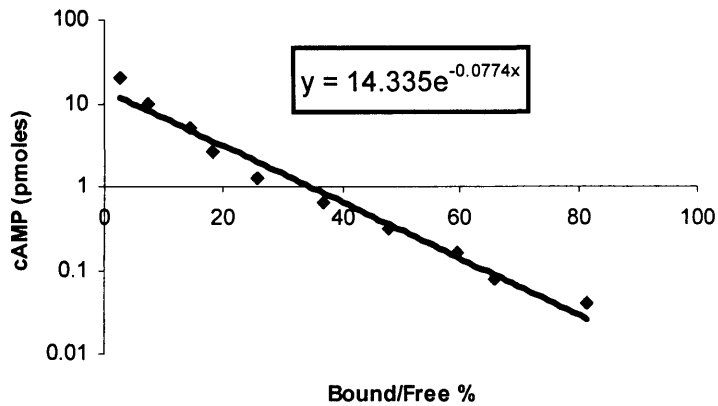
NSB	C <sub>0</sub>	C
400 µl buffer	400 µl buffer	400 µl standard or sample
10 µl triethylamine	10 µl triethylamine	10 µl triethylamine
5 µl acetic anhydride	5 µl acetic anhydride	5 µl acetic anhydride
100 µl cAMP label	100 µl cAMP label	100 µl cAMP label
100 µl dH <sub>2</sub> O	100 µl antibody	100 µl antibody
<b>Overnight at 4°C</b>		
100 µl gamma globulins	100 µl gamma globulins	100 µl gamma globulins
750 µl polyethylene glycol	750 µl polyethylene glycol	750 µl polyethylene glycol

**Table 2.12 Components for NSB, total binding (C<sub>0</sub>) and samples (C) for cAMP RIA.**

The percentage (ratio of counts bound/total counts bound) of each standard and unknown sample was calculated according to the following formula (average of the counts was used for each standard duplicates):

$$\frac{C - NSB}{C_0 - NSB} \times 100$$

A calibration curve was obtained by plotting this percentage verses log concentration (pmoles) of standards as an exponential curve using the scatter plot function of Microsoft Excel and the value of unknowns were calculated from the equation from each different set of experiments. An example of a calibration curve for cAMP measurement is shown in figure 2.3.



**Figure 2.3 Sample calibration curve obtained for cAMP RIA.**

## 2.6 Cellular protein quantification

### i. Principle

Total cellular protein was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Ltd., Hertfordshire, UK), a colourimetric assay based on the Bradford method (Bradford, 1976). This assay relies on the binding of proteins to Coomassie Brilliant Blue G-250 dye which can exist in three ionic forms: cationic (red), neutral (green), and anionic (blue) (Bradford, 1976). Under strongly acid conditions, the dye is predominantly in the doubly protonated red cationic form. When the dye binds to protein, however it is most stable as an unprotonated blue form that has an absorbance maximum at 590 nm (Compton and Jones, 1985). The quantity of protein in the sample can be estimated by measuring the amount of the blue protein-dye at 595 nm in a spectrophotometer.

### ii. Method

#### A. Collection of cellular protein

Cells were washed with 500  $\mu$ l of PBS and fixed with 500  $\mu$ l of 100% methanol for 1 minute. Subsequently, methanol was removed and the wells were

allowed to dry at room temperature. An appropriate volume (depending on the area of the well and the cell number) of 0.5 M sodium hydroxide (NaOH) was then added to each well and left for 30 minutes. Plates were stored for 24 hours at -20°C before collecting homogenates in 1.5 ml eppendorf tubes. Tubes were stored at -20°C for subsequent quantification of total cell protein.

### **B. Quantitation of Cellular Protein**

A standard curve was constructed by using known concentrations (2.5 to 20 µg/ml) of BSA in dH<sub>2</sub>O in a total volume of 800 µl in clean dry test tubes. Samples were also prepared by diluting in dH<sub>2</sub>O in a final volume of 800 µl based on the results of the test assay, whereas the blank contained 800 µl of dH<sub>2</sub>O. 200 µl of concentrated dye reagent was added to each tube (*i.e.* standards, test samples and blank) and mixed for a few seconds. Tubes were left for 10 minutes and the absorbance at 595 nm measured using a GENESYS 10UV spectrophotometer (Thermospectronic, Cambridge, UK). The total protein concentration in each unknown sample was determined by comparison with the standard curve.

## **2.7 Cell viability assay**

### **i. Principle**

A commercially available kit (CellTiter 96 AQueous solution cell proliferation assay; Promega, Southampton, UK) was used to measure cell number. This assay is referred to here as the MTS assay and comprises a metabolic colourimetric test, which utilises a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium, inner salt—MTS) and an electron coupling reagent, phenazine methosulfate (PMS). The MTS tetrazolium compound is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. Cellular metabolism by dehydrogenase enzymes in metabolically active cells via formation of reducing equivalents such as beta-nicotinamide adenine dinucleotide/reduced (NADH) or beta-nicotinamide adenine dinucleotide phosphate/reduced (NADPH) is thought to be responsible for the conversion of the tetrazolium compounds to the coloured formazan products that has been shown to be directly proportional to the number of living cells (Dunigan et al., 1995; Goodwin et al., 1995).

## **ii. Method**

Cells were seeded in 96 well tissue culture plates in 100 µl of normal growth medium, the outer wells were not used and contained only culture medium to prevent evaporation. After 24 hours the culture medium was replaced with fresh medium containing test compounds which was renewed every 3 days or as stated in the results. At the end of the experiment, cells were processed by addition of 20 µl of MTS directly to each well. The plate was then covered in aluminium foil (MTS is light sensitive) and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 hours. The absorbance of the formazan at 490 nm was measured directly from 96 well assay plates on a SpectraCount™ microplate photometer (Canberra Packard Ltd, Berkshire, UK) after 1 and 2 hours. The 1 hour reading was used in final data analysis, the 2 hour readings were used when the absorbance readings were particularly low.

## **2.8 Analysis of osteoblastic differentiation**

### **2.8.1 ALP enzyme activity**

#### **i. Principle**

The enzyme ALP is involved in osteoblast maturation and mineralisation in the early phase of bone calcification and was used here as a marker of osteoblastic development (Balcerzak et al., 2003). The assay for measuring the activity of ALP utilizes p-nitrophenylphosphate (p-NPP) as a substrate. Hydrolysis of this phosphomonoester releases inorganic phosphate and p-nitrophenol. The rate of p-nitrophenol liberation, which is measured photometrically at 405 nm, is proportional to the activity of ALP in the samples.

#### **ii. Method**

ALP activity was determined in 24 or 96 well plates using the phosphatase substrate with its supplied alkaline buffer (Sigma Aldrich, Poole, UK). Briefly, cells were washed with PBS and then a solution of phosphate substrate/alkaline buffer pH 10.3 at an appropriate ratio (depending on the level of ALP activity) was added to each well. The plate was incubated for 20 minutes at room temperature in the dark and absorbance was read at 405 nm on a SpectraCount™ microplate photometer (Canberra-Packard Ltd, Beaconsfield, Berks, UK). The ALP activity values were normalised to the relative number of viable cells in the same plate by prior determination using the MTS proliferation assay as described in section 2.7 or in a parallel set of experiments using total protein concentration if 24 well plates were used as described in section 2.6.

### 2.8.2 Mineralisation (Alizarin Red S staining)

#### i. Principle

Alizarin Red S staining is a common histochemical technique for visualisation of calcium mineral deposition in the extracellular matrix of osteoblast cultures *in vitro*. Calcium forms a complex with Alizarin red S in a chelation process which can be visualised as dark red colour.

#### ii. Method

The mineralised matrix was stained for calcium with Alizarin Red S. A working solution of stain was prepared by dissolving Alizarin Red S (1:100) in dH<sub>2</sub>O, adjusted to pH 4.2 and passed through a 150 mm filter paper (Whatman, Maidstone, UK) prior to use. Cells were washed with PBS and fixed in 4% formaldehyde in PBS (formal saline) for 15 minutes. The formal saline was removed, and the cells were washed with dH<sub>2</sub>O. The cells were then stained by complete immersion in the working solution of Alizarin Red S for 10 minutes at room temperature. The Alizarin Red S solution was removed and the cells were washed five times with 50% ethanol and then air-dried. The plates were scanned using an Epson Perfection 4990 photo flatbed scanner and the stained calcium deposits were assessed microscopically with a phase contrast ELWD 0.3 inverted microscope (Nikon) and images were captured by the Penguin 150 CL camera (Pixera Corporation, Surrey, UK) through Viewfinder 3.0.1 (Pixera Corporation). To quantify the level of mineralisation, cells were destained by an overnight incubation in 10% (w/v) cetylpyridinium chloride (CPC) at room temperature with continuous mechanical agitation and the obtained dye was transferred to a 96 well plate and the absorbance was

read at 562 nm using a SpectraCount™ microplate photometer (Canberra Packard Ltd, Oxfordshire, UK).

## **2.9 Analysis of adipogenic differentiation**

### **i. Principle**

Adipogenesis was quantified by measuring intracellular lipid accumulation by Oil Red O and Nile red staining. These lipophilic stains have a tendency to accumulate in the lipid containing vacuoles of adipocytes and can be used to distinguish adipogenic cells during differentiation (Fowler and Greenspan, 1985). Oil Red O is a common stain for neutral lipids and was used to determine relative amounts of lipid in treated and untreated adipocyte populations. Nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one), which is also a stain for lipids was used to identify the number of adipocytes in a given culture. The fluorescence of Nile red is quenched in an aqueous environment, whereas in a hydrophobic lipid environment Nile red fluoresces yellow to orange. After staining with Nile red, neutral lipids fluoresce yellow (peak fluorescence at 580 - 596, 590 nm), whereas polar lipids fluoresce orange (peak fluorescence at 597 - 620, 600 nm) (Greenspan et al., 1985).

### **ii. Methods**

#### **A. Oil Red O staining**

Accumulation of intracellular lipid was assessed by staining with Oil Red O. A stock solution of Oil Red O was prepared by dissolving 5 g of Oil Red O in 100 ml absolute isopropanol. This stock solution was stored at room temperature for up to 12 months. To achieve a working concentration, 3 parts of stock

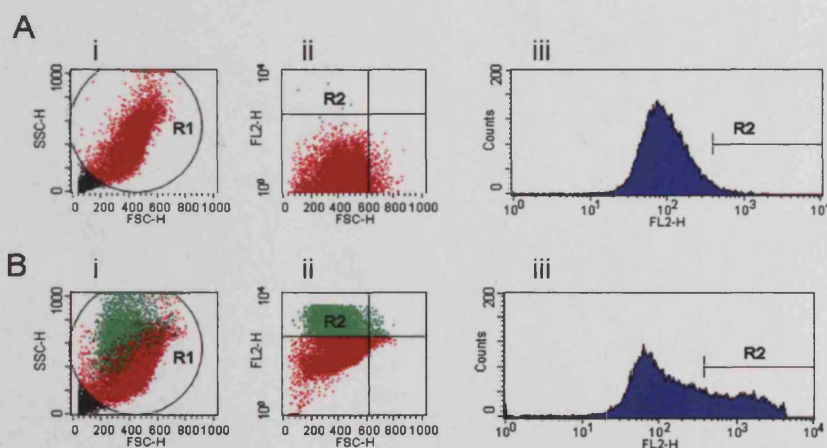
solution were mixed to 2 parts of dH<sub>2</sub>O, left for 15 minutes at room temperature, and filtered through a 150 mm Whatman filter paper prior to use. Cells were washed with PBS, fixed for 15 minutes with 4% formal saline, washed with dH<sub>2</sub>O and stained for 15 minutes by complete immersion in the Oil Red O working solution. The cells were washed once with 60% isopropanol and twice with PBS. The lipid accumulation was assessed microscopically with a phase contrast ELWD 0.3 inverted microscope (Nikon) and images were captured by the Penguin 150 CL camera (Pixera Corporation, Surrey, UK) through Viewfinder 3.0.1 (Pixera Corporation). In order to determine the extent of adipogenesis, the dye retained by the cells was extracted by incubation with 200  $\mu$ l of isopropanol, immediately transferred to a 96 well plate by gentle pipetting and the absorbance was read at 490 nm using a SpectraCount™ microplate photometer (Canberra Packard Ltd, Oxfordshire, UK).

### **B. Nile red staining**

To achieve a quantitative measure of adipocyte number, the Nile red staining method used was based on the method described by Sen (Sen et al., 2001). A stock solution was made by dissolving Nile red in DMSO (1 mg/ml) and stored at 4°C. A working solution was prepared immediately before use by diluting 1:100 in ice-cold PBS. Cells were detached carefully by trypsinisation and fixed for 30 minutes with 4% formal saline at 4°C. Cells were then centrifuged for 5 minutes at 200  $\times$  g at 4°C and the cell pellet was resuspended in 1 ml of Nile red working solution and stained for 30 minutes at 4°C. The samples were then analysed with a FACSCalibur flow cytometer



(Becton Dickinson Biosciences, Oxfordshire, UK) on the FL2 fluorescent emission channel at 585 nm. Firstly undifferentiated cells were used to set up the experiments. Using a forward scatter (FSC) versus side scatter (SSC) representation of events (based respectively on the size and granular content of the cell), a first R1 region (figure 2.4 Ai&Bi) was defined to exclude cellular debris from the analysis. The population within the R1 region was then used to plot the FL2 (emission channel at 585 nm that recognises the fluorescent emission by Nile red) versus FSC (size) (figure 2.4 Aii). The FL2 versus FSC plot was divided into quadrants using the undifferentiated cells (lower quadrants) as the base line of fluorescent emission. The lower quadrants therefore display events that are negative for Nile red fluorescent emission and represent undifferentiated cells (figure 2.4 Aii). The upper quadrants (R2) on the other hand contain events that are positive for Nile red fluorescent emission and therefore represent the adipocyte population (figure 2.4 Bii). Figures Aiii & Biii are histograms showing Nile red negative and positive populations respectively. For each sample 15,000 events were collected. The analysis and presentation of the data were done using the CellQuest™ 3.1 software (Becton Dickinson Biosciences, Oxfordshire, UK).



**Figure 2.4** Sample of flow cytometry plots of Nile red analysis. (A) undifferentiated 7F2 cells and (B) following differentiation to adipocytes.

## 2.10 Stable transfection of A<sub>1</sub> and A<sub>2B</sub> receptors into 7F2 cells

### 2.10.1 Plasmid constructs

Gene constructs for human A<sub>1</sub> and A<sub>2B</sub> receptors were a kind gift from K-N Klotz (Institute for Pharmacology und Toxicology, University of Würzburg, Germany). These were cloned or sub-cloned into the plasmid pcDNA3.1 by our group.

### 2.10.2 Isolation of plasmid constructs

*E.coli* (DH5 $\alpha$ , Invitrogen, Paisley, Scotland) containing pcDNA3/A<sub>1</sub> and pcDNA3/A<sub>2B</sub> constructs were incubated overnight on nutrient agar plates containing ampicillin (100  $\mu$ g/ml) at 37°C. A single bacterial colony was inoculated in 10 ml of Luria Bertani broth containing ampicillin at 100  $\mu$ g/ml and incubated overnight at 37°C in an orbital incubator with shaking. 600  $\mu$ l bacterial broth was used to isolate pcDNA3/A<sub>1</sub> and pcDNA3/A<sub>2B</sub> plasmid DNA using PureYield™ Plasmid Miniprep System (Promega, Southampton, UK). The culture was placed into a 1.5 ml microcentrifuge tube, 100  $\mu$ l of cell lysis buffer (containing NaOH) was added, and the complex was mixed by inverting the tube 6 times. Then, 350  $\mu$ l of cold (4°C) neutralisation solution was added, the complex was mixed as before and centrifuged at 16,000  $\times$  g for 3 minutes. The supernatant was transferred to a PureYield™ minicolumn, placed into a PureYield™ collection tube and centrifuged at 16,000  $\times$  g for 15 seconds. The flow-through was discarded and the column was washed with 200  $\mu$ l of endotoxin removal wash and centrifuged at 16,000  $\times$  g for 15 seconds. The column was then washed with 400  $\mu$ l of column wash solution and centrifuged at 16,000  $\times$  g for 30 second. Plasmid DNA was collected into a clean 1.5 ml

microcentrifuge tube by the addition of 50  $\mu$ l of elution buffer directly to the centre of the column, incubation at room temperature for 1 minute and a final centrifugation for 15 seconds at 16,000  $\times$  g.

### 2.10.3 Transfection of 7F2 cells

Transfection was performed using TransFast™ Transfection Reagent (Promega, Southampton, UK) according to manufacturer's instructions. Briefly, 7F2 cells were plated in a 6 well plate at a seeding density of  $1.5 \times 10^5$  cells per well in normal growth medium on the day before transfection. For each transfection, 5  $\mu$ g of pcDNA3/A<sub>1</sub>, pcDNA3/A<sub>2B</sub> or empty pcDNA3 vector (as the control) and 15  $\mu$ l of TransFast™ Reagent (DNA:TransFast™ Reagent ratio of 1:3) were added to 1 ml of pre-warmed serum free medium, mixed by vortexing and incubated at room temperature for 15 minutes. The culture medium was aspirated and the DNA/TransFast™ Reagent mixture was added to the well and the plate was incubated for 1 hour at 37°C in a 5% CO<sub>2</sub>:95% air atmosphere. At the end of the incubation period, 2 ml of normal growth medium was added to the well and the plate was incubated for further 48 hours. Cells containing the vector or vector/DNA were selected with G418 sulphate (0.4 mg/ml) and expanded prior to experimentation. Cells were maintained in media containing 0.2 mg/ml G418 sulphate.

### 2.11 Statistical Analysis

Data are presented as the mean  $\pm$  standard error of the mean using Origin 7 software unless otherwise stated. Statistical comparisons between means were made by one-way analysis of variance (ANOVA) using the Statistical

Package for Social Sciences (SPSS) version 16. When ANOVA provided a P-value less than 0.05, post hoc analyses were performed using the Tukey test to evaluate the differences among the mean values between groups. If comparisons were made only between two groups Student's t test (SPSS 16, SPSS) was used. A P-value of less than 0.05 was considered statistically significant. In figure legends n refers to the number of experiments.

## **Chapter 3**

# **Adenosine receptor expression and function in MSCs and during their differentiation to osteoblasts**

### **3.1 Introduction**

#### **3.1.1 Bone biology**

Bone is a specialised connective tissue that, together with cartilage, makes up the skeletal system. It is composed of an organic matrix that is strengthened by deposits of calcium salts in order to provide a high level of rigidity and strength to the skeleton while maintaining some degree of elasticity (Shea and Miller, 2005). This is important for both maintaining the structural integrity of the skeleton and calcium homeostasis (Gideon, 2003). To achieve its functions bone is continuously being resorbed and formed. This can either occur at different locations, in a process called modelling which alters the size, shape, or position of the bone or in a coupled process called remodelling in which bone resorption and formation are balanced. In remodelling, old bone is continuously replaced by new tissue. This ensures that the mechanical integrity of the bone is maintained without any changes in the morphology (Gideon, 2003; Hill, 1998; Pogoda et al., 2005). Imbalances of remodelling however can occur with ageing, metabolic bone diseases, states of increased or decreased mobility and certain therapeutic interventions. The long-term result of such imbalances in remodelling is a change in bone mass, strength, structure, and eventually a variety of skeletal disorders such as osteoporosis.

#### **3.1.2 Bone cells**

##### **3.1.2.1 Osteoclast lineage**

Osteoclasts are specialised cells responsible for bone resorption. They are tissue specific macrophage polykaryons formed from the differentiation of haematopoietic stem cells of monocyte/macrophage precursors in the

circulation and bone marrow (Boyle et al., 2003). Early haematopoietic progenitor cells give rise to osteoclasts when stimulated by the macrophage colony stimulating factor (M-CSF) and RANK-L that are expressed by MSCs or their osteoblastic derivatives (Khosla, 2001). M-CSF which acts via the c-fms receptor on osteoclast progenitors promotes their differentiation and proliferation (Franzoso et al., 1997; Yoshida et al., 1990). RANK-L is necessary for fusion of the mononuclear cells into an immature osteoclast (Franzoso et al., 1997). This occurs at or near the site of resorption where the circulating precursors exit the peripheral circulation and fuse with one another to form a multinucleated immature osteoclast. Further differentiation of the immature osteoclast occurs under the continued presence of RANK-L and the polypeptide growth factor CSF-1 is required for the expression of several genes, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and the  $\beta_3$ -integrin (Lacey et al., 1998). Once the transition to mature osteoclast is reached, the bone-resorbing activity and survival of the mature osteoclast are regulated by RANK-L, hormones and cytokines (Boyle et al., 2003).

The mature osteoclasts initiate the bone resorption by peripheral attachment to the mineralised bone surface; a process that involves matrix derived signals, transmitted by the cell attachment receptor  $\beta_3$ -integrin (McHugh et al., 2000). After attaching to the bone, osteoclasts form a micro-compartment between the ruffled basal border of the cell and the bone surface.  $H^+$  ions are pumped into the compartment by the osteoclast to solubilise the mineral component by acidification of the extracellular environment, which causes a

decrease in pH. Following this, CATK, a lysosomal protease, degrades the organic component (Downey and Siegel, 2006). This process produces irregular scalloped cavities on the bone surface, called Howship's lacunae. Osteoclasts will then detach from the site and osteoblasts will come into the area and replace the resorbed bone.

### 3.1.2.2 Osteoblast lineage

Osteoblasts are derived from MSCs that also give rise to chondrocytes, myoblasts and adipocytes as described in chapter 1. The process of osteoblast differentiation has been subdivided into three stages: 1) proliferation, 2) extracellular matrix development and maturation, and 3) mineralisation (figure 3.1.1). At the proliferation stage committed osteoprogenitors, *i.e.* progenitor cells restricted to osteoblast development and bone formation, divide rapidly to generate sufficient numbers of preosteoblasts for bone formation. During proliferation of preosteoblasts, mitotic activity is reflected by the expression of cell cycle regulated genes *e.g.*, histones and proto-oncogenes. Several genes associated with formation of the extracellular matrix such as type I collagen and fibronectin are expressed at this stage (Aubin, 2001).

As preosteoblasts differentiate into more mature osteoblasts, they begin to secrete bone matrix proteins. Type I collagen is the major protein in bone matrix, comprising 90 - 95% of the organic matrix. The network of type I collagen fibres provides the structure on which bone mineral is deposited. During matrix mineralisation osteoblasts also secrete a number of non-



collagenous proteins that are incorporated into the extracellular matrix (Downey and Siegel, 2006). The non-collagenous proteins include proteoglycans, sulfated glycoproteins and proteins modified to contain Gla amino acid residues (Gundberg, 2003). The main proteoglycans secreted by osteoblasts are decorin and biglycan that belong to the family of small leucine-rich proteoglycans (SLRPs). Biglycan binds to several growth factors, including TGF- $\beta$  and mediate their action on osteoblast precursors. Decorin on the other hand appears to regulate collagen fibrillogenesis. Osteonectin, bone sialoprotein, osteopontin, tenascin-C and fibronectin are among the glycoproteins present in the bone matrix, all of which are important in bone cell adhesion, migration, proliferation and/or differentiation. Other osteoblast derived proteins include osteocalcin and matrix Gla protein that are regulators of bone formation and mineralisation (Gundberg, 2003).

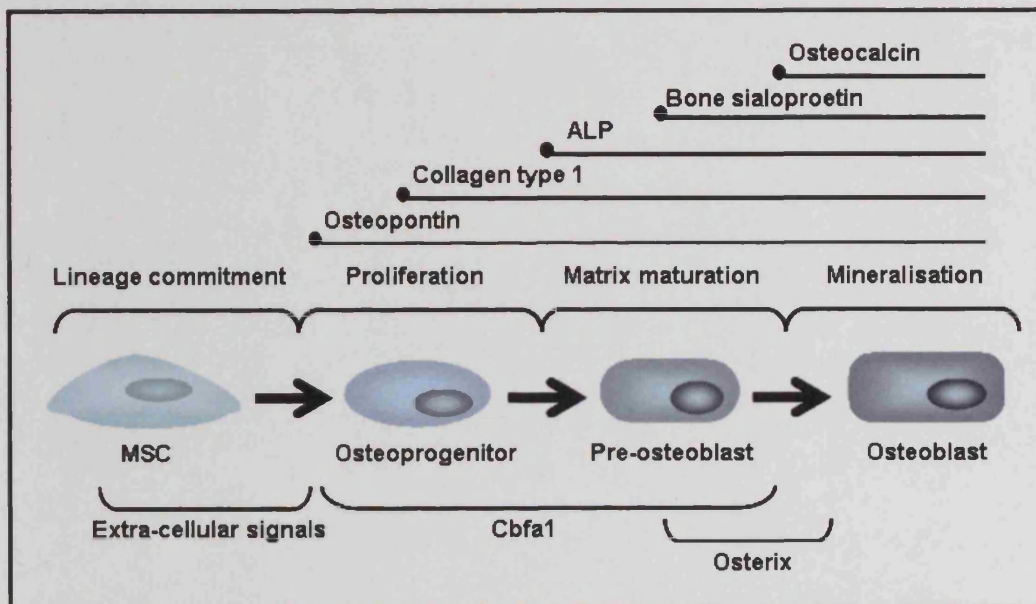
As a result of changes during differentiation, bone matrix undergoes a series of modifications in composition and organisation that facilitate mineralisation via the formation of hydroxyapatite. This is thought to occur through two possible mechanisms; i) the formation of matrix vesicles, small vesicles that bud from the plasma membrane and accumulate high concentrations of  $\text{Ca}^{2+}$  and inorganic phosphate that create an optimal environment for the formation of hydroxyapatite (Anderson, 2003), and/or ii) the nucleation of collagen, regulated by noncollagenous proteins that are incorporated into the bone matrix such as bone sialoprotein, osteocalcin and osteonectin (Boskey, 1998). Osteoblasts also contribute to the process of mineralisation by expression of ALP, a membrane-bound marker of osteoblast differentiation that hydrolyses

pyrophosphate, thereby generating the inorganic phosphate that is required for hydroxyapatite crystal formation (Balcerzak et al., 2003).

Once the osteoblasts have differentiated and accomplished their cycle of bone matrix formation, they can become trapped within the matrix as osteocytes (Knothe Tate et al., 2004). When incorporated in bone matrix, they begin to generate long cytoplasmic processes to remain in communication with surrounding cells and express E11, an early osteocyte marker (Wetterwald et al., 1996). As the matrix matures and mineralises, osteocytes become located deeper within the bone tissue, begin to mature and express a new set of genes, including matrix extracellular phosphoglycoprotein (MEPE) and sclerostin (Nampei et al., 2004; van Bezooijen et al., 2005). The mature osteocytes are thought to be important in cellular communication and nutrition within a mineralised matrix. They act as mechanosensory cells that communicate with other bone cells (osteoblasts and osteoclasts) to coordinate formation and resorption of the bone. Osteocytes are also involved in cell-mediated exchanges of minerals between the fluids in the bone and the vascular supply (Bonewald, 2007; Nakamura, 2007).

An alternative fate of some osteoblasts is differentiation into flat and inactive lining cells on the bone surface. Little is known about the function of these cells. It has been proposed that they secrete collagenase, which removes bone matrix and uncover a mineralised surface of the bone in preparation for osteoclastic resorption. Bone lining cells may also mediate the crystal growth

in bone, or function as a barrier between extracellular fluid and bone (Downey and Siegel, 2006).



**Figure 3.1.1** Postulated steps in the osteoblast differentiation process. Modified from Aubin (2001).

### 3.1.3 Osteoblastic induction

Osteoblastic differentiation of MSCs is well established *in vitro*. Differentiation is normally induced by addition of a combination of dexamethasone (Dex), ascorbic acid, and  $\beta$ -glycerophosphate ( $\beta$ -GP). Dex, a synthetic glucocorticoid, has been shown to induce osteoblastogenesis in both human and rat MSCs (Atmani et al., 2003; Cheng et al., 1996). Dex consistently induces ALP activity, expression of osteocalcin and bone sialoprotein, enhances PTH-induced cAMP production, and increases the rates of *in vitro* mineralisation in many *in vitro* models (Atmani et al., 2003; Jørgensen et al., 2004). However, the effects are not lineage specific, and Dex has also been shown to induce differentiation and maturation of adipocytes (see section 4.1.3). Ascorbic acid is thought to exert its effects on osteoblastogenesis by enhancing the production of the collagenous bone extracellular matrix. The

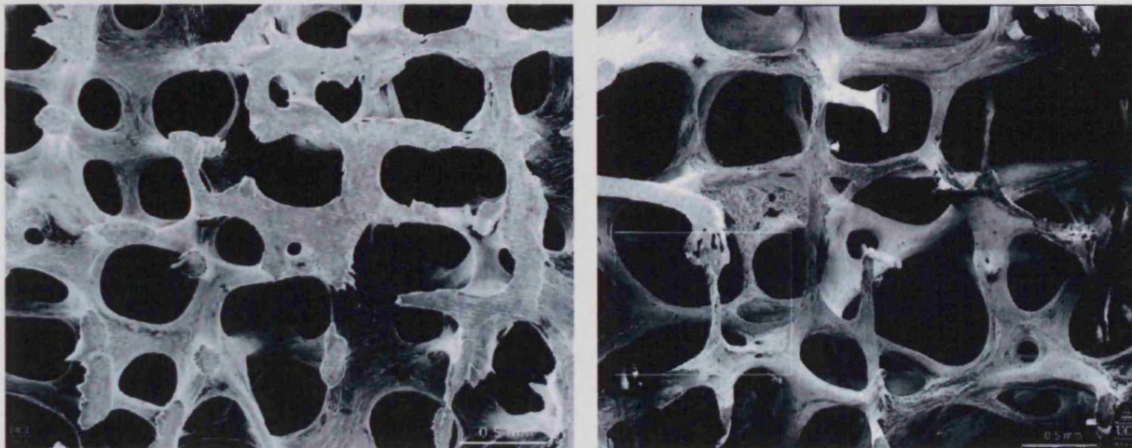
effect is mainly attributed to the essential role of ascorbic acid as a cofactor in the hydroxylation of proline and lysine residues and subsequent increase in the proline hydroxylation pool, the secretion and processing of type I procollagen and procollagen synthesis and gene expression (Alcaín and Burón, 1994; Franceschi et al., 1994). Ascorbic acid stimulated collagen synthesis is also shown to increase bone markers such as ALP and osteocalcin (Franceschi et al., 1994; Shiga et al., 2003; Takamizawa et al., 2004). One major disadvantage of ascorbic acid for use in culture system is its instability in solution. Therefore, the more stable ascorbic acid 2-phosphate (AA) with similar cofactor activity for collagen biosynthesis has been used instead of ascorbic acid in cultures of osteoblastic cells (Takamizawa et al., 2004). Although Dex and ascorbic acid are able to induce osteoblastic differentiation as evidenced by the expression of osteoblastic makers and extracellular matrix formation, in most cases  $\beta$ -GP is required for mineralisation of extracellular matrix. Mineralisation depends on a constant supply of phosphate ions, which is necessary for hydroxyapatite crystal formation (Balcerzak et al., 2003).  $\beta$ -GP is known to act as a substrate to produce high levels of local phosphate ions at sites of mineralisation when hydrolysed by ALP.  $\beta$ -GP has been also reported to induce osteogenesis and ALP activity (Coelho and Fernandes, 2000; Hamade et al., 2003).

### **3.1.4 Bone diseases**

#### **3.1.4.1 Osteoporosis**

Osteoporosis is characterised by low bone mass and microarchitectural deterioration of bone tissue (figure 3.1.2) resulting in enhanced bone fragility

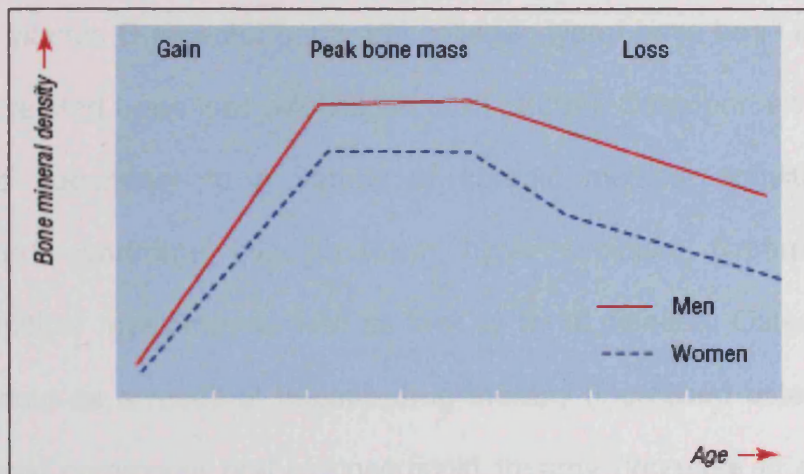
and a consequent increase in fracture risk (Christiansen, 1991). It is a well-recognised cause of morbidity and mortality in older men and women (Reginster and Burlet, 2006). The prevalence is higher in women than men, with 1 in 3 women beyond the age of 50 years being affected (Downey and Siegel, 2006). The lower incidence of osteoporosis in men is mainly due to greater bone mass and size as well as the absence of an equivalent male menopause (Olszynski et al., 2004).



**Figure 3.1.2 Normal (left) and osteoporotic (right) bones.** From Poole and Compston (2006).

In both men and women, peak bone density occurs early in life, when there is a combination of anabolic effects due to sex hormone and environmental influences leading to linear increases in bone mass favouring bone formation (Heaney et al., 2000). At peak bone mass the amount of bone formation is exactly matched by the amount of resorption (figure 3.1.3). However, with age a process of bone loss begins as a consequence of increased and imbalanced bone remodelling such that bone resorption exceeds bone formation (Simon, 2007). This occurs either as result of increase in the number and/or activity of osteoclasts, reduction in number and/or the function

of osteoblasts or a combination of the two, mainly due to changes in the production of local or systemic growth factors (Abdallah et al., 2006; Cheleuitte et al., 1998; Manolagas, 2000; Seck et al., 2001).



**Figure 3.1.3 Age related changes in bone mass throughout life, in women and men.** From Poole and Compston (2006).

In women, these changes often take place at the same time as the onset of menopause, which is associated with the decrease in the female sex hormone, estrogen, an important regulator of osteoblast and osteoclast formation and activity. The loss of estrogen elevates the rate of bone remodelling, by promoting the formation of osteoclasts and osteoblasts from their progenitors. This is sufficient to increase the loss of bone, as resorption always precedes and is faster than bone formation and new bone is less dense than older bone. In addition, estrogen deficiency increases the lifespan of osteoclasts that consequently leads to excessively deep cavities, which cannot be filled by the action of the osteoblasts. On the other hand, the loss of estrogen leads to shorter lifespan of osteoblasts that is unable to efficiently fill in normal sized lacuna (Manolagas, 2000; Manolagas et al., 2002).

The loss of bone in the elderly is also closely related to nutritional intake, especially vitamin D and calcium insufficiency, hormonal status such as decreased production of IGFs and also physical activity (Poole and Compston, 2006). Several genes that are involved in peak bone mass such as the vitamin D receptor gene and collagen type I have been also implicated in age related bone loss (McGuigan et al., 2002). Osteoporosis could also be caused secondary to a variety of chronic medical conditions such as Cushing's syndrome, hypogonadism, hyperthyroidism, hyperparathyroidism, RA, multiple myeloma, as well as liver or renal disease. Osteoporosis could also occur as a result of heparin drug therapy (increased osteoclast activity) and most commonly oral glucocorticoid therapy (increase in osteoclast and decrease in osteoblast life span) (Mauck and Clarke, 2006; Simon, 2007).

### **Pharmacological interventions**

Pharmacological therapies available for the treatment of osteoporosis are mostly drugs that target osteoclast induced bone resorption. Bisphosphonates (alendronate, risedronate and ibandronate) are the most potent agents available for treatment of osteoporosis that block bone resorption by inhibiting osteoclast activity (Mauck and Clarke, 2006; Simon, 2007). These compounds bind to hydroxyapatite crystals and coat the surface of the bone. However, as osteoclasts arrive at the site of resorption, the bisphosphonates detach from the bone and are taken up by osteoclasts, and subsequently inhibit their activity. It is also more difficult for osteoclasts to bind to and exert their action on the coated bone surface (Simon, 2007). Calcitonin is another anti-resorptive agent that inhibits the activity of osteoclasts, but not as effective as

bisphosphonates. It apparently acts by blocking the formation of the ruffled border by osteoclasts and also decreasing the number of osteoclasts (Zaidi et al., 2002).

Strontium ranelate that reduces osteoclastogenesis and promotes osteoblast replication, estrogen replacement and selective estrogen receptor modulators (Raloxifene) that act by reducing bone resorption, are among other anti-resorptive therapies available. Calcium and vitamin D are also often given as an adjunct to other therapies for osteoporosis (Compston, 2009; Meunier et al., 2004; Poole and Compston, 2006).

Recombinant human PTH *i.e.* teriparatide (1 - 34 PTH peptide) and Preotact (1 - 84 PTH peptide), both of which promote bone formation, are currently the only anabolic drug available (Compston, 2009). Low and intermittent doses of PTH have been shown to stimulate bone formation and increase bone mineral density (Blick et al., 2008; Cranney et al., 2006). PTH is thought to promote bone formation by increasing the life span of mature osteoblasts by reducing the rate of their apoptosis as well as inducing their differentiation from MSCs. These actions of PTH are mainly attributed to receptor coupling and the subsequent activation of adenylate cyclase, cAMP and PLC (Blick et al., 2008).



### 3.2 Aims

This chapter describes the expression and function of adenosine receptors in MSCs and during their differentiation to osteoblasts.

Specifically the aims are:

- To investigate the expression and function of adenosine receptors in MSCs.
- To induce differentiation of MSCs toward the osteoblast lineage and subsequently investigate the changes in expression and function of adenosine receptors at various stages of differentiation.
- To investigate the role of adenosine signalling pathways in the regulation of osteoblastic differentiation of MSCs.

### **3.3 Methods**

#### **3.3.1 Analysis of mRNA expression**

MSCs were cultured in 6 well multidishes at a seeding density of  $5 \times 10^4$  cells per well in normal growth medium. After 24 hours, the medium was replaced with osteoblastic differentiation medium consisting of normal growth medium supplemented with 50  $\mu\text{g/ml}$  AA and  $10^{-8}$  M Dex in the presence or absence of the indicated concentrations of adenosine or NECA as well as appropriate vehicle controls. The cells were treated for the indicated periods, with the media being changed every 2 - 3 days. At the end of the incubation periods, total RNA was isolated and DNase treated. The quality and quantity of RNA was determined spectrophotometrically by measuring absorbance at 260/280 nm. cDNA was synthesised from 1  $\mu\text{g}$  of total RNA and subjected to q-RTPCR amplification using specific primers for the target genes. Detailed methodology has been described in chapter 2.

#### **3.3.2 Analysis of protein expression**

MSCs were cultured as described above and cell lysates were prepared at indicated time points during osteoblastic differentiation by treating cells with RIPA buffer and the protein content determined using the Bio-Rad protein assay. Proteins were separated on 10% SDS-PAGE, transferred to PVDF membranes and expression of adenosine receptors detected. Blots were stripped and re-probed with  $\beta$ -actin antibody for normalisation. The ratios of adenosine receptor expression during the differentiation to the housekeeping control  $\beta$ -actin were calculated from densitometry and compared to control (undifferentiated cells). Detailed methodology is described in chapter 2.

### 3.3.3 Analysis of cAMP accumulation

MSCs were prepared as above. After differentiation for the indicated time points or following mineralisation, cells were stimulated for 15 minutes with various concentrations ( $10^{-8}$  M to  $10^{-4}$  M) of adenosine, NECA, or CGS 21680 in the presence of 10  $\mu$ M RO 20-1724 at 37°C. Reactions were stopped by rapid aspiration of media and the cyclic nucleotides extracted by the addition of 200  $\mu$ l of 0.1 M HCl. cAMP in the acid extract was measured using a radioimmunoassay as described in section 2.5.2. For undifferentiated MSCs, cells were allowed to reach 80% confluence before assaying for cAMP. To assess the effects of  $A_1$  receptor on cAMP, cells were pre-incubated for 15 minutes with the  $A_1$  receptor agonist CCPA at varying concentrations ( $10^{-8}$  M to  $10^{-4}$  M) in the presence of 10  $\mu$ M RO 20-1724 at 37°C. The cells were then incubated in the same medium for further 5 minutes in the presence of forskolin ( $0.5 \times 10^{-5}$  M) (to stimulate cAMP production).

### 3.3.4 Analysis of ALP activity

MSCs were cultured in 24 well multidishes at a seeding density of  $4 \times 10^3$  cells per well in normal growth medium. After 24 hours, cells were treated with adenosine receptor agonists and antagonists (as well as appropriate vehicle controls) during osteoblastic differentiation for the indicated periods, with the media being changed every 2 - 3 days. In some experiments MSCs were pre-differentiated with osteoblastic medium for 5, 7 and 9 days before treating with various concentrations of agonists for a further 2 days in the presence of osteoblastic differentiation medium. ALP activity was assessed colourimetrically as the release of the yellow chromogen p-nitrophenyl from p-

nitrophenyl phosphate substrate, as described in section 2.8.1. Protein content of cells was measured using the Bio-Rad protein assay in parallel experiments. The ALP results were corrected for protein content and expressed as percentage of control values.

### **3.3.5 Analysis of mineralisation**

MSCs were cultured in 12 well multidishes at a seeding density of  $2 \times 10^4$  cells per well in normal growth medium. After 24 hours, cells were incubated with osteoblastic differentiation medium supplemented with 2 mM  $\beta$ -GP in the presence of the indicated concentrations of adenosine receptor agonists and antagonists as well as appropriate vehicle controls for 10 to 12 days. Treatments were replenished every weekday since adenosine has a very short half life. Mineralisation was determined by staining for calcium deposits using Alizarin Red S (described in section 2.8.2)

### **3.3.6 Analysis of cell number**

MSCs were cultured in 96 well multidishes at a seeding density of  $1.5 \times 10^3$  cells per well in normal growth medium with reduced serum content (5% FCS) (optimum serum concentration for the proliferation assay was determined by plotting growth curves for several serum concentrations *i.e.* 1%, 5% and 10% FCS) (see figure 3.4.3 A). After 24 hours, the indicated concentrations of adenosine receptor agonists (as well as appropriate vehicle controls) were added for 2 and 5 days. Cell proliferation was measured by the MTS assay as in section 2.7.

## 3.4 Results

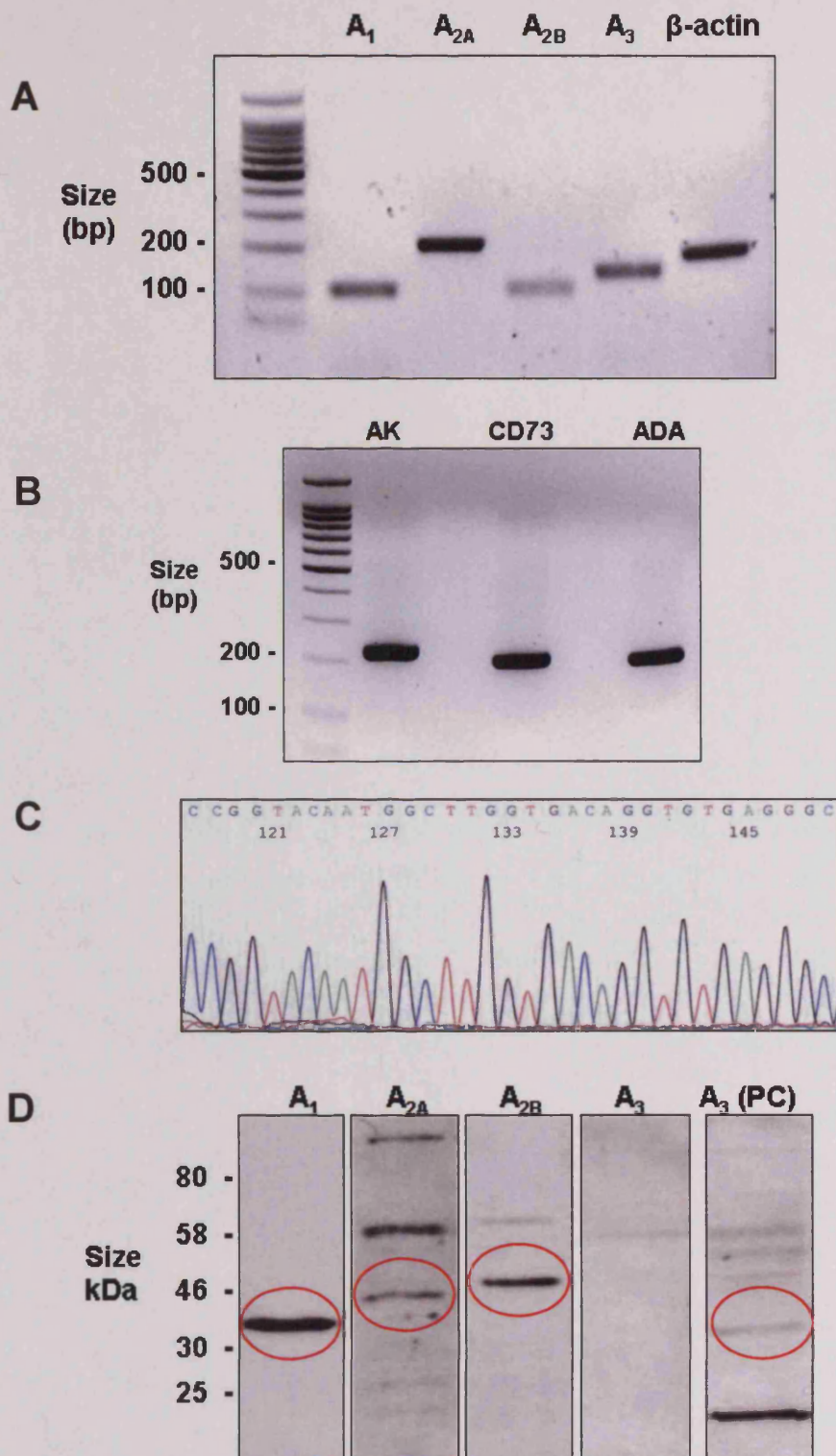
### 3.4.1 Adenosine receptor expression and function in MSCs

#### 3.4.1.1 mRNA expression

Initially the involvement of adenosine signalling in MSCs was studied by investigating the expression of mRNA for adenosine receptors and enzymes in the ATP - adenosine pathway by using standard RT-PCR. Amplification of mRNA gave rise to sharp bands of appropriate sizes ( $A_1$ : 100bp,  $A_{2A}$ : 200bp,  $A_{2B}$ : 100bp,  $A_3$ : 129bp, CD73: 192bp, ADA: 198bp, AK: 225bp,  $\beta$ -actin: 165bp) when visualised on an agarose gel stained with ethidium bromide (figure 3.4.1 A&B). Sequencing of amplicons confirmed gene expression, an example is shown in figure 3.4.1 C.

#### 3.4.1.2 Protein expression

Although RT-PCR demonstrated that mRNA for  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  receptors are expressed in MSCs, it does not predict the presence of adenosine receptors at the protein level. The effective translation of mRNA into the corresponding protein was therefore investigated by Western blotting of whole cell lysates using affinity-purified rabbit polyclonal antibodies for each receptor (figure 3.4.1 D). The antibody against the adenosine  $A_1$  receptor recognised a single band with the expected molecular weight of around 38 kDa. The antibody against the  $A_{2A}$  adenosine receptor recognised a specific band of around 46 kDa, corresponding to its expected molecular weight; in addition to two non-specific bands with higher molecular mass of about 60 and 130 kDa were also detected.



**Figure 3.4.1** Expression of adenosine receptors and enzymes on rat MSCs. RT-PCR analysis of rat MSC mRNA using primers (A) for A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, A<sub>3</sub> receptors and (B) adenosine metabolic (CD73) and catabolic enzymes ADA and AK. Left margin indicates the sizes (bp) of DNA ladder. (C) Example of DNA sequencing fragment for a PCR product (A<sub>2A</sub> receptor). (D) Western blot analysis of adenosine receptor protein expression. Positive control for the A<sub>3</sub> receptor is shown. Left margin indicates the sizes (kDa) of protein marker.

The antibody against the A<sub>2B</sub> adenosine receptor recognised a band of around 52 kDa, consistent with the published data for the A<sub>2B</sub> protein by other research groups (Peyot et al., 2000; Puffinbarger et al., 1995; Trincavelli et al., 2004). An extra band of low intensity at 65 kDa was also seen on the A<sub>2B</sub> immunoblots. There was no detectable band for the A<sub>3</sub> receptor. To confirm the absence of the A<sub>3</sub> receptor a cell line (GH3 pituitary cells) transfected with the A<sub>3</sub> receptor was used as a positive control and this showed a faint band with the expected molecular weight of around 36 kDa, in addition to bands of around 17 and 55 kDa. These data show that MSCs express A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> but undetectable levels of the A<sub>3</sub> receptor at the protein level.

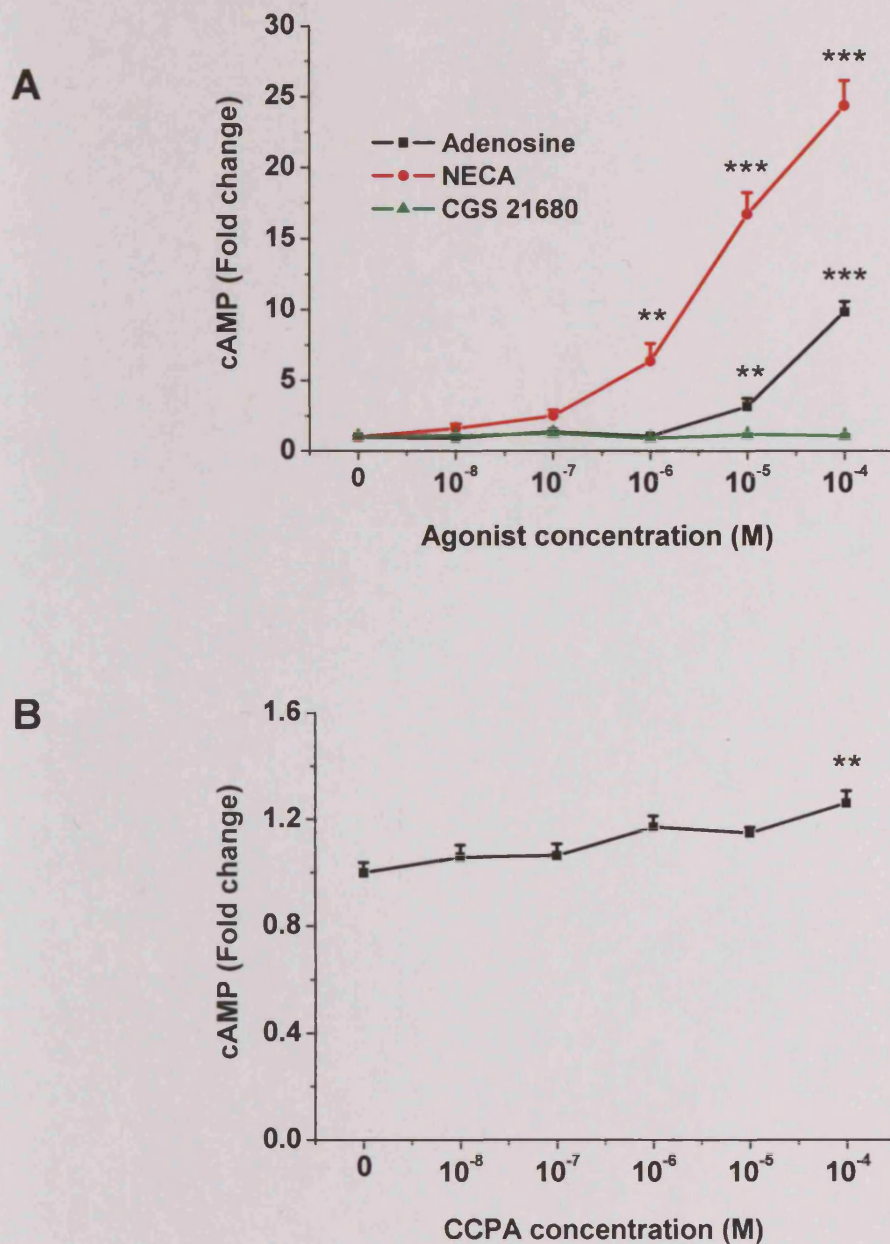
#### **3.4.1.3 Adenosine mediated cAMP responses in MSCs**

Adenosine receptors can be functionally identified using selective adenosine receptor agonists and their coupling to cAMP. Adenosine produced a dose dependent accumulation of cAMP in MSCs, suggesting that the A<sub>2</sub> rather than A<sub>1</sub> or A<sub>3</sub> receptors are functionally predominant (figure 3.4.2 A). This does not however rule out the presence of A<sub>1</sub> and A<sub>3</sub> receptors in MSCs. To find out whether the observed cAMP responses to adenosine were due to A<sub>2A</sub> or A<sub>2B</sub> receptor activation, cells were stimulated with CGS 21680, a high-affinity selective A<sub>2A</sub> receptor agonist, and NECA, a synthetic universal adenosine receptor agonist. No sufficiently selective A<sub>2B</sub> receptor agonist is currently commercially available. Therefore NECA when used in conjunction with CGS 21680 can be used to demonstrate the presence of A<sub>2B</sub> receptors; a greater potency of NECA compared with CGS 21680 indicates the existence of A<sub>2B</sub> receptors.

Treatment of MSCs with adenosine and NECA produced dose dependent accumulations of cAMP. NECA was the most efficacious agonist; at 100  $\mu$ M, it stimulated endogenous cAMP levels by 24 fold ( $P < 0.001$ ), whereas similar concentrations of adenosine showed a 9 fold ( $P < 0.001$ ) increase. In contrast, CGS 21680 was ineffective and failed to elicit a cAMP response at concentrations up to 100  $\mu$ M (figure 3.4.2 A). These findings indicate a rank order of potency of NECA > adenosine > CGS 21680. This potency order is consistent with the published pharmacology profile of  $A_{2B}$  receptors (Feoktistov and Biaggioni, 1998).

Although these results suggest the predominance of the  $A_{2B}$  receptor, it does not preclude the co-expression of functional  $A_1$  receptors as MSCs were shown to express this receptor at the mRNA and protein level. The functional presence of the  $A_1$  receptor was therefore investigated by measuring the changes in cAMP production in response to incubation with the  $A_1$  receptor agonist CCPA followed by stimulation with forskolin. Since the  $A_1$  receptor is negatively coupled to adenylate cyclase a reduction in forskolin stimulated cAMP could be expected. In MSCs CCPA at a concentration of  $10^{-4}$  M resulted in an apparent increase in cAMP production (figure 3.4.2 B) whereas at lower concentrations there was little or no effect on forskolin stimulated cAMP. A possible explanation for this is that CCPA loses receptor selectivity at high concentrations and is able to bind to  $A_2$  receptors (Klotz et al., 1997). These data, together, indicate a dominance of functional  $A_{2B}$  receptors when compared with  $A_1$ ,  $A_{2A}$  and  $A_3$  receptors in MSCs.





**Figure 3.4.2 Effects of adenosine receptor agonists on intracellular cAMP production in MSCs.** MSCs were incubated (A) with the indicated concentrations of adenosine, NECA and CGS 21680 for 15 minutes in the presence of the cAMP PDEs inhibitor RO 20-1724 ( $10^{-4}$ M) and cAMP was measured by an in-house RIA (n=3; total of 12 replicates). (B) To assess the presence of  $A_1$  receptors, cells were pre-incubated for 15 minutes with CCPA ( $10^{-8}$  M to  $10^{-4}$  M) in the presence of 10  $\mu$ M RO 20-1724 at 37°C and for a further 5 minutes with forskolin ( $0.5 \times 10^{-5}$  M) (n=2; total of 8 replicates). \*\*P < 0.01, and \*\*\*P < 0.001 when compared with untreated control.

#### **3.4.1.4 Effects of adenosine receptor agonists on MSC cell number**

Since the adenosine signalling pathway has been linked to cell death and proliferation in various tissues (Hofer et al., 2008; Lelievre et al., 1998; Merighi et al., 2002b; Olah et al., 2008; Rees et al., 2002; Shimegi, 1998), the effect of adenosine receptor agonists on the number of MSCs was investigated. Cells were treated with various concentration of adenosine, NECA, CGS 21680 and CCPA in a reduced serum condition (5% FCS was shown to support moderate growth of the cells as determined by growth curve studies (figure 3.4.3 A) for 2 and 5 days and cell number was assessed by the MTS assay. Except for the highest concentration of NECA *i.e.*  $10^{-4}$  M, which caused a 10% reduction in the number of MSCs after 5 days, there was no significant difference in cell number between control and agonist treated cells (figure 3.4.3 B). The observed effects of  $10^{-4}$  M NECA may be due to cell toxicity.

#### **3.4.1.5 Effects of adenosine receptor agonists on ALP expression and activity in MSCs**

As described previously, MSCs expressed functional  $A_{2B}$  adenosine receptors. To examine whether activation of these receptors alone is sufficient for inducing differentiation to osteoblasts, MSCs were grown in normal growth medium in the presence of adenosine receptor agonists for 2, 5, 7, 9 and 12 days and ALP mRNA expression determined.

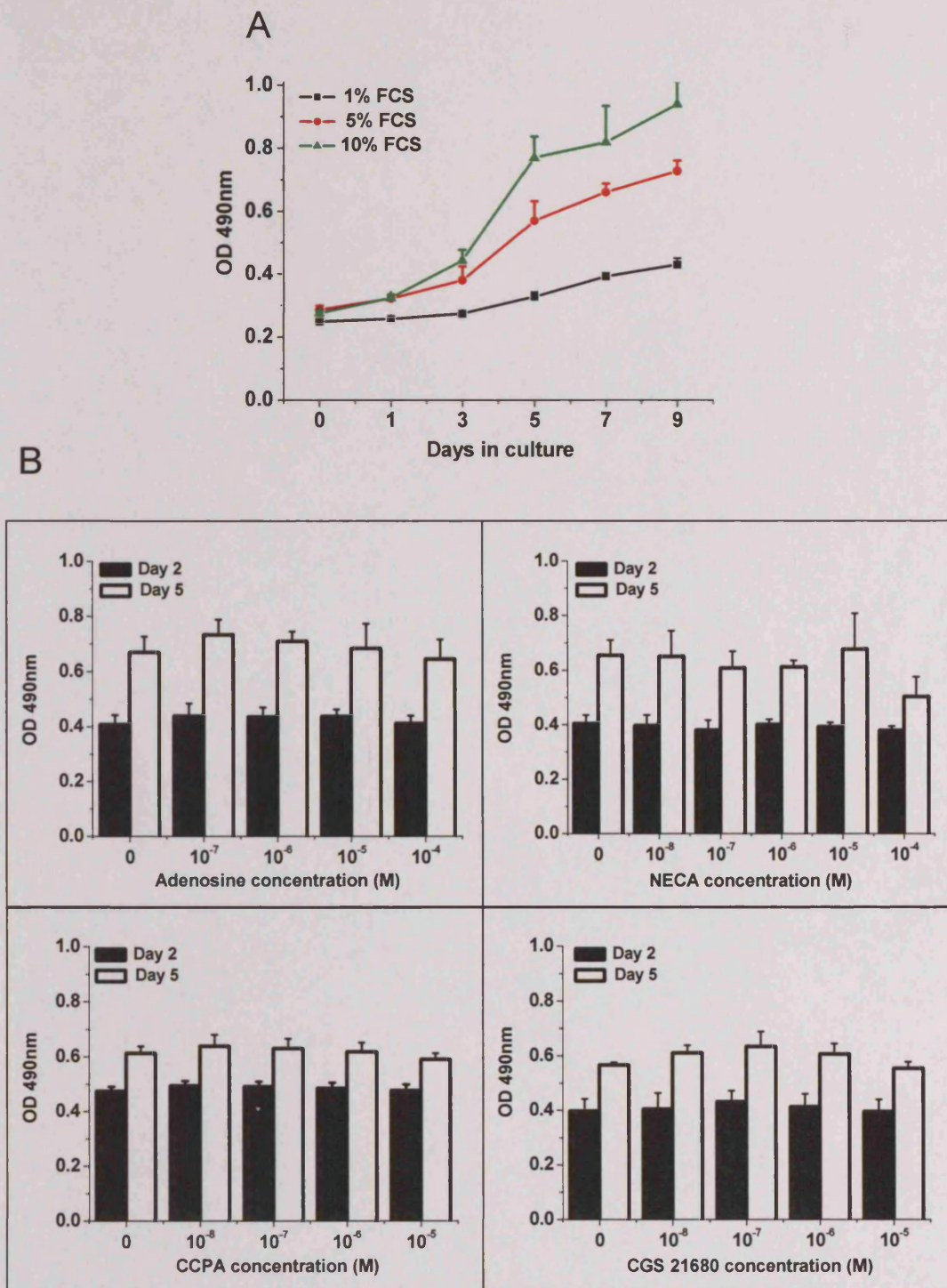
Time course experiments using q-RTPCR showed that NECA at ( $10^{-5}$  M) significantly induced ALP mRNA expression by 6 fold at days 5 - 7 when compared with untreated MSCs (figure 3.4.4 A). NECA induction of ALP

mRNA expression was accompanied with increased enzyme activity (figure 3.4.4 B). A dose dependent effect was observed when MSCs were treated with NECA for 2 and 5 days with a significant effect being detectable at concentrations as low as  $10^{-6}$  M. Consistent with the ALP mRNA results the enzyme response was most evident following 5 days of treatment. Consistent with the lack of  $A_{2A}$  receptors, CGS 21680 had no effect on ALP enzyme activity; a similar finding was seen when adenosine was used as the agonist (figure 3.4.5 A&B). The lack of effect of adenosine is unclear but may reflect its rapid metabolism.

### **3.4.2 Adenosine receptor expression during osteoblastogenesis**

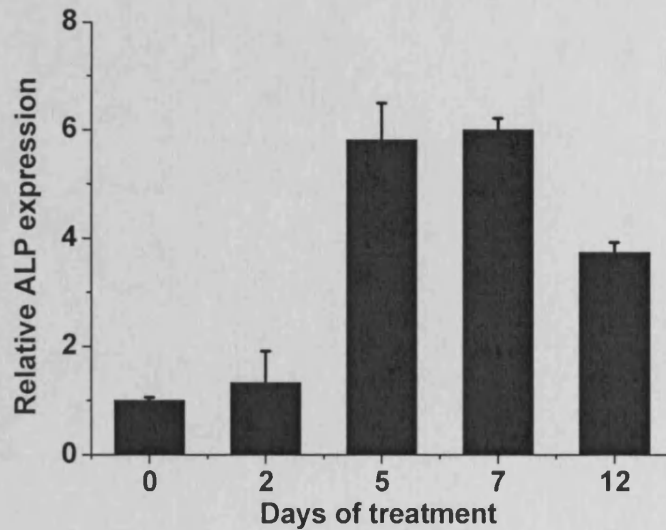
#### **3.4.2.1 Differentiation of MSCs into osteoblasts**

In order to study the expression and function of adenosine receptors during osteoblastic differentiation, it was necessary to first determine the osteoblastic differentiation potential of MSCs derived from rat bone marrow. MSCs have been shown to differentiate into osteoblasts by osteogenic medium (containing Dex and AA) and to undergo matrix mineralisation by addition of  $\beta$ -GP. Expression of mRNA of markers of various stages of osteoblast differentiation namely Cbfa1, collagen type I, ALP and osteocalcin was investigated during a time course of 0, 2, 7 and 12 days of osteogenic induction. Under basal conditions (day 0), MSCs expressed some of these markers. Cbfa1, type I collagen and ALP mRNAs were all easily detectable. In contrast, osteocalcin, the late marker of differentiation, was barely detectable. When treated with osteogenic medium mRNA expression of all the markers,

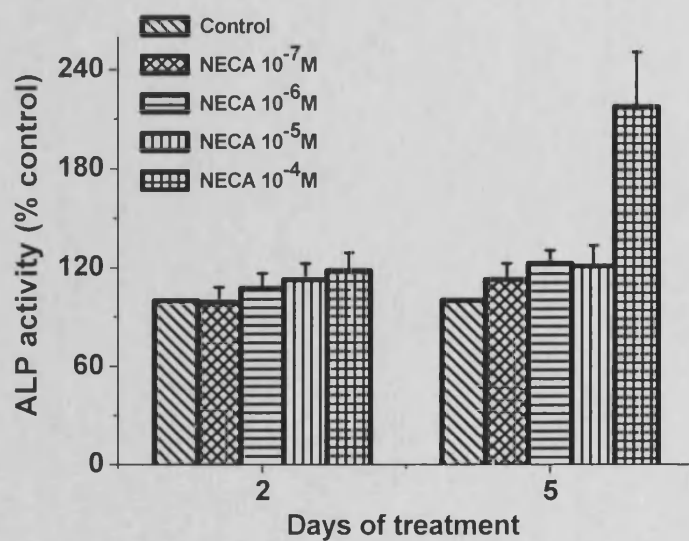


**Figure 3.4.3 Effects of adenosine receptor agonists on MSC cell number.** (A) Growth curves for MSCs using 1%, 5% and 10% FCS. (B) MSC cell number following 2 and 5 days of treatment with indicated concentrations of adenosine, NECA, CCPA and CGS 21680 (n=2; total of 24 replicates). Data are expressed as mean  $\pm$  standard deviation of the mean (SDM).

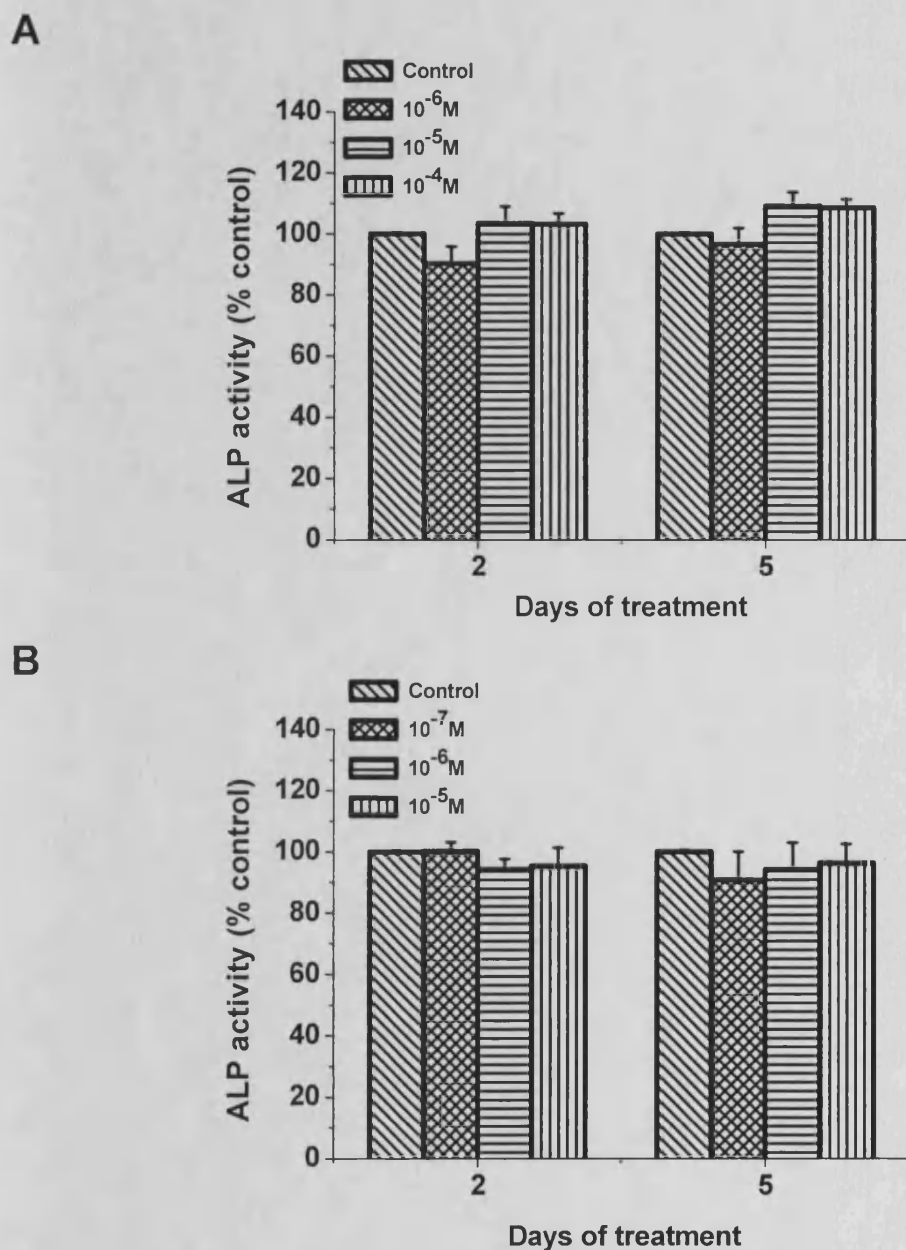
A



B



**Figure 3.4.4 Effects of NECA on the induction of ALP mRNA expression and enzyme activity in MSCs.** MSCs were treated with the indicated concentrations of NECA or DMSO (vehicle control) for the indicated period. Total cellular RNA was isolated and **(A)** mRNA expression of ALP was quantified with q-RT-PCR. Expression was normalised to  $\beta$ -actin and presented as the relative mRNA expression level to untreated MSCs ( $n=2$ ; total of 4 replicates). **(B)** ALP activity was measured and normalised to protein content ( $n=2$ ; total of 8 replicates). Data are expressed as mean  $\pm$  SDM.



**Figure 3.4.5 Effects of adenosine and CGS 21680 on ALP activity in MSCs.** MSCs were treated with indicated concentrations of (A) adenosine, (B) CGS 21680 or DMSO (vehicle control) for the indicated times. ALP activity was measured and normalised to protein content ( $n=2$ ; total of 8 replicates).

as expected, increased strongly with time when compared with day 0 time point after normalisation to  $\beta$ -actin (figure 3.4.6 A). *Cbfa1*, type I collagen and ALP mRNA expression were significantly increased at day 7 and reached 7 - 20 fold by day 12. Osteocalcin mRNA expression was barely detectable at day 2, but was massively induced ( $>10^4$  fold) at day 7 and  $0.5 \times 10^5$  fold after day 12 of osteoblastogenesis.

Parallel to osteoblastic marker expression, terminal differentiation of osteoblasts derived from MSCs was examined by their ability to undergo mineralisation, when cultured in the presence of osteogenic medium supplemented with  $\beta$ -GP. The first signs of mineralisation become apparent under microscopic visualisation after about 7 - 8 days and increased rapidly thereafter. Alizarin Red S positive calcium deposits were detectable in treated cultures, but not in those with basal medium (figure 3.4.6 B).

#### **3.4.2.2 Adenosine receptor and adenosine metabolic enzyme mRNA expression during osteoblastogenesis**

Having demonstrated the presence of functional adenosine receptors in undifferentiated MSCs, possible alterations in adenosine receptor expression associated with varying stages of differentiation was investigated. The relative mRNA expression of the four adenosine receptors during osteoblastogenesis is shown in figure 3.4.7. Expression of  $A_1$  receptor mRNA was upregulated by up to 8 fold between days 5 and 12 whereas the  $A_{2A}$  and  $A_{2B}$  receptors showed small increases (2 fold). Interestingly  $A_{2B}$  receptor expression also appeared to decrease beyond day 7. There was no significant difference in

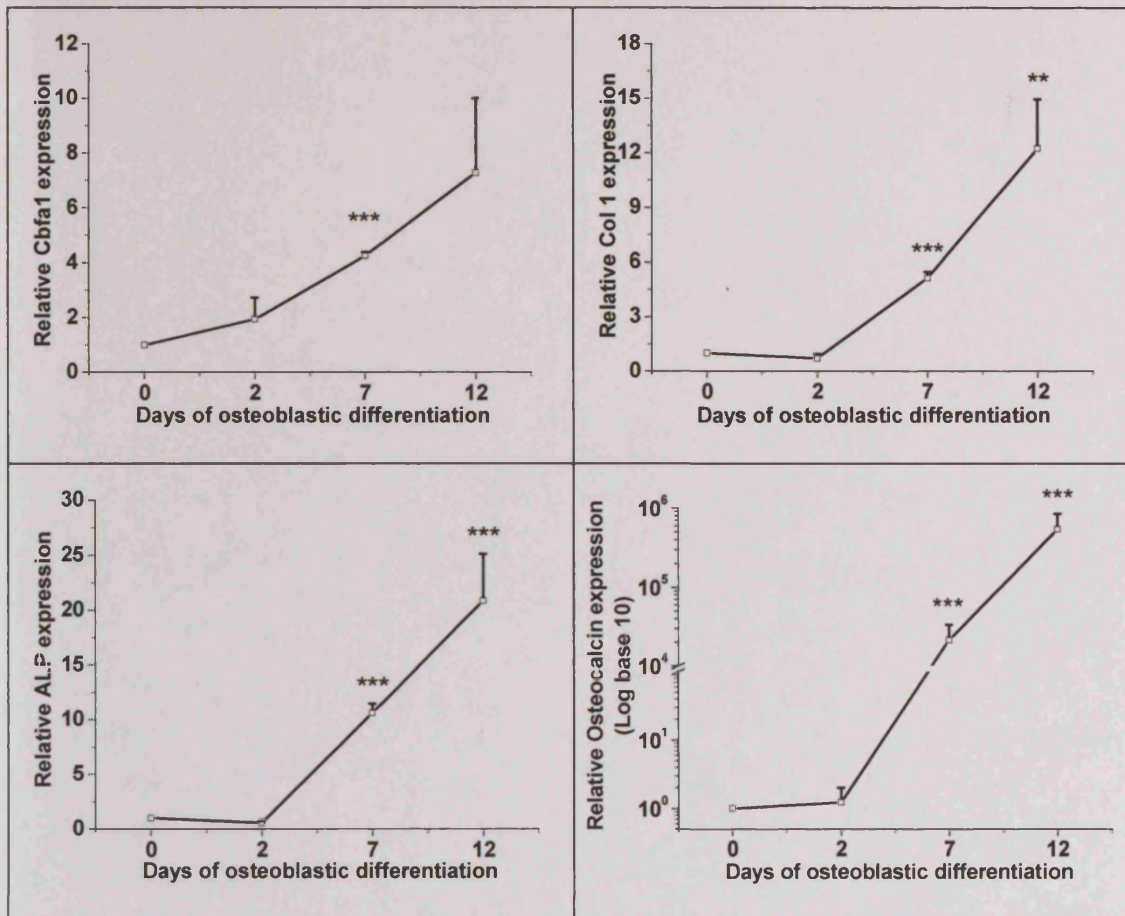
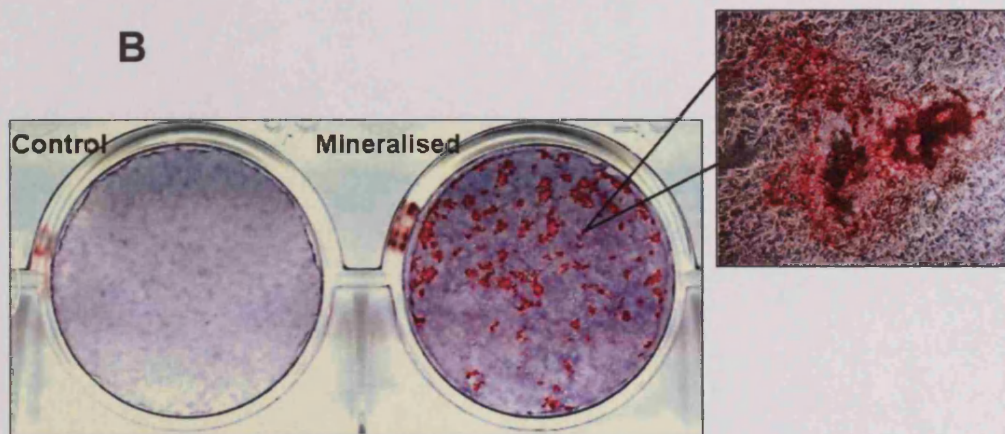
expression of A<sub>3</sub> receptor mRNA at any time point during osteoblastic differentiation.

Since CD73 and ADA are vital for metabolism and catabolism of extracellular adenosine, the changes in mRNA expression of these enzymes were also investigated during the process of differentiation. CD73 mRNA decreased (50% P < 0.05) by day 2 of osteoblastic differentiation and then progressively returned back to levels seen in undifferentiated cells (figure 3.4.7 E). No significant change in the expression of ADA was observed during osteoblast differentiation (figure 3.4.7 F).

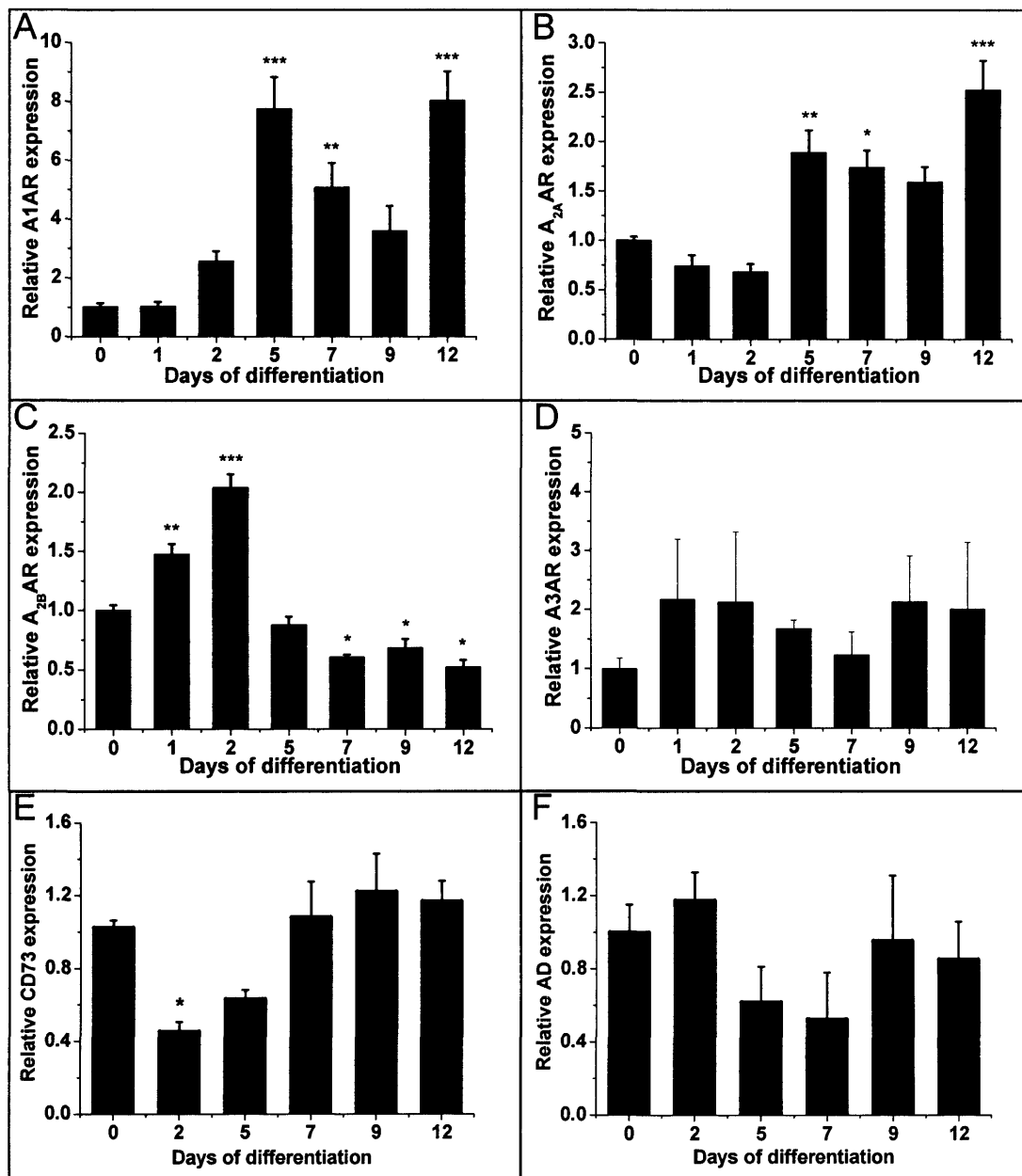
### **3.4.2.3 Adenosine receptor protein expression during osteoblastogenesis**

Representative blots for Western analysis of A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptors in MSCs during osteoblast differentiation for 0, 1, 2, 5, 7, 9 and 12 days are shown in figures 3.4.8 - 10. To facilitate comparisons between samples, receptor band densities were corrected for background and normalised to β-actin. The day 0 time point was used as the reference to which other band densities were compared. In contrast to changes in A<sub>1</sub> receptor mRNA expression during osteoblastogenesis the A<sub>1</sub> receptor protein remained relatively unchanged or increased slightly during differentiation (figure 3.4.7). The reason for this difference may be related to the already high expression of the protein in undifferentiated MSCs which could limit further translation of mRNA.



**A****B**

**Figure 3.4.6 Osteoblastic differentiation of MSCs.** Cells were incubated with osteoblastic differentiation medium containing AA (50  $\mu\text{g}/\text{ml}$ ) and Dex ( $10^{-8}$  M) for 2, 7 and 12 days. **(A)** mRNA expression of osteoblast specific markers *i.e.* Cbfa1, Col type 1, ALP and osteocalcin was determined by q-RT-PCR and presented as the relative expression to untreated MSCs after normalisation to  $\beta$ -actin ( $n=3$ ; total of 6 replicates). \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with undifferentiated control. **(B)** Mineralisation was induced by addition of 2 mM  $\beta$ -GP and calcified extracellular matrix was visualised after 12 days by Alizarin Red S staining.



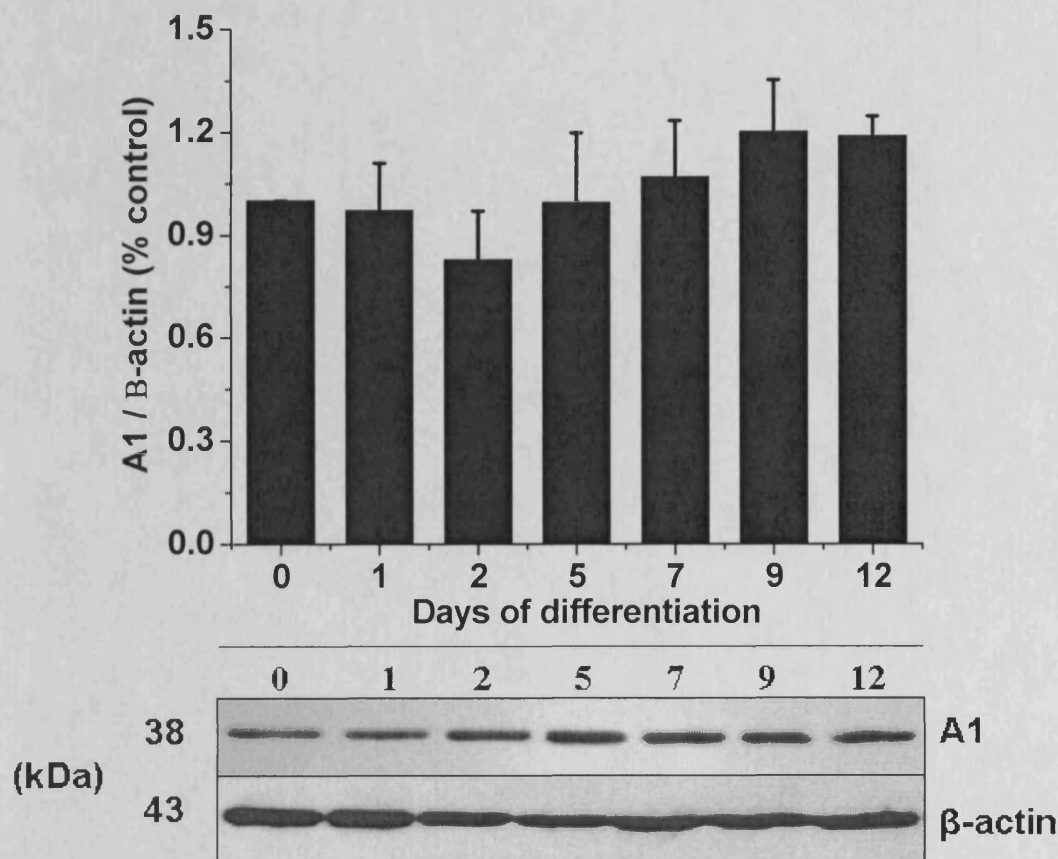
**Figure 3.4.7 Adenosine receptor and adenosine metabolic enzyme mRNA expression during the differentiation of MSCs into osteoblasts.** MSCs were cultured for indicated times in osteoblastic medium. mRNA expression of (A) A<sub>1</sub>, (B) A<sub>2A</sub>, (C) A<sub>2B</sub>, (D) A<sub>3</sub> receptors, (E) CD73 and (F) ADA were quantified with q-RT-PCR. Expression of each receptor was normalised to  $\beta$ -actin and values compared to untreated MSCs (*i.e.* day 0), which was assigned a value of 1 ( $n=3$ ; total of 6 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with day 0.

Changes in  $A_{2A}$  receptor protein expression paralleled changes in mRNA expression (figure 3.4.7 B) and there was direct correlation between protein and mRNA expression during differentiation. A relatively faint band for protein was detected in undifferentiated MSCs which increased gradually in intensity during osteoblast differentiation and was maximal at days 7 - 12 (figure 3.4.9). The  $A_{2B}$  protein expression (figure 3.4.10) remained unchanged at day 1, but was strongly upregulated at day 2 and 7, by day 12 expression had fallen back to basal levels. A similar pattern of expression was seen for  $A_{2B}$  receptor mRNA during osteoblastogenesis but the increase in protein level persists beyond that of the mRNA; this is probably because the protein signal is sustained after the mRNA has been degraded.

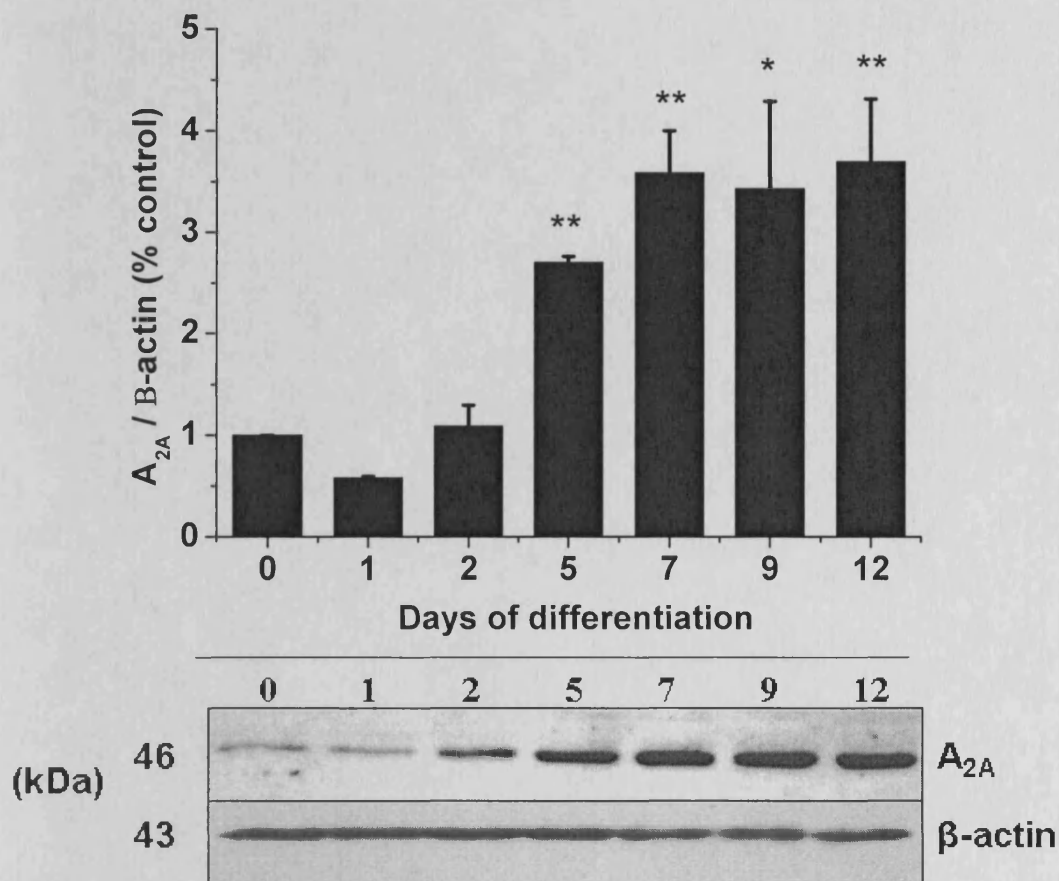
#### **3.4.2.4 Adenosine mediated cAMP responses during osteoblastogenesis**

Examination of adenosine receptor expression both at the mRNA and protein level by q-RTPCR and Western blotting identified differential expression of adenosine receptors during osteoblastogenesis. To check that these receptors were functional, cAMP responses to adenosine receptor agonists were investigated.

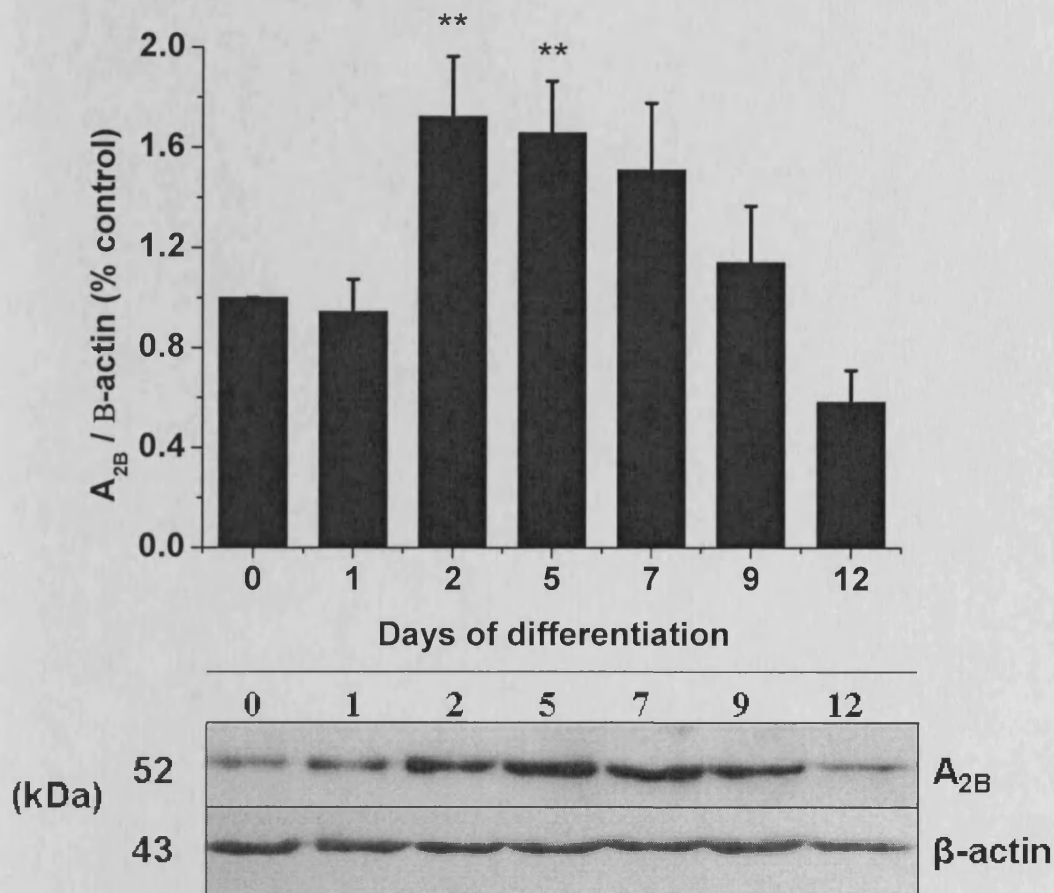
Treatment with adenosine and NECA increased cAMP accumulation in a dose related manner at all time points, indicating the predominance of  $A_2$  receptors at all stages of osteoblastogenesis (figure 3.4.11 A&B). When compared to undifferentiated MSCs (day 0), the efficiency of adenosine and NECA to stimulate cAMP production increased during the time of osteoblastic induction.



**Figure 3.4.8** A<sub>1</sub> receptor protein expression during the differentiation of MSCs into osteoblasts. MSCs were cultured for 1, 2, 5, 7, 9 and 12 days in osteoblastic medium. Cells were lysed in RIPA buffer and equal amount of protein lysates were separated on 10% SDS-PAGE. Blots were probed for the A<sub>1</sub> receptor, stripped and re-probed for  $\beta$ -actin for normalisation. The ratio of A<sub>1</sub> receptor to  $\beta$ -actin was calculated from densitometry of Western blots and compared to control. Representative blots and combined densitometric values from at least 3 separate experiments are shown above.



**Figure 3.4.9 A<sub>2A</sub> receptor protein expression during the differentiation of MSCs into osteoblasts.** MSCs were cultured for 1, 2, 5, 7, 9 and 12 days in osteoblastic medium. Cells were lysed in RIPA buffer and equal amount of protein lysates were separated on 10% SDS-PAGE. Blots were probed for the A<sub>2A</sub> receptor, stripped and re-probed for  $\beta$ -actin for normalisation. The ratio of A<sub>2A</sub> receptor to  $\beta$ -actin was calculated from densitometry of Western blots and compared to control. Representative blots and combined densitometric values from at least 3 separate experiments are shown above. \*P < 0.05, \*\*P < 0.01 when compared with day 0.



**Figure 3.4.10 A<sub>2B</sub> receptor protein expression during the differentiation of MSCs into osteoblasts.** MSCs were cultured for 1, 2, 5, 7, 9 and 12 days in osteoblastic medium. Cells were lysed in RIPA buffer and equal amount of protein lysates were separated on 10% SDS-PAGE. Blots were probed for the A<sub>2B</sub> receptor, stripped and re-probed for β-actin for normalisation. The ratio of A<sub>2B</sub> receptor to β-actin was calculated from densitometry of Western blots and compared to control. Representative blots and combined densitometric values from at least 3 separate experiments are shown above. \*\*P < 0.01 when compared with day 0.

At day 0  $10^{-4}$  M adenosine stimulated cAMP levels by 9 fold, this was increased to 22, 31, 153 and 25 fold respectively after 5, 7, 9 and 12 days of differentiation. The efficacy of NECA to increase cAMP was also increased significantly from 25 in MSCs to 42, 75, 275 and 125 fold at 5, 7, 9 and 12 days of osteoblastogenesis respectively.

As shown previously, there was no evidence of functional coupling of  $A_{2A}$  receptors to adenylate cyclase in undifferentiated MSCs (day 0). The selective  $A_{2A}$  receptor agonist, CGS 21680, failed to elicit a cAMP response. However, upon osteoblastic induction CGS 21680 significantly stimulated cAMP production in a dose dependent manner (figure 3.4.11 C). The efficacy of CGS 21680 to increase cAMP accumulation was increased further during the differentiation process with the maximum response at day 9. However the maximal stimulation of cAMP accumulation by NECA was about 20 fold more than CGS 21680 at all stages of osteoblastogenesis. Together, these data indicate that  $A_{2A}$  and  $A_{2B}$  receptor expression increases during osteoblastogenesis of MSCs and concur with the previous data showing increased mRNA and protein expression. Nevertheless the  $A_{2B}$  receptor still remains to be the dominant receptor in MSC derived osteoblasts.

#### **3.4.2.5 Adenosine receptor agonist mediated cAMP responses in mineralised osteoblasts derived from MSCs**

The stimulatory effects of adenosine and adenosine receptor agonists on cAMP accumulation were also investigated following mineralisation of MSC derived osteoblasts. As with MSC derived osteoblasts, mineralised

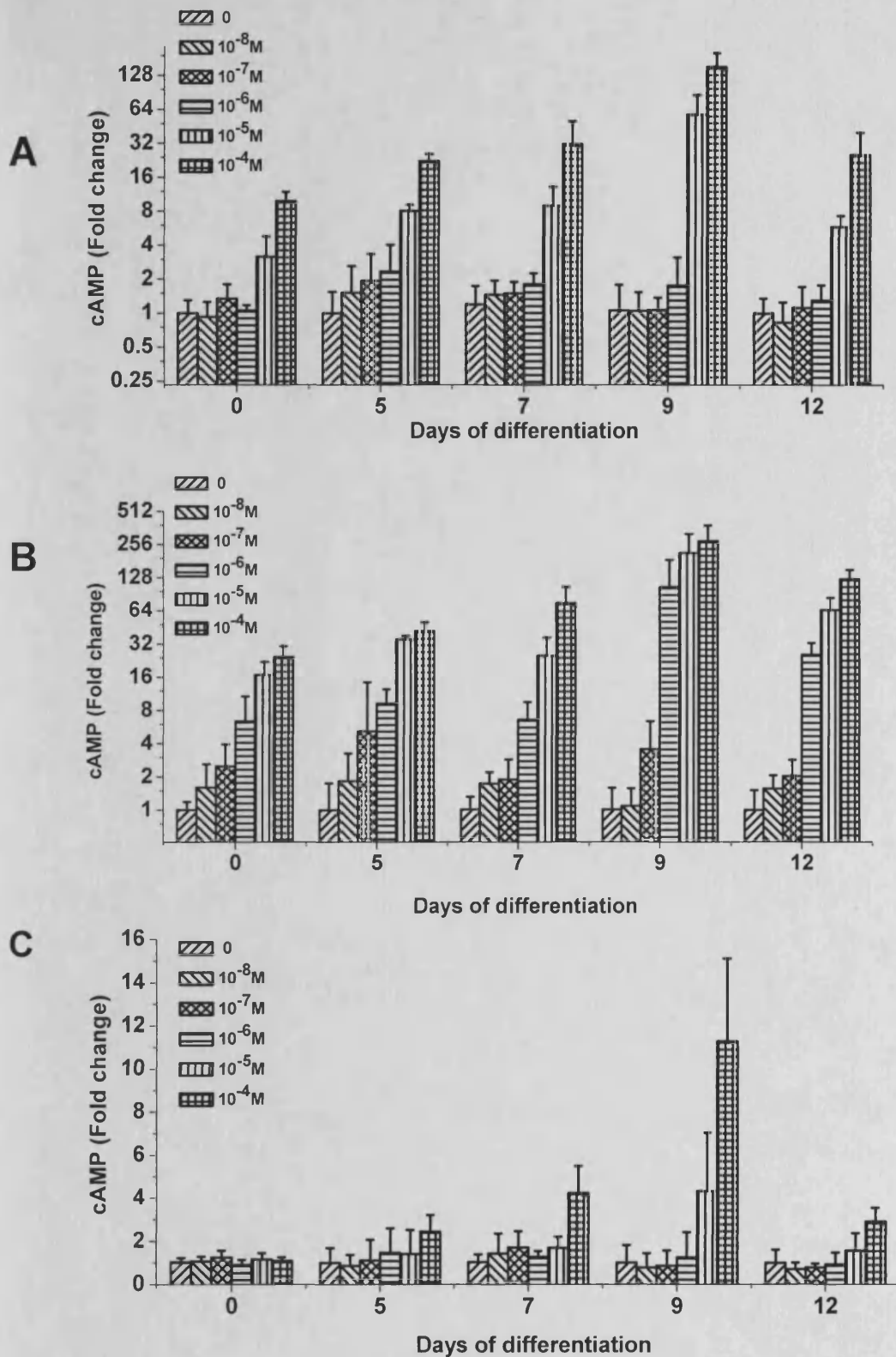
osteoblasts also responded (in terms of cAMP changes) to CGS 21680, this was however not observed in unmodified MSCs. Adenosine, NECA and CGS 21680 ( $10^{-4}$  M) increased cAMP concentrations from 9, 25 and 0 fold in MSCs to 16, 30 and 3 fold following 12 days of mineralisation respectively (figure 3.4.12). The difference was most noticeable when CGS 21680 was used as the agonist confirming an increase in  $A_{2A}$  receptor expression on osteoblastogenesis.

### **3.4.3 Effects of adenosine and NECA on markers of osteoblastogenesis during the differentiation of MSCs.**

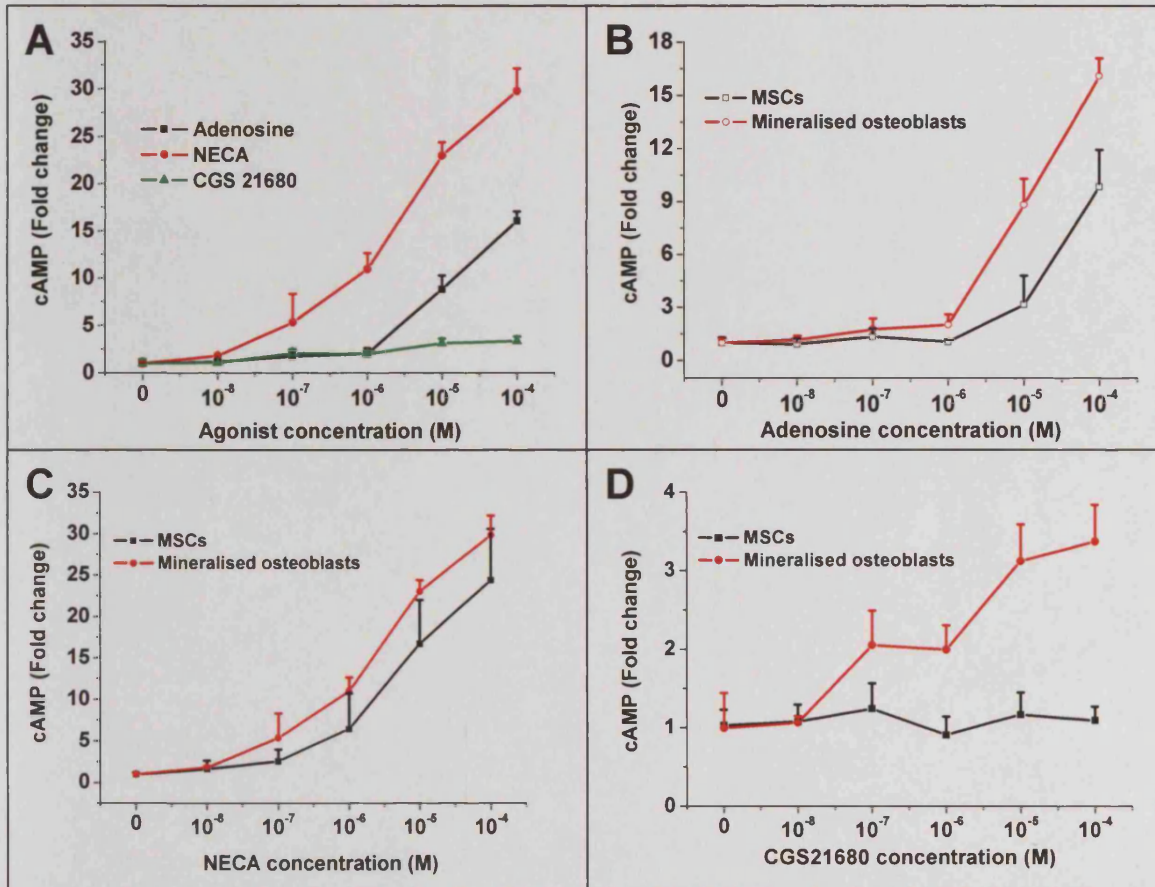
#### **3.4.3.1 Cbfa1, ALP, collagen type I and osteocalcin mRNA expression**

Previous data showed that expression of the  $A_{2A}$  and  $A_{2B}$  receptors in particular increased on osteoblastogenesis of MSCs. All four of the osteoblast markers, cbfa1, ALP, collagen type I and osteocalcin increased within 2 - 5 days of induced osteoblastogenesis (figure 3.4.13 to 3.4.16). The late osteoblast marker, osteocalcin showed a particularly large change with > 10,000 fold increase in mRNA after 7 days of induced osteoblastogenesis (figure 3.3.16). Cbfa1 mRNA was increased 4 fold at day 7 in osteoblast medium and further enhanced by NECA to 7 fold (figure 3.4.13 B), no effect however was seen when adenosine was used in place of NECA (figure 3.4.13 A). NECA and adenosine also stimulated ALP mRNA expression above that seen with osteoblastic medium alone (figure 3.4.14 A - C) but appeared to have little effect on the expression of collagen type I (figure 3.4.15 A&B) or osteocalcin mRNA (figure 3.4.16 A&B).





**Figure 3.4.11 Adenosine receptor agonist induced cAMP production in MSCs and at various time points during their differentiation into osteoblasts.** MSCs were cultured in osteoblastic medium for 0, 5, 7, 9 and 12 days. cAMP accumulation was measured after exposure to **(A)** adenosine **(B)** NECA and **(C)** CGS 21680 for 15 minutes in the presence of RO 20-1724 ( $10^{-4}M$ ) ( $n=2$ ; total of 8 replicates). Data are expressed as mean  $\pm$  SDM.



**Figure 3.4.12 Comparison of the effects of adenosine receptor agonists on cAMP production in MSCs and mineralised osteoblasts.** Mineralised osteoblasts were prepared from MSCs cultured for 12 days in osteoblastic differentiation medium supplemented with 2 mM  $\beta$ -GP. cAMP accumulation was measured in MSCs and mineralised osteoblasts. **(A)** MSCs treated with adenosine receptor agonists for 15 minutes in the presence of RO 20-1724 ( $10^{-4}$ M). **(B)** Adenosine, **(C)** NECA and **(D)** CGS 21680 induced cAMP accumulation in mineralised osteoblasts compared with MSCs ( $n=2$ ; total of 8 replicates). Data are expressed as mean  $\pm$  SDM.

### 3.4.3.2 Effects of adenosine receptor agonists on ALP enzyme activity

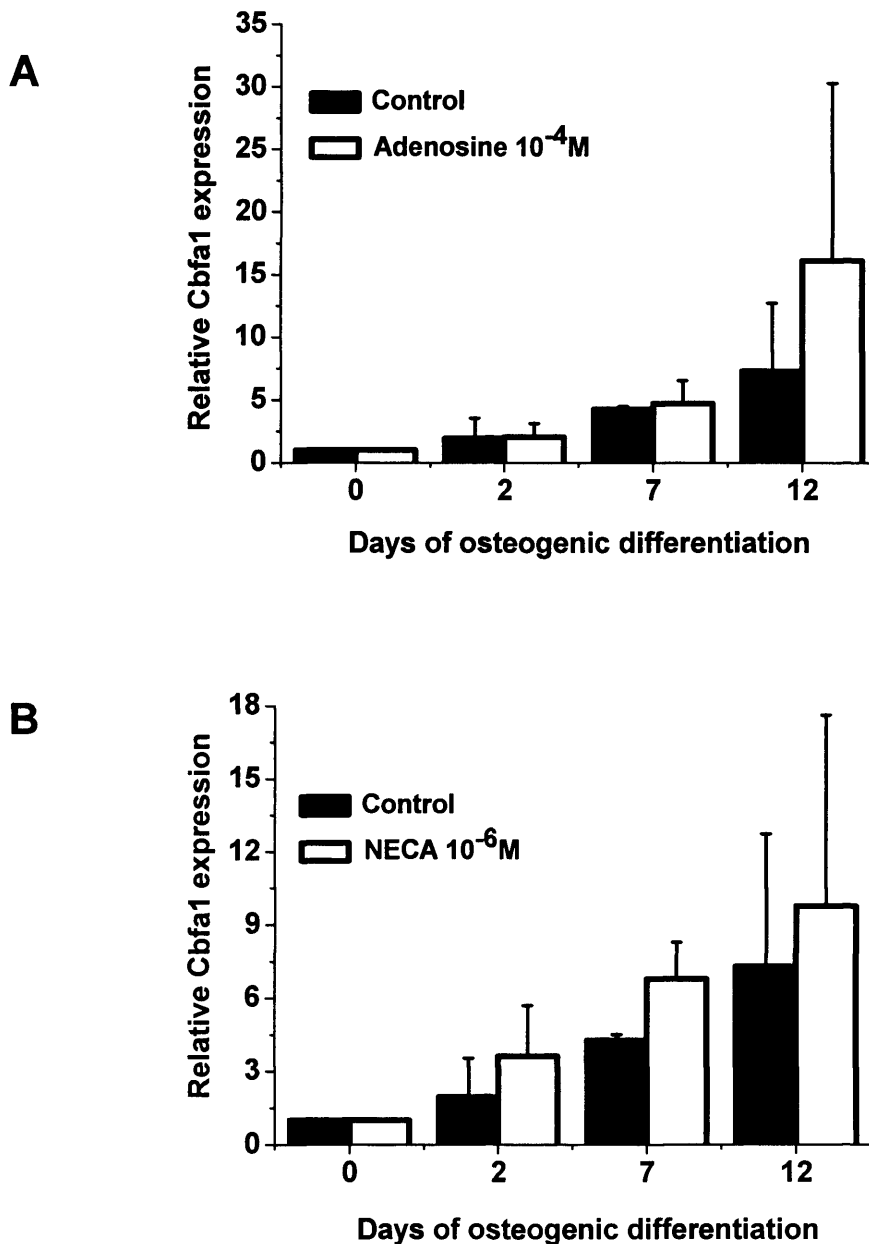
ALP mRNA expression was increased in a time related manner when MSCs were treated with adenosine or NECA during their differentiation to osteoblasts. In this set of experiments adenosine receptor agonists were used to investigate their effects on ALP enzyme activity. Adenosine and NECA both induced a dose dependent increase in ALP activity over 5 - 7 days of exposure (figure 3.4.17 A - B). The effects of NECA seemed to occur earlier (day 2) and appeared more potent than adenosine and this is likely to reflect the stability of NECA in comparison with adenosine. Beyond day 7 of agonist exposure, adenosine and NECA had little effect on ALP enzyme activity. Similar stimulatory effects on ALP enzyme activity were seen with the  $A_{2A}$  receptor agonist CGS 21680, interestingly this increase in enzyme activity seemed to persist up to at least 12 days (figure 3.4.18 A). The selective  $A_1$  receptor agonist, CCPA, also had an apparent stimulatory effect on ALP enzyme activity (figure 3.4.18 B) but this occurred only at  $10^{-5}$  M and is probably due to activation of other adenosine receptors.

In addition to investigating the effects of adenosine receptor agonists during osteoblast differentiation of MSCs the effects of these compounds on ALP enzyme activity in pre-differentiated (osteoblasts) cells were also carried out. In these experiments the pre-differentiated cells were only exposed to adenosine receptor agonists for 2 days; it is possible that in the differentiation experiments desensitisation to long term exposure (up to 12 days) to adenosine receptor agonists could occur. In the pre-differentiated experiments adenosine, NECA and CGS 21680 stimulated ALP enzyme activity at 7, 9 and

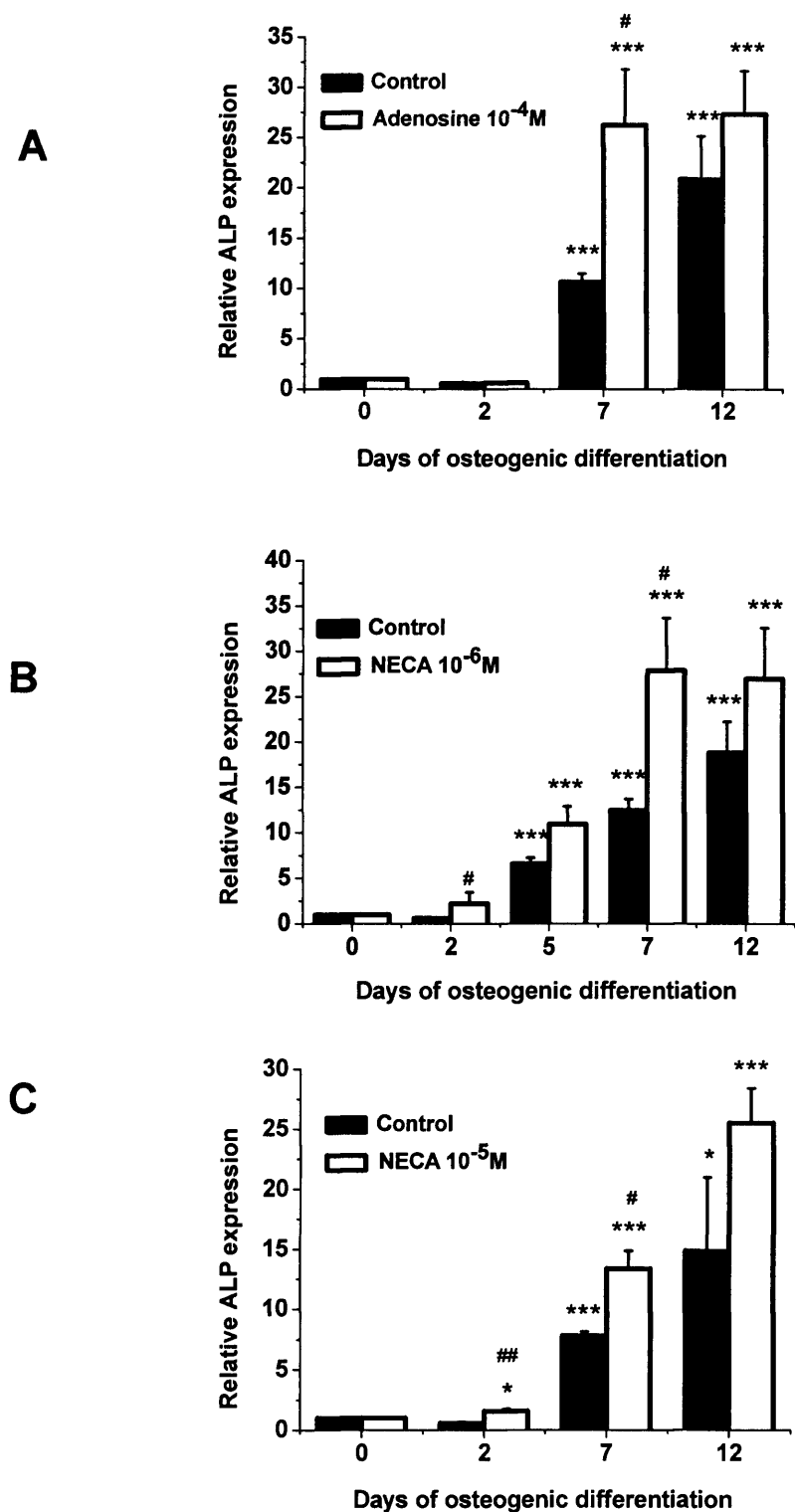
12 days (figure 3.4.19 A - C) and the effects were more potent than when adenosine receptor agonists were included for the whole duration of differentiation. This might suggest that prolonged exposure to agonists can lead to desensitisation of adenosine receptors.

### **3.4.3.3 Effects of adenosine receptor antagonists on NECA induced ALP mRNA expression and enzyme activity**

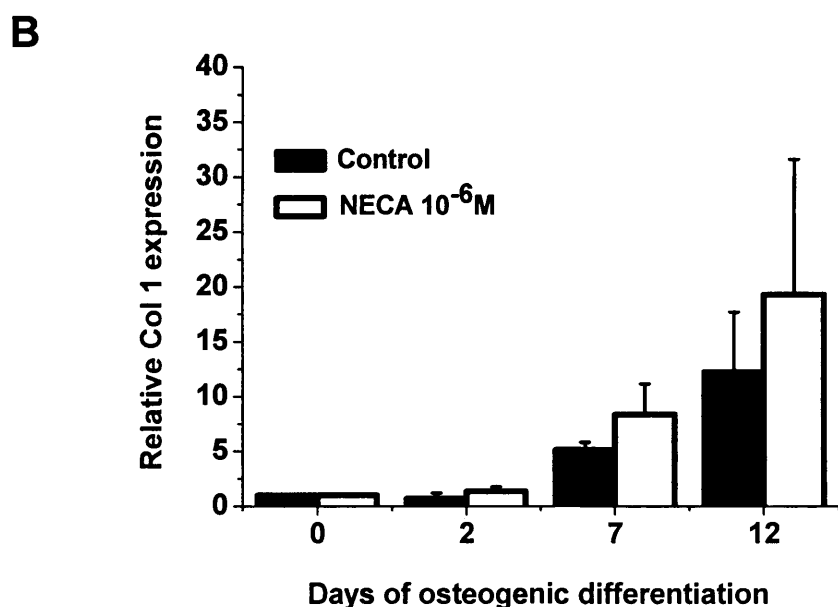
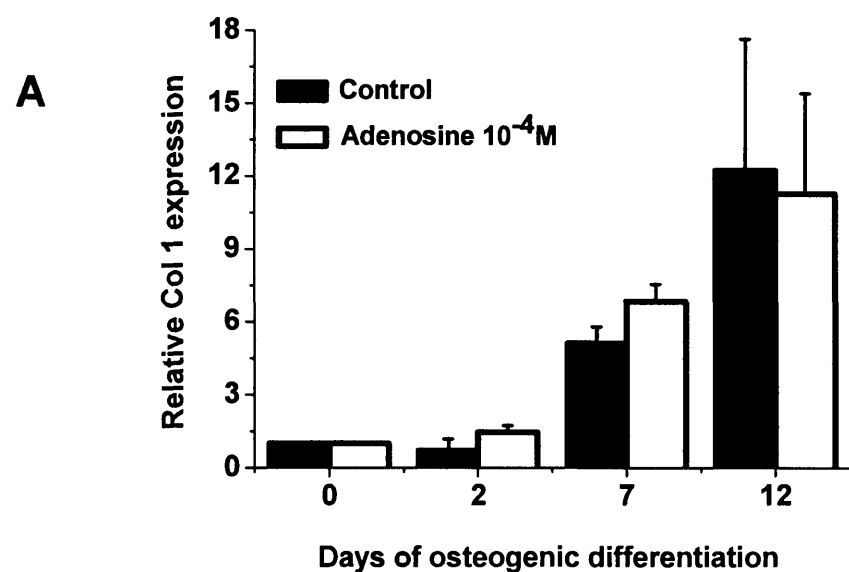
Previous experiments showed that  $A_{2A}$  and  $A_{2B}$  receptors are increased during osteoblastic differentiation of MSCs and activation of such receptors also stimulate expression of osteoblast marker genes. To further confirm the identities of the receptors involved, antagonist compounds ( $A_{2A}$  receptor, SCH 442416;  $A_{2B}$  receptor, MRS 1706) were used to inhibit the effects of NECA on ALP mRNA expression and enzyme activity. Initial experiments were carried out to see if these antagonists alone inhibited cell growth and using the MTS assay it was clear that concentrations up to  $10^{-6}$  M were without effect (data not shown). These concentrations were subsequently used and exposure to antagonists alone for 2 or 5 days induced only a negligible change in the ALP expression or activity as MSCs differentiated into osteoblasts when compared to untreated control (figures 3.4.20 - 21). In the presence of SCH 442416, NECA induced ALP expression and activity during osteoblastogenesis was partially but significantly reversed by approximately 75% ( $P < 0.001$ ) (figures 3.4.20 - 21). In addition the  $A_{2B}$  receptor antagonist, MRS 1706, abolished the increase in ALP expression and activity induced by NECA at day 2 and 5 of differentiation (figures 3.4.20 - 21).



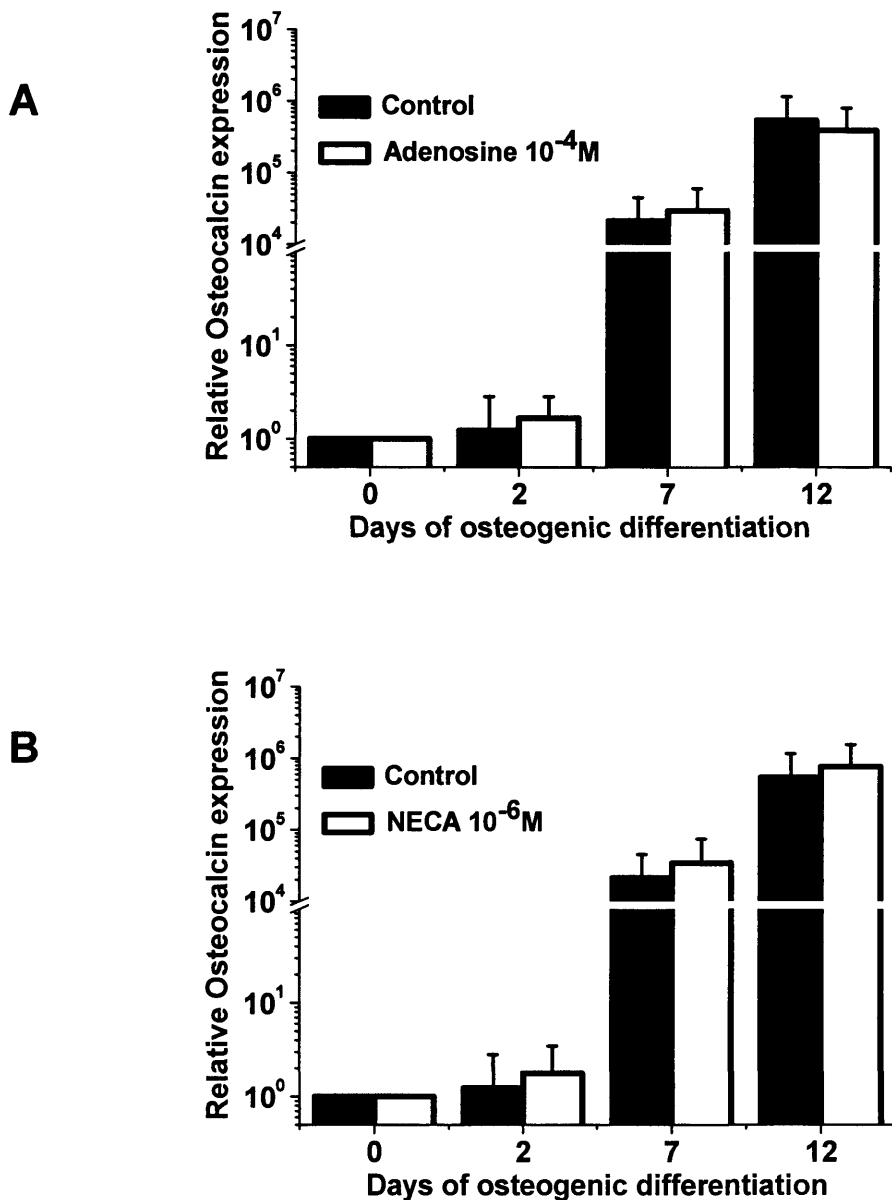
**Figure 3.4.13 NECA and adenosine mediated Cbfa1 mRNA expression during differentiation of MSCs into osteoblasts.** MSCs were incubated with osteoblastic differentiation medium in presence or absence of (A) adenosine ( $10^{-4}$  M) and (B) NECA ( $10^{-6}$  M) for 2, 7 and 12 days. Cbfa1 mRNA was quantified with q-RTPCR and normalised to  $\beta$ -actin and presented as mRNA expression relative to undifferentiated MSCs (day 0) ( $n=2$ ; total of 4 replicates). Data are expressed as mean  $\pm$  SDM.



**Figure 3.4.14 NECA and adenosine mediated ALP mRNA expression during differentiation of MSCs into osteoblasts.** MSCs were incubated with osteoblastic differentiation medium in presence or absence of (A) adenosine ( $10^{-4}M$ ), (B) NECA ( $10^{-6}M$ ) and (C) NECA ( $10^{-5}M$ ) for 2, 7 and 12 days. ALP mRNA was quantified with q-RT-PCR and normalised to  $\beta$ -actin and presented as mRNA expression relative to undifferentiated MSCs (day 0) (n=3; total of 6 replicates). \*P < 0.05 and \*\*\*P < 0.001 when compared with day 0. #P < 0.05 and ##P < 0.01 when compared with vehicle at the same time point.

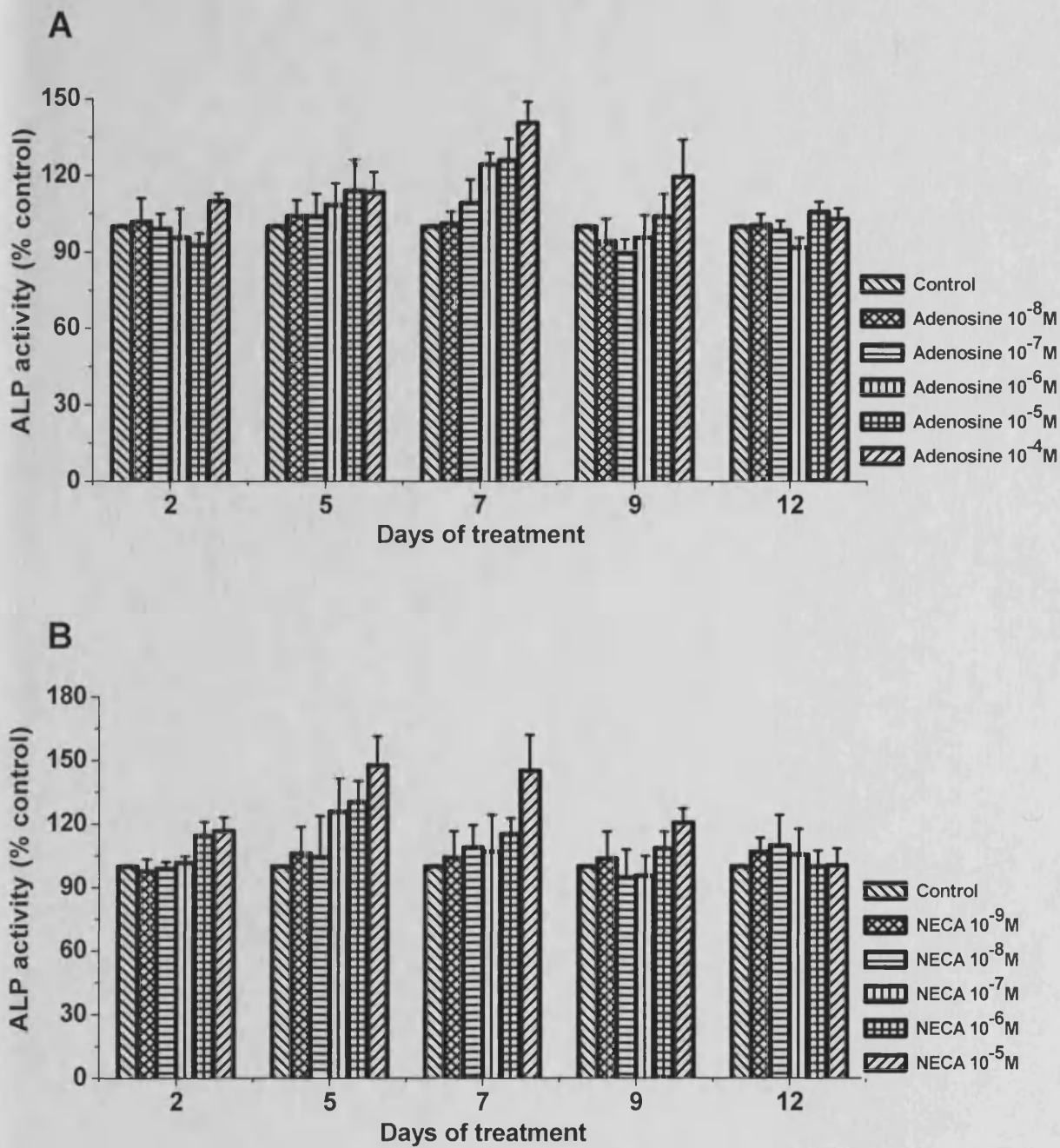


**Figure 3.4.15 NECA and adenosine mediated collagen type I mRNA expression during differentiation of MSCs into osteoblasts.** MSCs were incubated with osteoblastic differentiation medium in presence or absence of (A) adenosine ( $10^{-4}$ M) and (B) NECA ( $10^{-6}$  M) for 2, 7 and 12 days. Collagen type I mRNA was quantified with q-RTPCR and normalised to  $\beta$ -actin and presented as mRNA expression relative to undifferentiated MSCs (day 0) (n=2; total of 4 replicates). Data are expressed as mean  $\pm$  SDM.

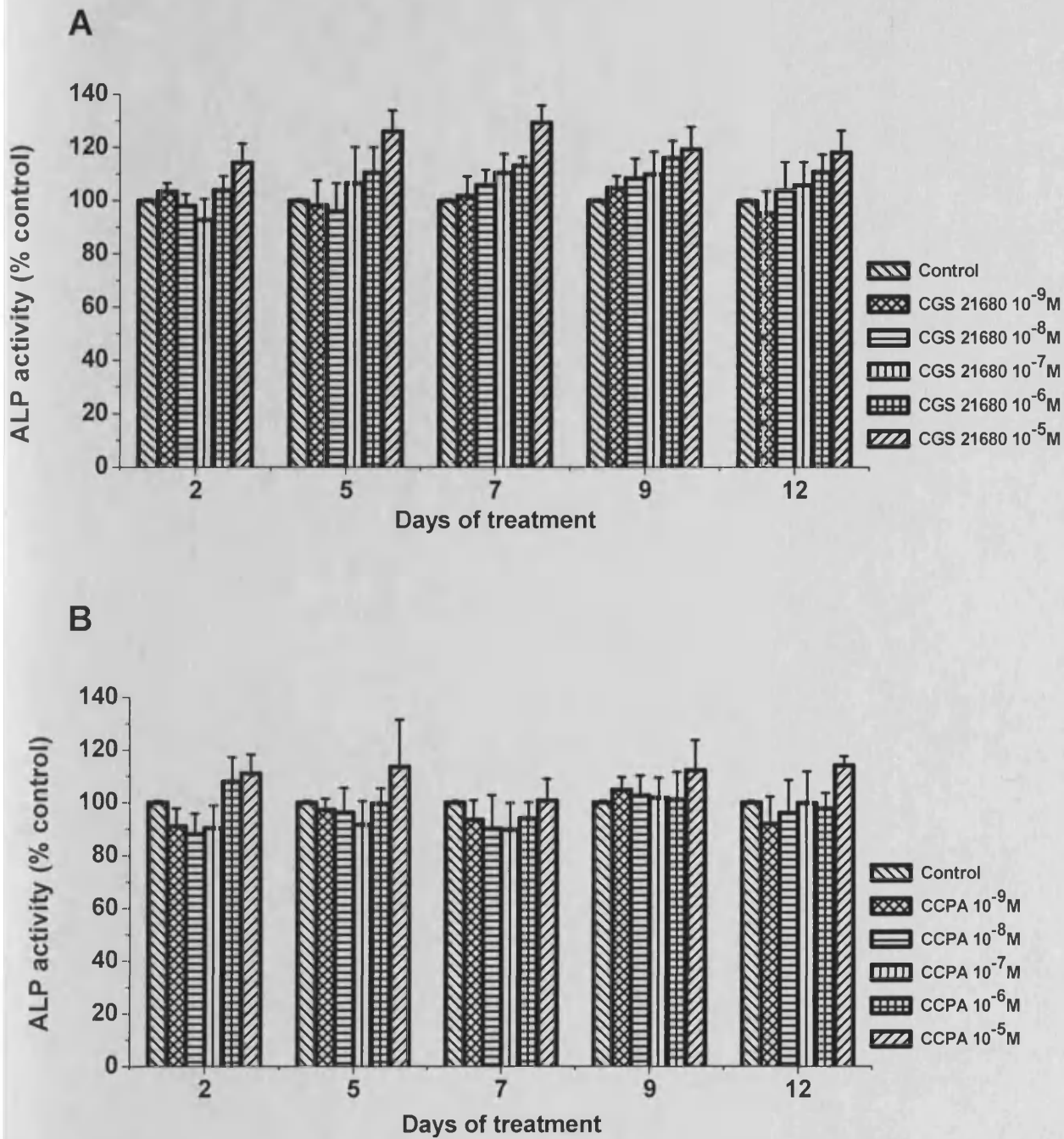


**Figure 3.4.16 NECA and adenosine mediated osteocalcin mRNA expression during differentiation of MSCs into osteoblasts.** MSCs were incubated with osteoblastic differentiation medium in presence or absence of (A) adenosine ( $10^{-4}$ M) and (B) NECA ( $10^{-6}$  M) for 2, 7 and 12 days. Osteocalcin mRNA was quantified with q-RTPCR and normalised to  $\beta$ -actin and presented as mRNA expression relative to undifferentiated MSCs (day 0) ( $n=2$ ; total of 4 replicates). Data are expressed as mean  $\pm$  SDM.

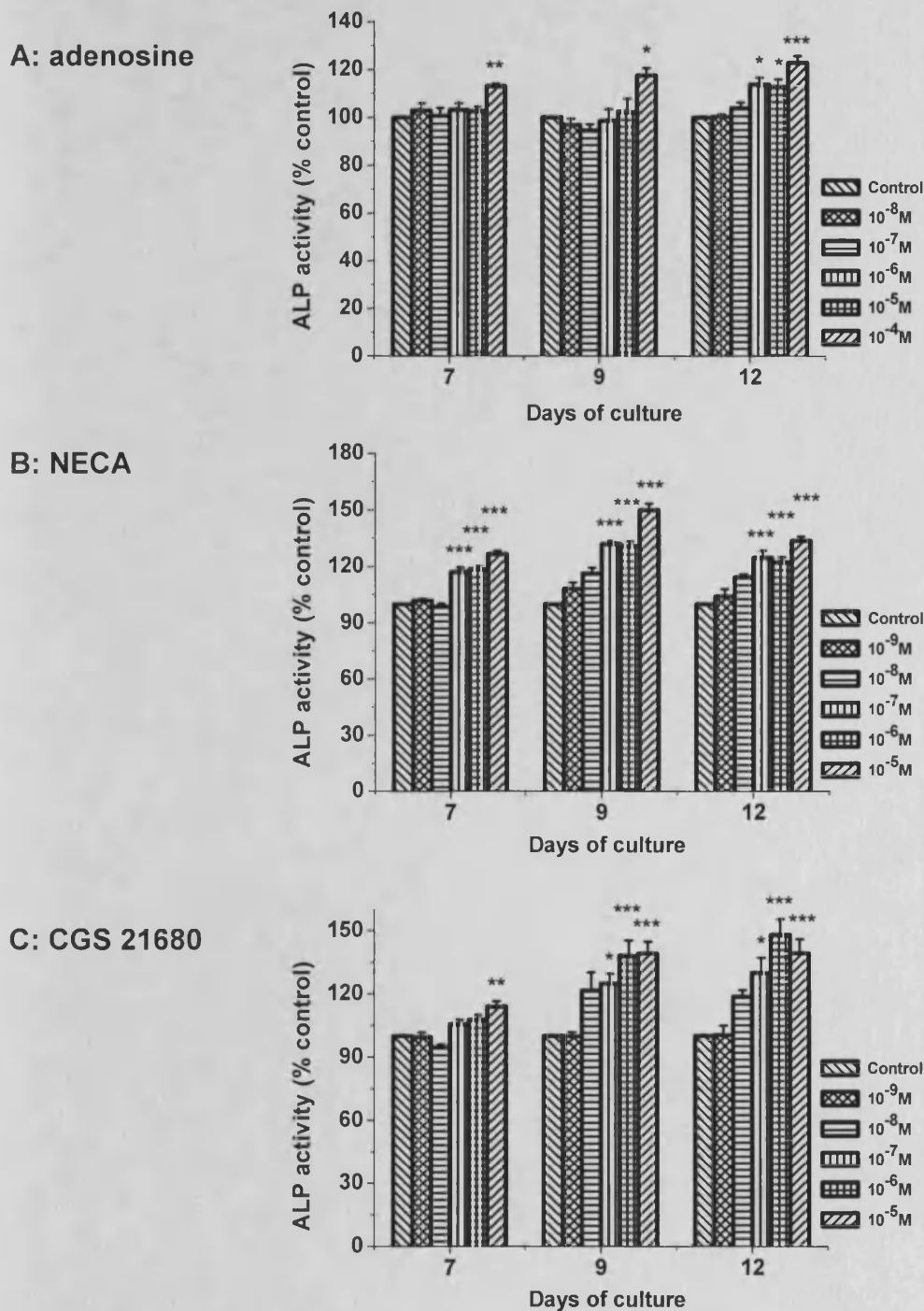




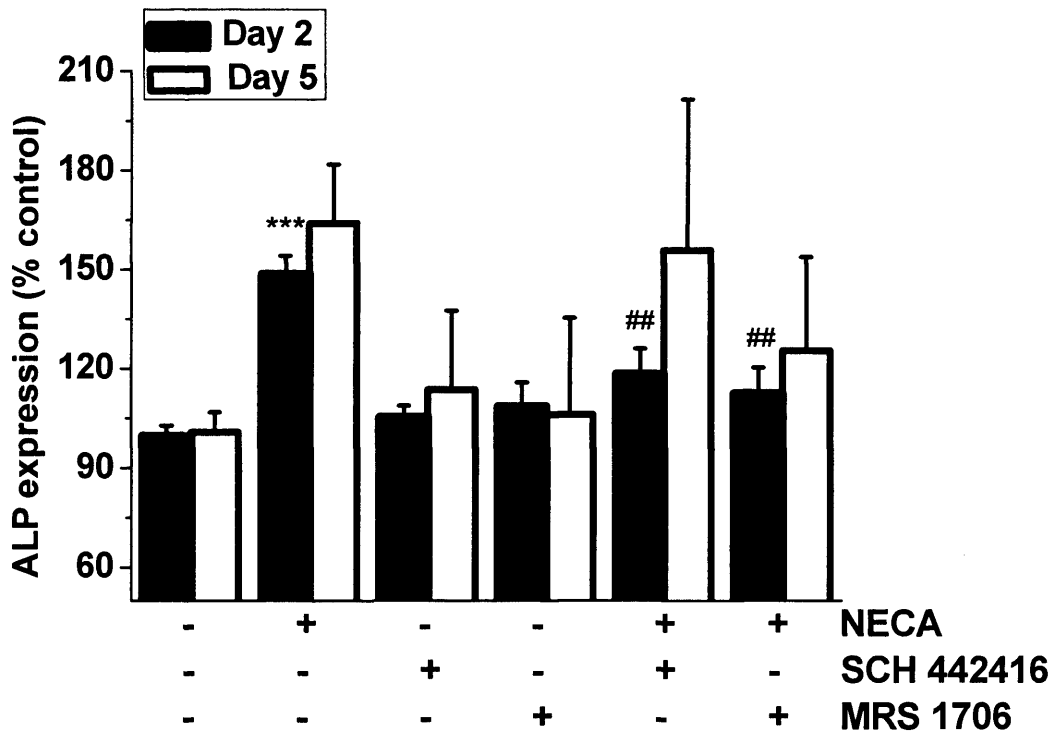
**Figure 3.4.17 Effects of adenosine and NECA on ALP enzyme activity at various stages of osteoblastic differentiation.** MSCs were treated with the indicated concentrations of (A) adenosine and (B) NECA or vehicle in osteoblastic differentiation medium for 2, 5, 7, 9 and 12 days. ALP activity was measured and normalised to protein content ( $n=2$ ; total of 8 replicates). Data are expressed as mean  $\pm$  SDM.



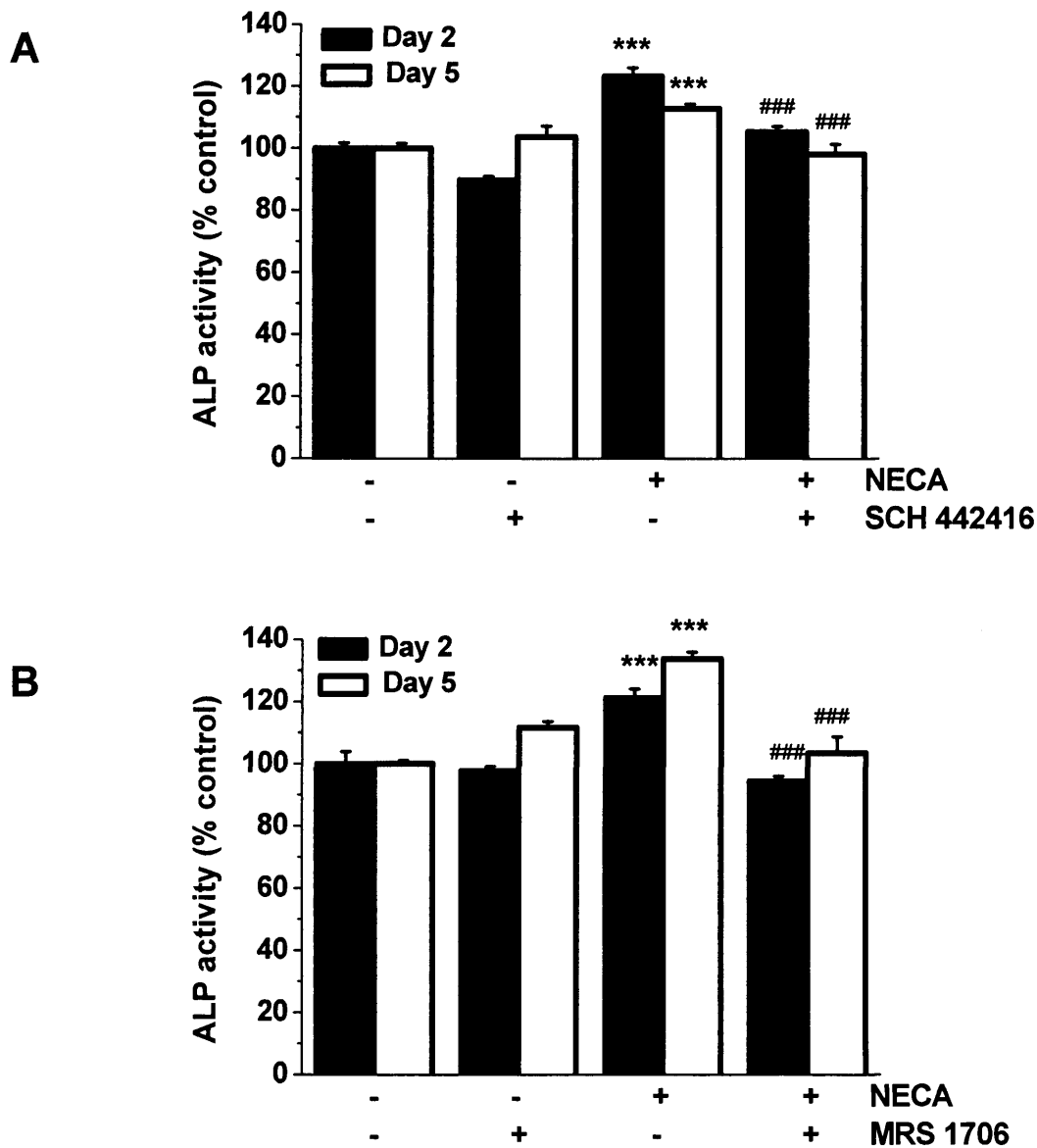
**Figure 3.4.18** Effects of CGS 21680 and CCPA on ALP enzyme activity at various stages of osteoblastic differentiation. MSCs were treated with the indicated concentrations of (A) CGS 21680 and (B) CCPA or vehicle in osteoblastic differentiation medium for 2, 5, 7, 9 and 12 days. ALP activity was measured and normalised to protein content ( $n=2$ ; total of 8 replicates). Data are expressed as mean  $\pm$  SDM.



**Figure 3.4.19 Dose dependent effects of adenosine receptor agonists on ALP enzyme activity on pre-differentiated osteoblasts.** MSCs were pre-differentiated with osteoblastic medium for 5, 7 and 9 days before treating with various concentrations of (A) adenosine, (B) NECA, and (C) CGS 21680 for a further 2 days in osteoblastic differentiation medium. ALP activity was measured and normalised to protein content ( $n=3$ ; total of 12 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with vehicle at the same time point.



**Figure 3.4.20** Effects of  $A_{2A}$  and  $A_{2B}$  receptor antagonists SCH 442416 and MRS 1706 on NECA induced expression of ALP mRNA during differentiation of MSCs into osteoblasts. MSCs were treated with  $10^{-6}$  M SCH 442416 and MRS 1706 alone or in the presence of NECA ( $10^{-6}$  M) in osteoblastic differentiation medium for 2 and 5 days. ALP mRNA expression was quantified with q-RT-PCR and normalised to  $\beta$ -actin and compared to untreated cells at the same time point ( $n=3$ ; total of 6 replicates). \*\*\* $P < 0.001$  when compared with untreated cells. ## $P < 0.01$  when compared with NECA alone stimulated cells at the same time point.

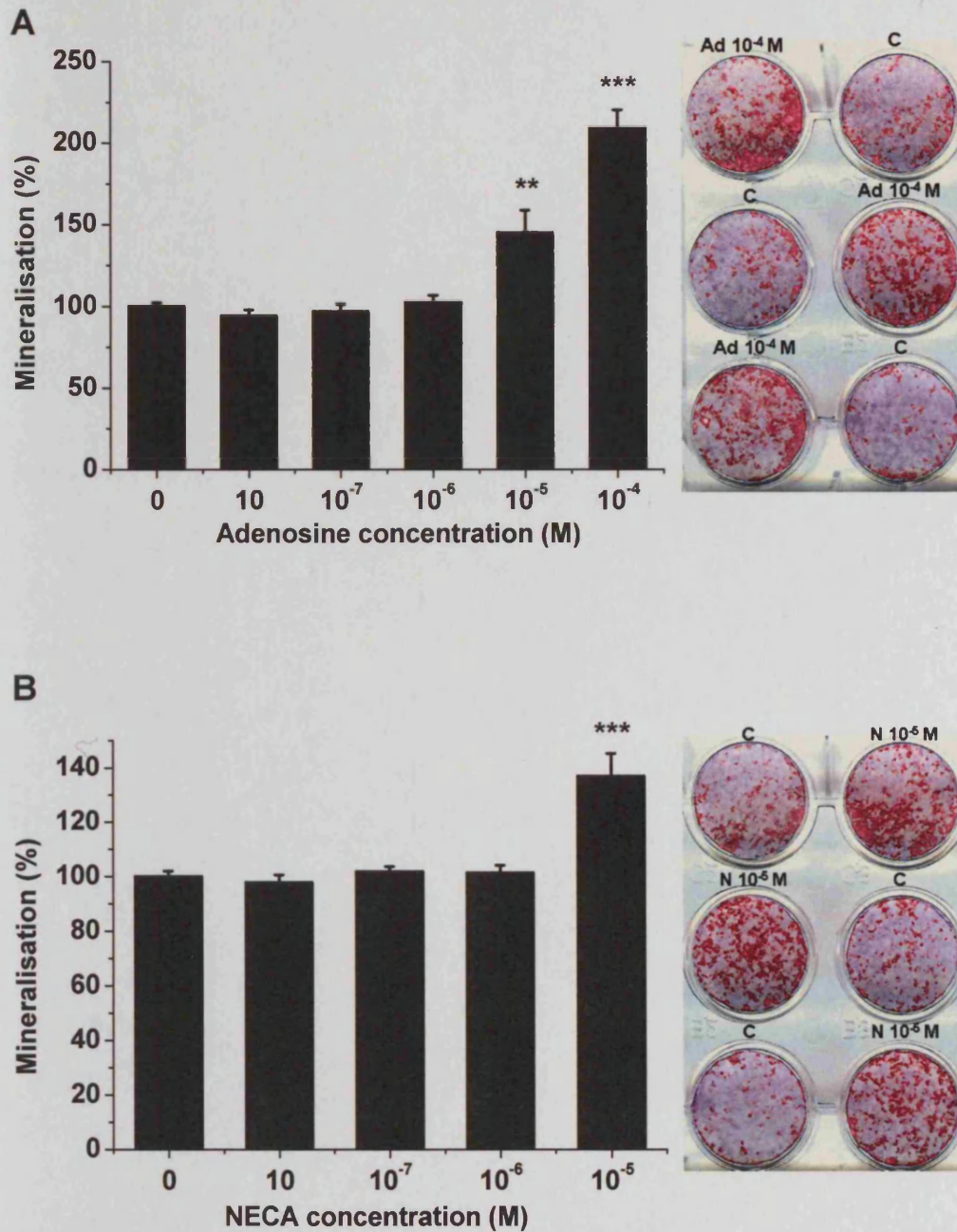


**Figure 3.4.21** Effects of  $A_{2A}$  and  $A_{2B}$  receptor antagonists SCH 442416 and MRS 1706 on NECA induced ALP enzyme activity during differentiation of MSCs into osteoblasts. MSCs were treated with  $10^{-6}$  M SCH 442416 (A) or MRS 1706 (B) alone or in combination with NECA ( $10^{-6}$  M) in osteoblastic differentiation medium for 2 and 5 days. ALP activity was measured and normalised to protein content ( $n=3$ ; total of 12 replicates). \*\*\* $P < 0.001$  when compared with untreated cells. ### $P < 0.001$  when compared with NECA alone stimulated cells at the same time point.

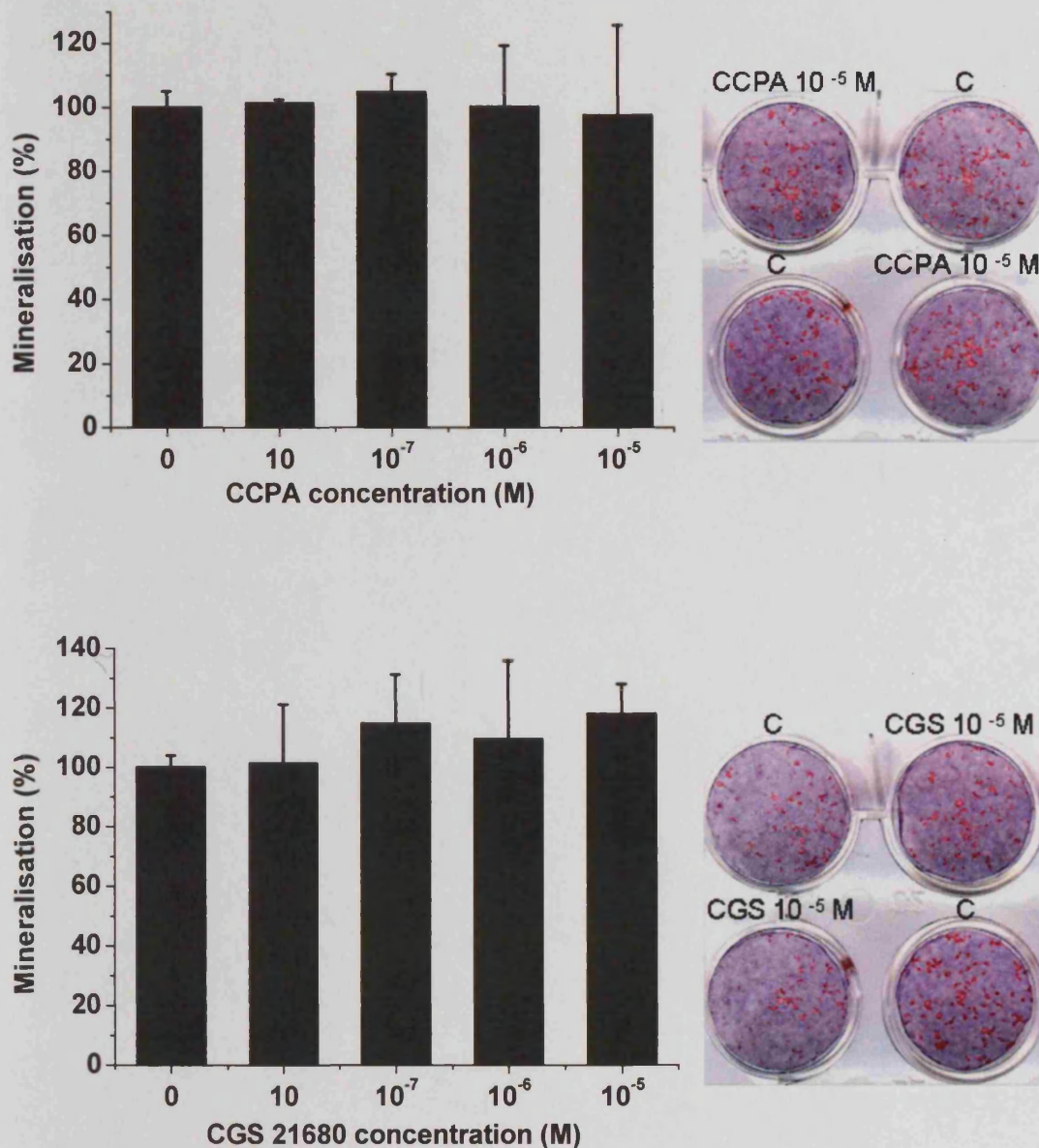
These data combined with previous observations indicate that both  $A_{2A}$  and  $A_{2B}$  receptors are responsible for the induction of osteoblast markers in MSCs and during the differentiation into osteoblasts.

#### **3.4.3.4 Effects of adenosine receptor agonists and antagonists on mineralisation of MSC derived osteoblasts**

Current findings show that the  $A_{2A}$  and  $A_{2B}$  receptors play an important role in osteoblastogenesis of MSCs and in these experiments I have investigated whether activation of the same receptors also stimulates matrix mineralisation. Moderate Alizarin Red S staining was observed in untreated MSCs in the presence of 2 mM  $\beta$ -GP after 10 to 12 days and this was further enhanced ( $P < 0.001$ ) respectively by 109%, 50% and 37% when adenosine ( $10^{-4}$  M), adenosine ( $10^{-5}$  M) or NECA ( $10^{-5}$  M) (figure 3.4.22 A&B) was included in the culture medium. Mineralisation was however unaffected by treatment with lower concentrations of NECA or adenosine. Surprisingly, CGS 21680 (previously shown to induce ALP activity) had no effect on mineralisation (figure 3.4.23 A) and as expected, mineralisation was also unaffected by treatment with the  $A_1$  receptor agonist, CCPA (figure 3.4.23 B). The  $A_{2B}$  receptor antagonist, MRS 1706, inhibited the increase in mineralisation induced by adenosine by up to 95% ( $P < 0.001$ ) (figure 3.4.24 B), whereas the  $A_{2A}$  receptor antagonist, SCH 442416 had no significant effect (figure 3.4.24 A). The lack of effect by CGS 21680 ( $A_{2A}$  receptor selective agonist) and the lack of inhibition of adenosine induced mineralisation by SCH 442416 suggests that the  $A_{2B}$  receptor is responsible for the induction of mineralisation.

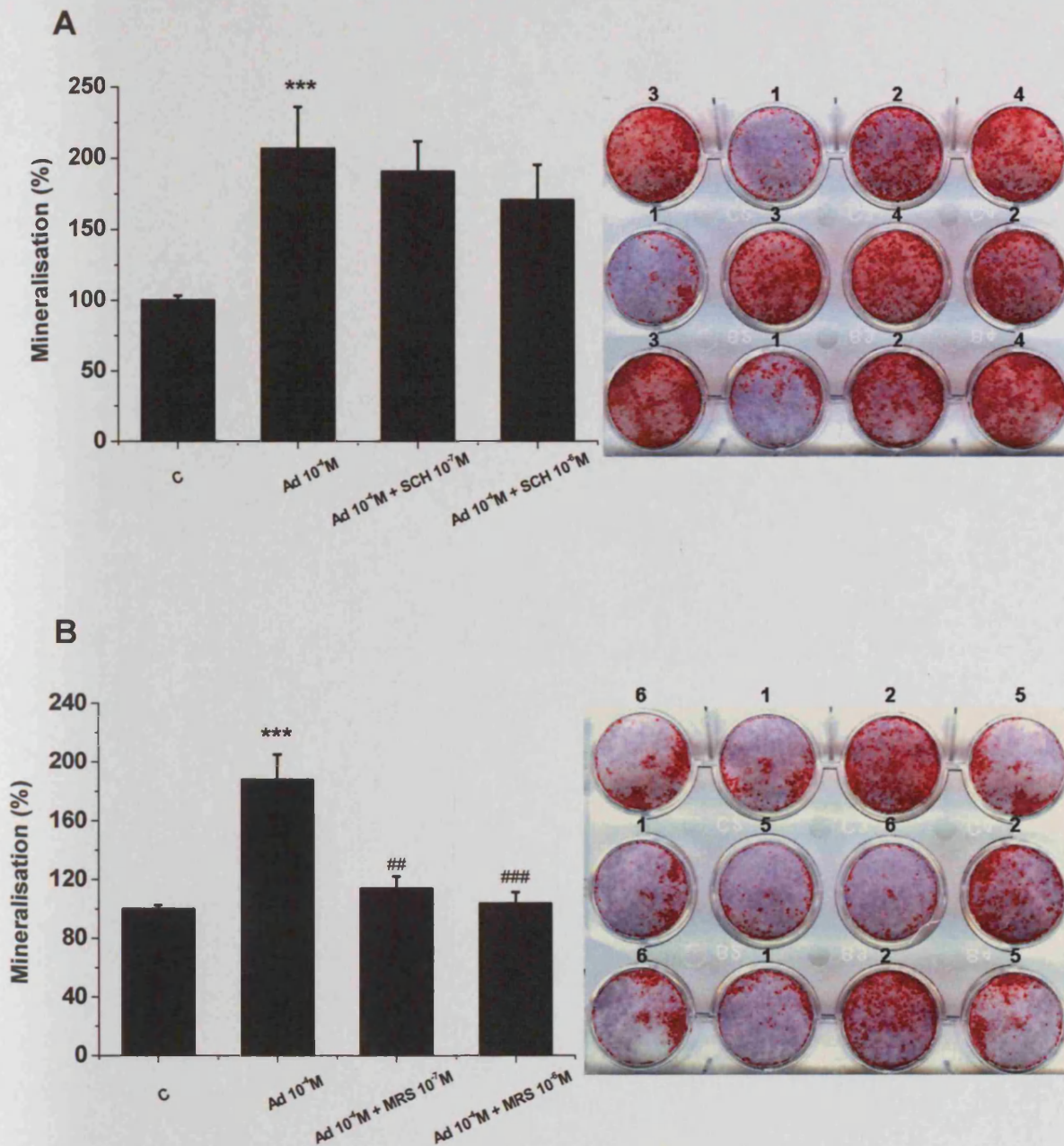


**Figure 3.4.22 Effects of adenosine and NECA on mineralisation of MSCs.** Cells were treated for 10 - 12 days with the indicated concentrations of (A) adenosine or (B) NECA or appropriate vehicle control in mineralisation medium and calcium deposition was determined by Alizarin Red S staining. (n=5; total of 15 replicates). \*\*P < 0.01, and \*\*\*P < 0.001 when compared with vehicle control.



**Figure 3.4.23** Effects of CCPA and CGS 21680 on mineralisation of MSCs. Cells were treated for 10 - 12 days with the indicated concentrations of (A) CCPA or (B) CGS 21680 or appropriate vehicle control in mineralisation medium and calcium deposition was determined by Alizarin Red S staining (n=3; total of 9 replicates).

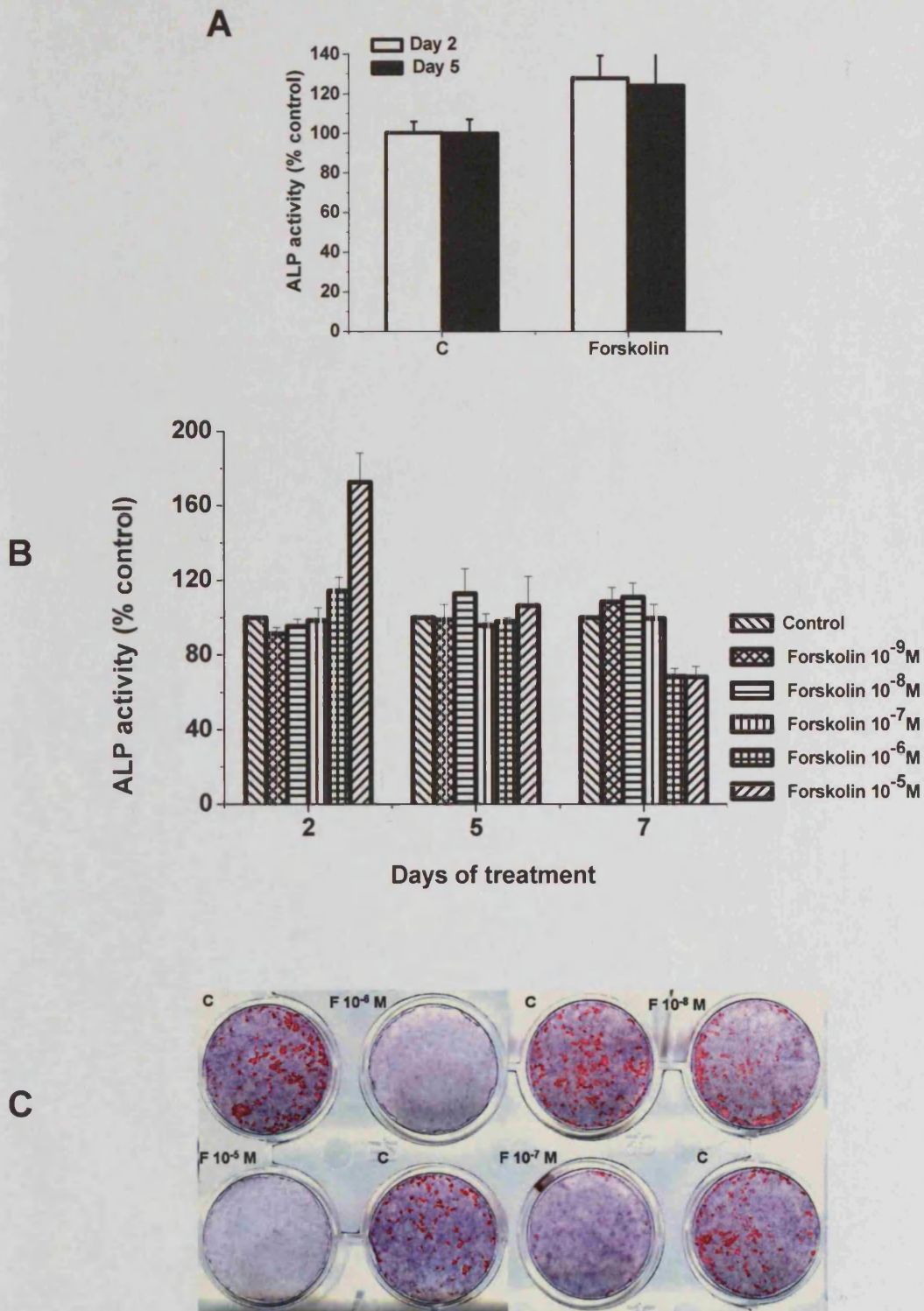




**Figure 3.4.24 Effects of  $A_{2A}$  and  $A_{2B}$  receptor antagonists SCH 442416 and MRS 1706 on adenosine induced mineralisation.** MSCs were treated with adenosine ( $10^{-4}$  M) in mineralisation medium alone or in combination with  $10^{-6}$  M to  $10^{-7}$  M (A) SCH 442416 or (B) MRS 1706 and calcium deposition was determined by Alizarin Red S staining ( $n=3$ ; total of 9 replicates). \*\*\* $P < 0.001$  when compared with vehicle control. ## $P < 0.001$  and ### $P < 0.001$  when compared with adenosine stimulated. (For pictures; 1= control, 2= adenosine ( $10^{-4}$  M), 3= adenosine ( $10^{-4}$  M) + SCH 442416 ( $10^{-7}$  M), 4= adenosine ( $10^{-4}$  M) + SCH 442416 ( $10^{-6}$  M), 5= adenosine ( $10^{-4}$  M) + MRS 1706 ( $10^{-7}$  M) and 6= adenosine ( $10^{-4}$  M) + MRS 1706 ( $10^{-6}$  M)).

#### **3.4.3.5 Effects of forskolin on osteoblastogenesis**

The primary signalling molecule for  $A_2$  receptors is the activation of cAMP and figure 3.4.25 A shows that forskolin, an activator of adenylate cyclase is able to stimulate ALP enzyme activity in MSCs. Forskolin also stimulated ALP activity during early stages of osteoblastogenesis, whereas prolonged treatment had little effect or even reduced ALP activity (figure 3.4.25 B). Prolonged exposure to forskolin also inhibited mineralisation. These data could suggest that osteoblastogenesis is mediated via a transient rise in cAMP but prolonged cAMP is inhibitory. Prolonged forskolin treatment had a negative effect on ALP activity and on mineralisation, the mechanisms behind these observations are however unclear. These inhibitory effects of prolonged exposure to cAMP may be due to the induction of PDEs as seen in other cells (Conti et al., 2003; Hu et al., 2008).



**Figure 3.4.25 Effects of forskolin on ALP enzyme activity and mineralisation.** MSCs were treated with (A) 10<sup>-5</sup> M forskolin in normal growth medium and (B) varying concentrations of forskolin in osteoblastic differentiation medium. ALP activity was measured and normalised to protein content (n=2; total of 8 replicates). Data are expressed as mean  $\pm$  SDM. (C) Mineralisation following staining with Alizarin Red S in cells treated with forskolin (F) or DMSO (C).

### 3.5 Discussion

Adenosine receptors are ubiquitously expressed throughout the body in many cells and tissues. Previously the presence of these receptors was demonstrated by our group in human osteoprogenitor cells (Evans et al., 2006) and recently by Katebi and colleagues in mouse bone marrow derived MSCs (Katebi et al., 2009). In line with these results, we found here that MSCs derived from rat bone marrow also express mRNA for all four receptors and for enzymes involved in adenosine synthesis and metabolism. In addition to gene expression, Western blot analysis confirmed the presence of A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptor protein, whereas the A<sub>3</sub> receptor protein was not detected.

It is important to note that based on protein sequences, the wild type A<sub>2B</sub> receptor is predicted to have a molecular mass of 36 kDa. However, in rat MSCs, A<sub>2B</sub> receptors appeared as a band with apparent molecular mass of about 52 kDa which is similar to the 50 - 55 kDa reported by others in various tissues and cells in human, mouse and rat (Peyot et al., 2000; Puffinbarger et al., 1995; Trincavelli et al., 2004). This greater molecular mass may be due to the formation of complexes or alternate splicing (Feoktistov et al., 2003).

Although the protein for the A<sub>3</sub> receptor was undetectable in MSCs, the presence of A<sub>3</sub> receptor protein cannot be completely excluded as Western blotting analyses are entirely dependent on the quality of the antibody used. Nonetheless it is likely that the expression of A<sub>3</sub> receptor protein in MSCs is either not or lowly expressed. This is compatible with studies that reported a lack or low level of A<sub>3</sub> receptor expression in various cell types (Gessi et al.,

2004; Madi et al., 2004; Phelps et al., 2006; Rivkees et al., 2000). For example, in the MG-63 osteoblastic cell line, mRNA and protein for the A<sub>3</sub> receptor is absent (Russell et al., 2007). In contrast to normal cells, A<sub>3</sub> receptors are prominently expressed in tumour cell lines such as melanoma, lymphoma, pineal gland, colon, and prostate carcinoma (Gessi et al., 2004; Madi et al., 2003; Madi et al., 2004).

Although RT-PCR and Western blotting confirm the presence of A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptor mRNA and protein, these results do not necessarily correlate with the presence of functional adenosine receptors on the cell surface. The functional relevance of adenosine receptors in MSCs was determined by measuring changes in cAMP expression. NECA, in the  $\mu$ M range, stimulated cAMP production, similar to that previously reported for cells expressing functional A<sub>2B</sub> receptors (Feoktistov and Biaggioni, 1998; Lynge et al., 2003; Phelps et al., 2006; Zeng et al., 2003) and for cells transfected with the human A<sub>2B</sub> receptor (Klotz et al., 1997; Schulte and Fredholm, 2000). The potency order for cAMP production was NECA > adenosine > CGS 21680 and is in agreement with that reported previously for the A<sub>2B</sub> receptor (Feoktistov and Biaggioni, 1998; Klotz et al., 1997). The A<sub>2B</sub> receptor is a low affinity receptor that is inactive under physiological concentrations of adenosine and activated by high levels of adenosine such as that seen during high metabolism or in disease states such as ischemia. The inability of CGS 21680 to induce a cAMP response in MSCs indicates that the A<sub>2A</sub> receptor is either not or weakly present or it is not coupled to adenylate cyclase. The lack of a cAMP response to CGS 21680 also indicates that the A<sub>2B</sub> receptor is

dominant. The  $A_1$  receptor is also weakly expressed in MSCs as inhibition of forskolin stimulated cAMP could not be demonstrated although mRNA and protein studies show that the receptor is indeed expressed. The small stimulation of cAMP at high CCPA concentrations is probably due to non-specific activation of  $A_{2B}$  receptors (Klotz et al., 1997).

The apparent discord between the expression of the  $A_1$  and  $A_{2A}$  receptors and their functional activity has been previously reported by other groups e.g. the  $A_1$  receptor in folliculostellate cells, the  $A_1$  and  $A_{2A}$  receptors in the glioblastoma cell line U87MG and in skeletal muscle cells and the  $A_{2A}$  receptor in HMEC-1 cells. In these examples, although receptors were present, they failed to couple to the cAMP transduction pathway (Feoktistov et al., 2002; Lynge et al., 2003; Rees et al., 2002; Zeng et al., 2003). One plausible explanation for this phenomenon is the occurrence of post-translational modifications that result in alterations in the level of functional protein. On the other hand there could be alterations in sub-cellular localisation of the receptors that result in diminished signal transduction of the expressed receptor proteins (Zeng et al., 2003).

The  $A_{2B}$  receptor is dominantly expressed in MSCs and is also important in their differentiation to osteoblasts as NECA alone but not adenosine or CGS 21680 can stimulate ALP mRNA expression and enzyme activity in undifferentiated MSCs. The lack of effect of adenosine however could be attributed to the short half-life of adenosine (shown by our group to be <1 h in culture). Receptor expression is not static and may change as undifferentiated

cells undergo lineage differentiation and this has been reported for P2 receptors where there is a loss of P2X and an increase in P2Y, specifically the P2Y<sub>2</sub> receptor in mature osteoblasts (Orriss et al., 2006). I therefore investigated the changes in expression of adenosine receptors during osteoblastogenesis.

MSCs expressed both osteoblast (Cbfa1, collagen type I and ALP) and adipocyte (PPAR $\gamma$  and C/EBP $\alpha$ ) markers indicating their progenitor status and multilineage differentiation capacity (Rickard et al., 1996). In line with studies in rodent and human bone marrow derived progenitor cells, osteogenic induction in MSCs was accompanied by significant increases in osteoblast genes as well as mineralised matrix formation (Aubin, 1998).

Osteoblastogenesis of MSCs were also accompanied by increases in A<sub>2A</sub> and A<sub>2B</sub> receptor expression. The A<sub>2B</sub> receptor mRNA fell back to basal levels by day 5 of induced differentiation. The protein expression for A<sub>2B</sub> receptors followed a similar pattern but it persisted beyond that of the mRNA, possibly because the protein signal is sustained after the mRNA has been degraded. On the other hand A<sub>1</sub> receptor mRNA also increased significantly during osteoblastogenesis, this however did not appear to translate to protein which showed little change. Increased expression of A<sub>2A</sub> and A<sub>2B</sub> receptors on osteoblastogenesis were also demonstrated by increased cAMP responses to NECA and CGS 21680; the response to NECA remained dominant showing that the A<sub>2B</sub> receptor is still the major subtype. However, it should be noted

that MSC cultures undergoing differentiation contain both undifferentiated cells as well as cells at various stages of osteoblastic differentiation.

The differential expression of adenosine receptors suggests that they might be important at various stages of differentiation of MSCs or in regulating the activities of osteoblasts. The rapid induction of  $A_{2B}$  receptor mRNA (day 2) prior to changes in the mRNA for osteoblast markers (day 7) could suggest that its expression is mainly involved in the early commitment stage of osteoblastogenesis, but is less important in later stages of differentiation where there is a tailing off of expression. Similar observations have been reported for the PTH/PTHrP receptor in which the expression was associated with early osteoblastic differentiation while cAMP production in response to PTH was elevated at the late stage of differentiation (Kondo et al., 1997). This contrasts with the  $A_{2A}$  receptor which is upregulated at later stages of differentiation suggesting that this receptor may be related to differentiation or functional activities of osteoblasts.

Differentiation to osteoblasts was also associated with an early transient reduction in expression of the message for CD73 and no change for ADA. The association between CD73 and  $A_2$  receptors has been previously described; for example CD73 and the  $A_{2A}$  receptor can regulate expression of each other (Katebi et al., 2009; Napieralski et al., 2003) and a coordinated increase in expression of  $A_{2B}$  receptor and CD73 was observed under hypoxic conditions (Eltzschig et al., 2003). Coordinate regulation of CD73 and  $A_2$  receptors has been suggested to be a mechanism for balancing receptor



expression and extracellular adenosine levels. In osteoblastogenesis of MSCs, CD73 and  $A_{2B}$  receptor expression were inversely proportional and in this case one could speculate that activity at the  $A_{2B}$  receptor can be limited by reducing the production of adenosine. However the downregulation of CD73 mRNA should be treated with caution as parallel changes in protein have not been determined. Furthermore the levels of adenosine have not been measured thus the ultimate end point of CD73 downregulation cannot be determined.

The importance of  $A_2$  receptors in osteoblastogenesis was also demonstrated as NECA and adenosine both increased expression of ALP and Cbfa1 mRNA. NECA, adenosine and CGS 21680 were also strong activators of ALP enzyme activity in MSCs that had been pre-differentiated to osteoblasts. These findings were clearly mediated by  $A_{2A}$  and  $A_{2B}$  receptors, as disruption of signalling through these receptors by co-incubation with specific antagonists significantly reduced NECA induced ALP mRNA expression. In contrast to ALP and Cbfa1, collagen type I and osteocalcin were not significantly affected by treatment with adenosine or NECA during differentiation into osteoblasts.

Although Cbfa1 is a key transcription factor in the regulation of osteoblast differentiation, Cbfa1 independent pathways have also been demonstrated. Both *in vivo* and *in vitro* studies suggest that Wnt signalling through the LRP5 receptor controls osteoblast differentiation and function by increasing ALP expression in a Cbfa1 independent manner (Kato et al., 2002; Rawadi et al.,

2003). Thus it is possible that adenosine could activate multiple signalling pathways that are dependent or independent of Cbfa1 to regulate osteoblast differentiation. Further investigations are needed to establish the precise mechanisms involved in adenosine stimulated osteoblast differentiation.

The rapid induction of ALP enzyme activity by NECA is compatible with early upregulation of  $A_{2B}$  receptor expression and function shown by increases in mRNA, protein and cAMP accumulation. The reversibility of NECA induced ALP activity by MRS 1706 confirms that osteo-inducing activity can be mediated mainly through activation of  $A_{2B}$  receptors. The action of CGS 21680 on ALP activity during osteoblastogenesis seemed to occur at later time points than NECA and is consistent with the later time frame for  $A_{2A}$  receptor up regulation. One could perhaps speculate that  $A_{2B}$  receptors are important for commitment to osteoblast differentiation and activation of the higher affinity  $A_{2A}$  receptors maintains the differentiation process. Both receptors appear to be involved in osteoblastogenesis as NECA induced ALP activity is reduced by  $A_{2A}$  and  $A_{2B}$  receptor antagonists.

Preliminary experiments were also carried out to investigate if cAMP is a prime stimulator of osteoblastogenesis as cAMP is the primary signalling molecule for  $A_2$  receptors. Forskolin, an activator of adenylate cyclase, clearly induced ALP enzyme activity in MSCs and during osteoblast differentiation. Prolonged exposure to forskolin however inhibited ALP enzyme activity; the reason for this is unknown but may be related to downregulation of adenylate cyclase and upregulation of PDEs. It should be noted that adenosine

receptors can also act through other signal transduction pathways, such as the MAPK pathway; both cAMP and ERK signalling have been reported to be involved in osteoblast differentiation and activity (Lai et al., 2001; San Miguel et al., 2005; Siddappa et al., 2008; Yang et al., 2008).

The data presented here shows that the  $A_{2A}$  and  $A_{2B}$  receptors are strongly implicated in osteoblastogenesis of MSCs; whether these receptors are also involved in calcium deposition was also investigated. Adenosine and NECA but perhaps surprisingly not CGS 21680 stimulated mineralisation. Adenosine/NECA induced mineralisation is unlikely to be due to changes in cell number because we were unable to observe any significant effects of these compounds on cell numbers or on protein content. The lack of  $A_{2A}$  effect is surprising because it appears to be involved in the regulation of ALP activity during the later stages of differentiation.

In summary the data presented in this chapter show that the  $A_{2B}$  receptor is the dominant subtype in MSCs and during their osteoblastogenesis. Osteoblast differentiation is associated with increases in the  $A_{2A}$  and  $A_{2B}$  receptor expression and that these receptors stimulate osteoblastogenesis. The  $A_{2B}$  receptor in particular is the major subtype in the differentiation process as it enhances the three stages of initiation, maturation and mineralisation in osteoblastogenesis of MSCs.

## **Chapter 4**

# **Adenosine receptor expression and function during the differentiation of MSCs to adipocytes**

## 4.1 Introduction

### 4.1.1 Adipose tissue

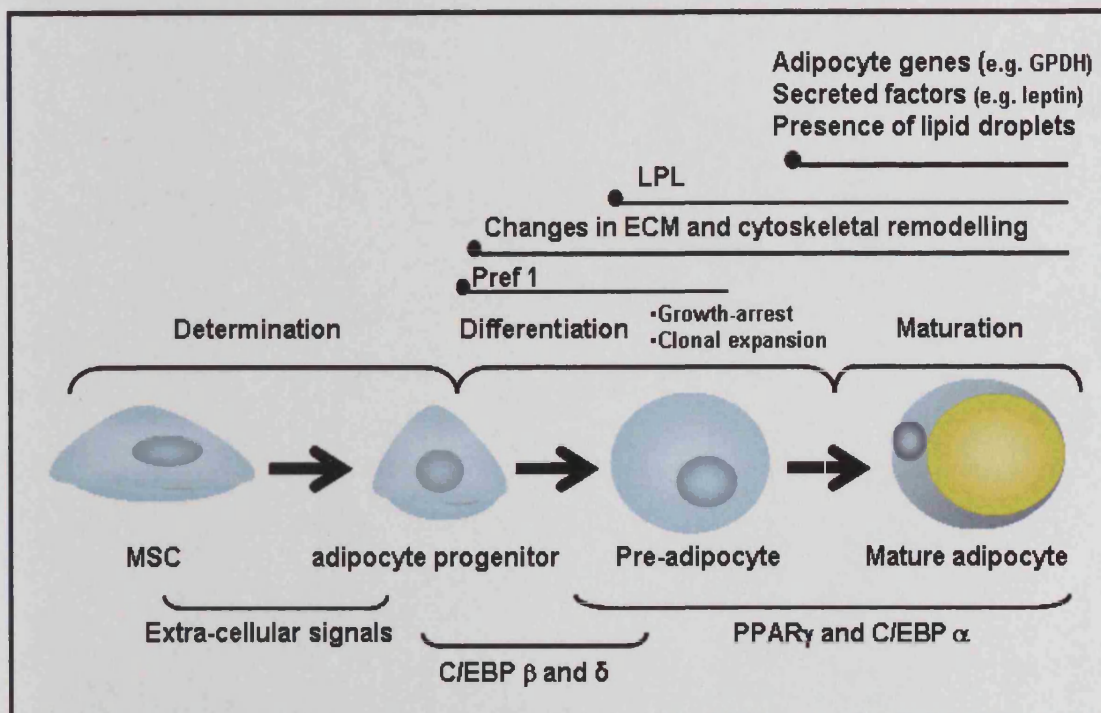
Adipose tissue was traditionally regarded as an inactive energy store and a source of insulation. In most mammals there are two forms, brown adipose tissue, which generates heat in newborns through mitochondrial uncoupling of lipid oxidation and white adipose tissue which form the majority of adipose tissue in adult humans and is responsible for storing energy, providing insulation and mechanical protection (Cinti, 2005). In addition, white adipose tissue is now known to secrete a variety of factors (*e.g.* leptin, adiponectin and IL-6) and express many receptors (*e.g.* leptin, adenosine A<sub>1</sub>, PPAR $\gamma$ ) that suggests they have important autocrine, paracrine and endocrine actions (Ronti et al., 2006; Waki and Tontonoz, 2007). An excess of white adipocytes is found to be associated with a number of adverse metabolic consequences such as insulin resistance, hyperglycemia, dyslipidemia, hypertension and proinflammatory states (Eckel et al., 2005). In addition there is considerable evidence of links between increased adipocyte number in the bone marrow and decreased bone mineral density. Conditions associated with bone loss, including ageing, glucocorticoid treatment, increased cortisol production and osteoporosis also coincide with increased marrow adiposity. This may be due to preferential differentiation of MSCs into adipocytes at the expense of osteoblasts (see section 1.2.2). On the other hand factors secreted from adipocytes could affect the differentiation and function of stem cell precursors as well as other mature cell types such as osteoblasts and osteoclasts (Zhao et al., 2008). Therefore, understanding the cellular and molecular

mechanisms behind the differentiation process and function of adipocytes could provide a basis for therapies aimed at improving bone formation.

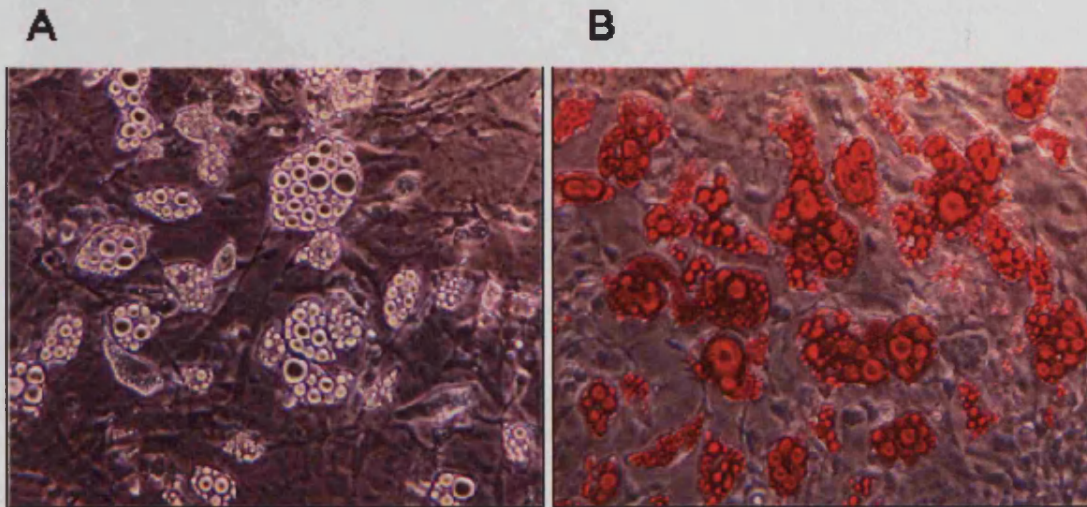
#### 4.1.2 The process of adipogenesis

The development of adipocytes from MSCs is a complex process, which involves changes in gene expression, hormone sensitivity and cell morphology. Adipogenesis is a multistage process (figure 4.1.1) and is regulated by transcription factors such as PPAR $\gamma$  and C/EBPs. The first stage is determination, in which cells become committed to the adipocyte lineage and lose their ability to differentiate into other lineages. Committed cells undergo an initial period of growth arrest at the G0/G1 cell cycle boundary which is followed by mitotic clonal expansion in response to mitogenic and adipogenic signals and the formation of mature adipocytes (Gregoire et al., 1998; Otto and Lane, 2005). These early events are accompanied by changes in gene expression and cell morphology which are initiated by transient expression of C/EBP $\beta$  and C/EBP $\delta$  and followed by an accumulation of PPAR $\gamma$  and C/EBP $\alpha$ . These latter two molecules remain high for the rest of the differentiation process and throughout the life of the mature adipocyte (Farmer, 2006; Gregoire et al., 1998; Lefterova and Lazar, 2009; Wu et al., 1996). Several other genes are expressed during the early stages of adipocyte differentiation and these include preadipocyte factor-1 (Pref-1), sterol regulatory element binding protein-1c (SREBP-1c) and LPL (Gregoire et al., 1998). Upregulation of LPL, in the early stages of differentiation, is thought to be associated with growth-arrest (Amri et al., 1986) whereas its expression in mature adipocytes is related to fatty acid and glycerol formation. In addition

to these molecular events, remarkable changes also occur in cell morphology, cytoskeletal components and the amount and type of extracellular matrix (ECM). Reorganisation of ECM and cytoskeletal proteins causes cells to change their shape from fibroblast-like to a more spherical appearance (Gregoire et al., 1998). These changes are followed by terminal differentiation, in which preadipocytes become mature and gain all the machinery necessary for adipocyte function. Mature adipocytes accumulate, in addition to PPAR $\gamma$  and C/EBP $\alpha$  (Gregoire et al., 1998), proteins that are involved in triglyceride metabolism, such as GPDH, LPL and fatty acid translocase (FAT). Morphologically, cells become spherical and accumulate small lipid droplets that subsequently fuse together to give a rounded appearance (figure 4.1.2 shows the accumulation of fat droplets in mature adipocytes *in vitro*).



**Figure 4.1.1 Postulated steps in adipocyte differentiation process.**  
Modified from Gregoire et al (1998) and Frith and Genever (2008).



**Figure 4.1.2 Mature adipocytes derived from rat MSCs.** (A) in culture (phase contrast microscopy) and (B) stained with Oil Red O.

#### 4.1.3 *In vitro* adipogenesis

Several cell culture models, including committed preadipocyte cell lines (3T3-L1 and Ob17), multipotent stem cell lines (C3H10T1/2) and primary cultures of MSCs, have been used to study the molecular and cellular events that occur during the process of adipocyte differentiation. Adipogenesis of such cells is induced by the addition of a cocktail of compounds that include insulin, Dex, IBMX and indomethacin. Insulin appears to increase the number of cells that differentiate into adipocytes as well as the amount of lipid accumulated; this effect of insulin, which only occurs at supraphysiological concentrations has been shown to be mediated through cross-activation of the IGF-1 receptor (Girard et al., 1994; Smith et al., 1988). Dex (a synthetic glucocorticoid agonist) has been shown to increase adipocyte differentiation, lipid accumulation and expression of C/EBP $\alpha$  and PPAR $\gamma$  by activation of the glucocorticoid receptor (Wu et al., 1996). IBMX, a PDEs inhibitor, is known to induce adipogenesis by increasing the levels of cAMP (described in section 1.3.6). Indomethacin, which has cyclooxygenase inhibitory activity, appears to



stimulate the activation of adipose differentiation related protein (ADRP) and PPAR $\gamma$  (Lehmann et al., 1997; Ye and Serrero, 1998). In other studies, PPAR $\gamma$  agonists (such as troglitazone, pioglitazone, thiazolidinedione and rosiglitazone) have been added to the adipogenic cocktail (Abdallah et al., 2006; Zhang et al., 2006). The above mentioned adipogenic inducing agents are most effective when used in combination and are unlikely to induce a significant effect on adipogenesis when used alone.

## 4.2 Aims

This chapter describes the expression and function of adenosine receptors during the differentiation of MSCs to adipocytes.

Specifically the aims are:

- To induce differentiation of MSCs toward the adipocyte lineage and subsequently investigate the changes in expression and function of adenosine receptors at various stages of differentiation.
- To investigate the role of adenosine signalling pathways in the regulation of differentiation of MSCs to adipocytes.

### **4.3 Methods**

#### **4.3.1 Analysis of adenosine receptors expression and function**

MSCs were seeded in multiwell plates at a density of  $5.5 \times 10^3$  cells per  $\text{cm}^2$  and cultured in normal growth medium until 50% confluent. This medium was then changed to adipogenic differentiation medium consisting of fresh growth medium supplemented with  $10^{-8}$  M Dex, 60  $\mu\text{M}$  indomethacin and 10  $\mu\text{g/ml}$  insulin. Adenosine receptor agonists and antagonists were sometimes included. Cultures were maintained in differentiation media for up to 12 days and fresh media were added every 2 - 3 days. mRNA and protein expression were assessed by q-RTPCR and Western blotting, and cAMP accumulation determined by radioimmunoassay as described in chapters 2 and 3.

#### **4.3.2 Oil Red O analysis of lipid accumulation**

For Oil Red O analysis, MSCs were plated into 12 well multidishes at a density of  $5.5 \times 10^3$  cells per  $\text{cm}^2$  and cultured and treated as above. Lipids were visualised with Oil Red O and quantified spectrophotometrically (absorbance at 490nm) following dye extraction with 100% isopropanol as described in section 2.9.

#### **4.3.3 Nile red analysis of adipogenesis**

For Nile red analysis MSCs were seeded in 6 well multidishes at a density of  $5.5 \times 10^3$  cells per  $\text{cm}^2$  and cultured and treated as above. Labelled cells were identified by flow cytometry analysis as described in section 2.9.

## 4.4 Results

### 4.4.1 Adenosine receptor expression during the differentiation of MSCs to adipocytes

#### 4.4.1.1 Differentiation of MSCs to adipocytes

Adipogenic inducing mixes usually contain IBMX. IBMX is however a methylated xanthine derivative and is a non-selective adenosine receptor antagonist and could therefore potentially interfere with adenosine signalling pathways. Three adipogenic cocktail mixes were therefore tested in the presence or absence of IBMX for their ability to induce the differentiation of rat MSCs to adipocytes; these were 1: Dex ( $10^{-8}$  M), indomethacin (60  $\mu$ M), insulin (10  $\mu$ g/ml) and IBMX ( $10^{-4}$  M), 2: same as 1 but without IBMX and 3: biotin (33  $\mu$ M), panthothenate (17  $\mu$ M), tri-iodothyronine (1 nM), Dex (100 nM), thiazolidinedione (1  $\mu$ M), (a PPAR $\gamma$  agonist) and insulin (500 nM). MSCs were treated with normal culture medium and cocktail media, 1, 2 or 3 for 10 days; lipid content was assessed by Oil Red O staining of cultures.

As shown in figure 4.4.1, lipid accumulation was increased when all three adipogenic media were used, with media 1 and 3 possibly inducing slightly higher adipogenesis than media 2. However, media 2 was chosen for all of my MSC studies; as this does not contain IBMX which potentially could interfere with adenosine signalling.

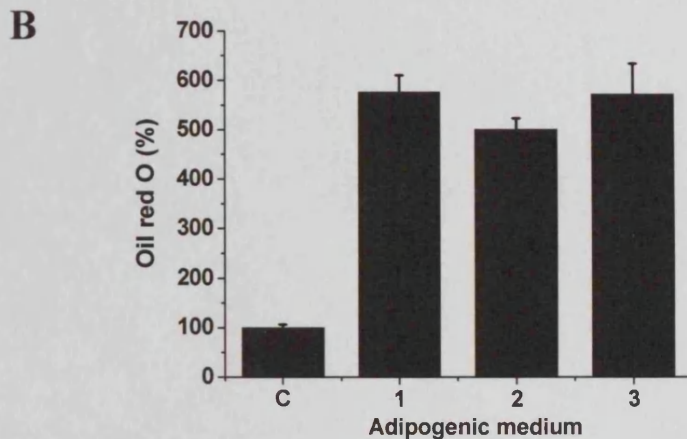
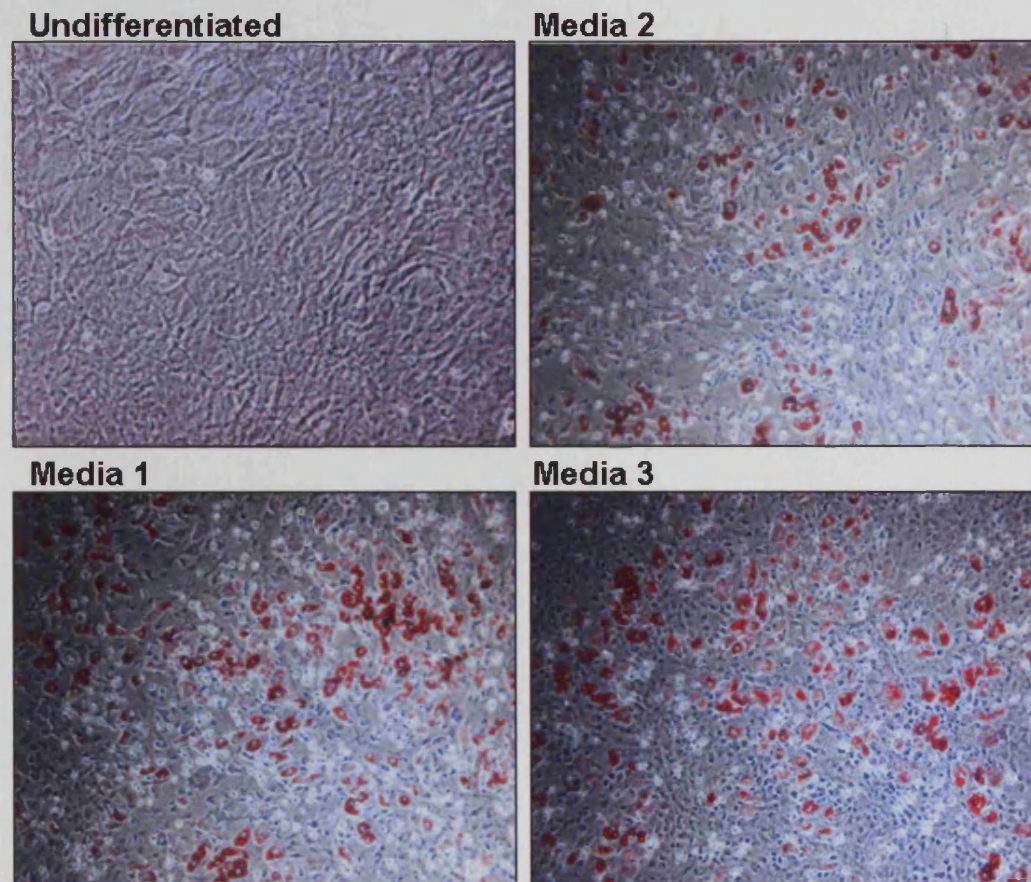
In the presence of adipogenic differentiation, intracellular lipid droplets were microscopically detectable after about 4 days. The number of adipocytes and the size of the lipid vesicles increased progressively throughout the

differentiation process as assessed by Oil Red O staining and by flow cytometry of Nile red stained cells (figures 4.4.2 A&B). Differentiation to adipocytes was further demonstrated by q-RTPCR analysis of the mRNAs for PPAR $\gamma$ , C/EBP $\alpha$  and LPL. The mRNAs for these genes were all easily detectable in undifferentiated MSCs maintained under basal conditions, and on adipogenesis there was a progressive increase in their expression. As shown in (figure 4.4.2 C) PPAR $\gamma$  was increased significantly by 3, 6 and 10 fold; CEB/P $\alpha$  by 4, 9 and 11 fold and LPL by 3, 23 and 29 fold after respectively 2, 7 and 12 days of adipogenesis.

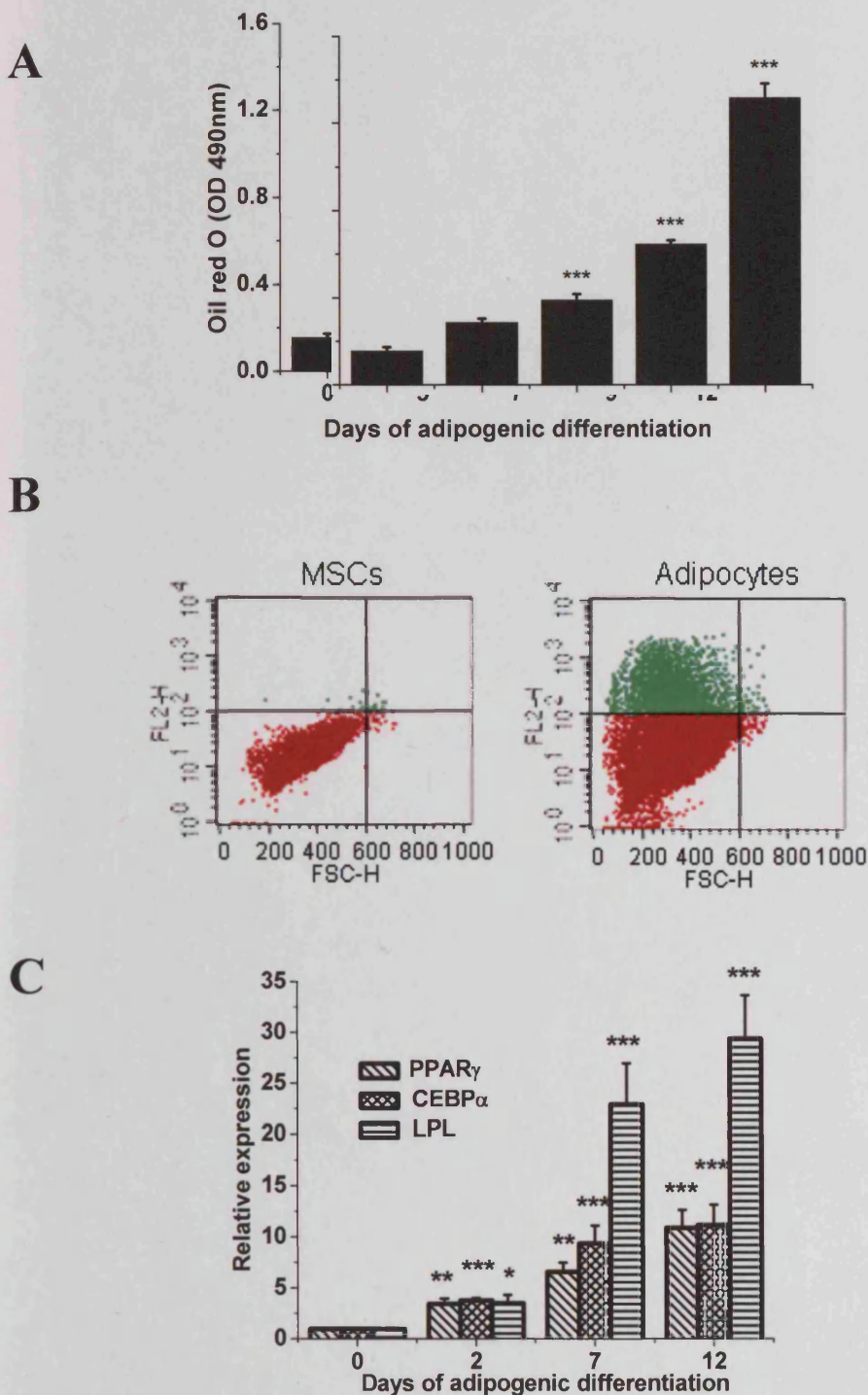
#### 4.4.1.2 Adenosine receptor mRNA expression during adipogenesis

RNA was extracted from undifferentiated MSCs (day 0) and at different time points (1, 2, 5, 7, 9 and 12 days) after the initiation of adipogenesis. Expression of adenosine receptor mRNA was analysed using q-RTPCR, normalised to  $\beta$ -actin and compared with day 0 (undifferentiated MSCs) that was assigned a value of 1.

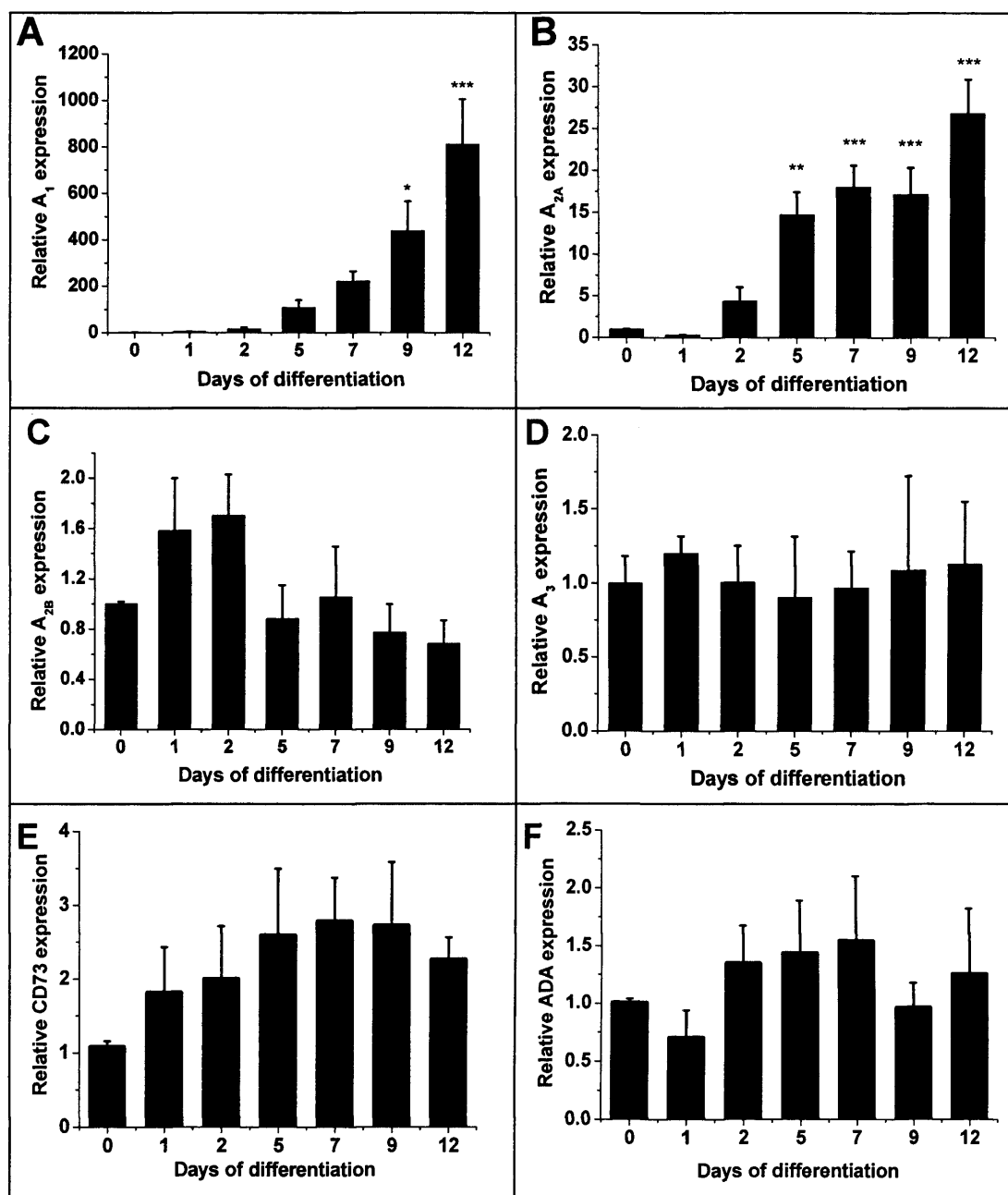
The relative mRNA expression of the four adenosine receptors during adipogenesis is shown in (figure 4.4.3). A<sub>1</sub> receptor mRNA expression was strongly up regulated during adipogenesis with an 800 fold increase at day 12 (P < 0.001). Changes in A<sub>2A</sub> mRNA paralleled that of A<sub>1</sub> receptor but only showed a 15 - 20 fold increase at 12 days (P < 0.001). There was no significant difference in expression of A<sub>2B</sub> and A<sub>3</sub> receptor mRNA during the differentiation process. Both CD73 and ADA remained unchanged during adipogenesis (figure 4.4.3).



**Figure 4.4.1 Examination of Oil Red O stained MSCs under various adipogenic culture conditions.** MSCs were cultured for 10 days in normal growth medium, adipogenic media 1 containing Dex ( $10^{-8}$  M), indomethacin ( $60 \mu\text{M}$ ), insulin ( $10 \mu\text{g/ml}$ ) and IBMX ( $10^{-4}$  M), adipogenic media 2 containing Dex ( $10^{-8}$  M), indomethacin ( $60 \mu\text{M}$ ) and insulin ( $10 \mu\text{g/ml}$ ), adipogenic media 3 containing thiazolidinedione ( $1 \mu\text{M}$ ), biotin ( $33 \mu\text{M}$ ), panthothenate ( $17 \mu\text{M}$ ), tri-iodothyronine ( $1 \text{nM}$ ), Dex ( $100 \text{nM}$ ) and insulin ( $500 \text{nM}$ ) and stained for lipid with Oil Red O ( $\times 100$ ). **(E)** The retained dye was measured spectrometrically ( $490 \text{ nm}$ ) and compared with cells in normal growth medium ( $n=2$ ; total of 6 replicates). Data are expressed as mean  $\pm$  SDM.



**Figure 4.4.2 Adipogenic differentiation of MSCs.** Cells were cultured with adipogenic medium. **(A)** Oil Red O labelling, measured at 490nm. **(B)** Flow cytometry analysis of Nile red stained cells after 7 days. **(C)** q-RTPCR of PPAR $\gamma$ , C/EBP $\alpha$  and LPL mRNA, normalised to  $\beta$ -actin mRNA and compared with day 0 that was assigned a value of 1 (n=3; total of 6 replicates). \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 when compared with undifferentiated MSCs.



**Figure 4.4.3** Changes in adenosine receptor and adenosine metabolic enzyme mRNA expression during the differentiation of MSCs to adipocytes. MSCs were cultured in adipogenic medium for the times shown and mRNA expression for (A)  $A_1$ , (B)  $A_{2A}$ , (C)  $A_{2B}$  and (D)  $A_3$  receptors determined. (E) and (F) show the expression of CD73 and ADA mRNA respectively ( $n=3$ ; total of 6 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with day 0.

#### 4.4.1.3 Adenosine receptor protein expression during adipogenesis

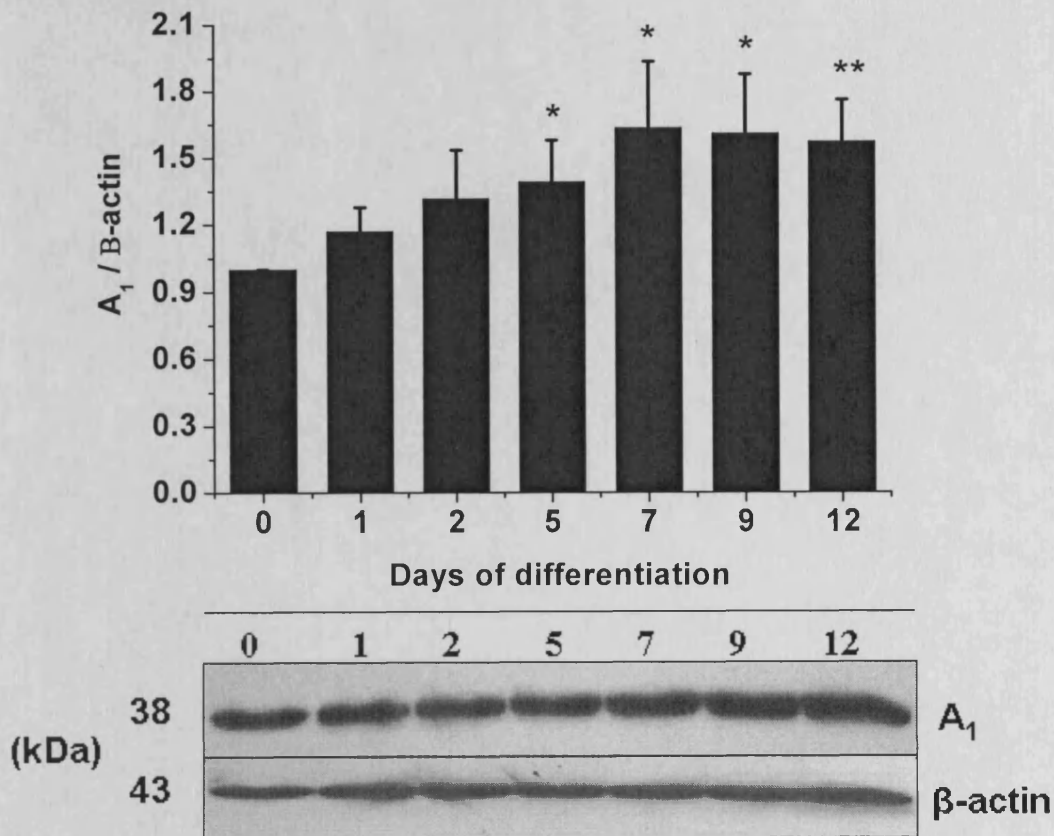
Western blot analyses were performed to investigate adenosine receptor protein expression during adipogenesis (up to 12 days) of MSCs. Representative Western blots for  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptors together with densitometric histograms for combined experiments are shown in figures 4.4.4 - 6 respectively. For the densitometric histograms, band densities for each adenosine receptor were corrected for background and normalised to  $\beta$ -actin and compared with day 0 (assigned a value of 1).

In parallel with changes in mRNA expression the proteins for the  $A_1$  and  $A_{2A}$  receptors also increased on MSC differentiation to adipocytes as judged by individual Western blotting analyses and by densitometric profiles. There were no changes in  $A_{2B}$  receptor protein expression during adipogenesis, consistent with a lack of change in its mRNA. The  $A_3$  receptor protein was not detectable.

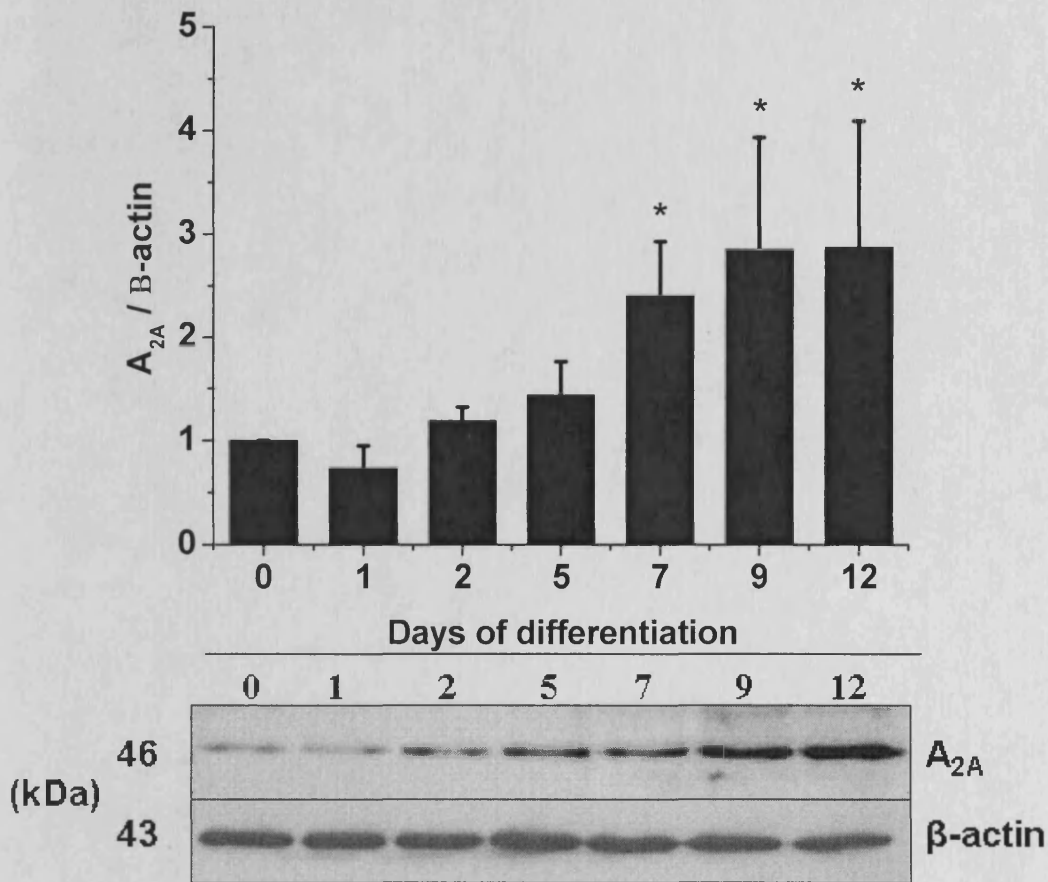
#### 4.4.1.4 Adenosine mediated cAMP responses during adipogenesis

Putative changes in adenosine receptor expression during adipogenesis of MSCs were also determined functionally by investigating cAMP responses to adenosine, NECA, CGS 21680 and CCPA. Figure 4.4.7 shows the changes in cAMP in MSCs and adipocytes, cultured respectively in normal growth medium and adipocyte differentiation medium for 9 days. At a concentration of  $10^{-4}$  M the fold increases in cAMP for MSCs and adipocytes were respectively

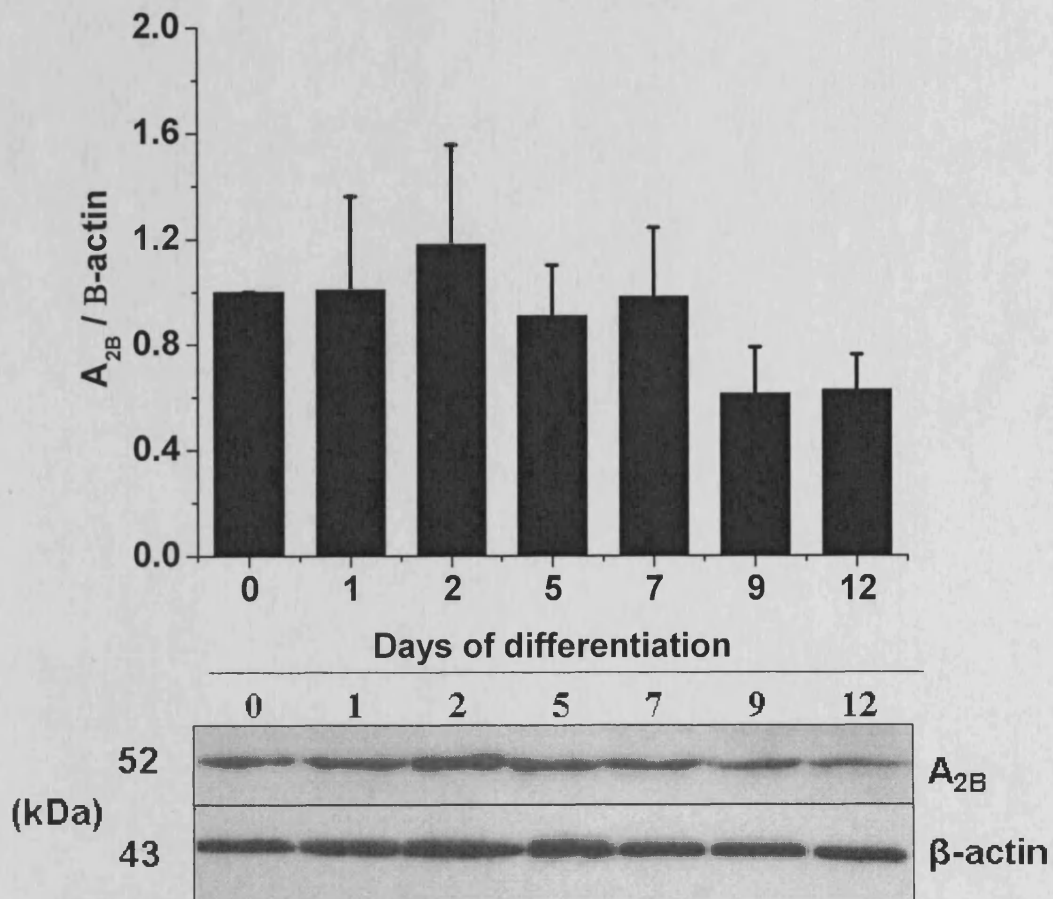




**Figure 4.4.4 A<sub>1</sub> receptor protein expression during the differentiation of MSCs into adipocytes.** MSCs were cultured in adipogenic medium. Protein lysates were separated by SDS-PAGE, probed for A<sub>1</sub> receptor and then stripped and re-probed for  $\beta$ -actin. Representative blots and combined densitometric values from at least 3 separate experiments are shown above. \* $P < 0.05$  and \*\* $P < 0.01$ , when compared with day 0 (assigned a value of 1).



**Figure 4.4.5 A<sub>2A</sub> receptor protein expression during the differentiation of MSCs into adipocytes.** MSCs were cultured in adipogenic medium. Protein lysates were separated by SDS-PAGE, probed for A<sub>2A</sub> receptor and then stripped and re-probed for β-actin. Representative blots and combined densitometric values from at least 3 separate experiments are shown above. \*P < 0.05 when compared with day 0 (assigned a value of 1).



**Figure 4.4.6** A<sub>2B</sub> receptor protein expression during the differentiation of MSCs into adipocytes. MSCs were cultured in adipogenic medium. Protein lysates were separated by SDS-PAGE, probed for A<sub>2B</sub> receptor and then stripped and re-probed for β-actin. Representative blots and combined densitometric values from at least 3 separate experiments are shown above.

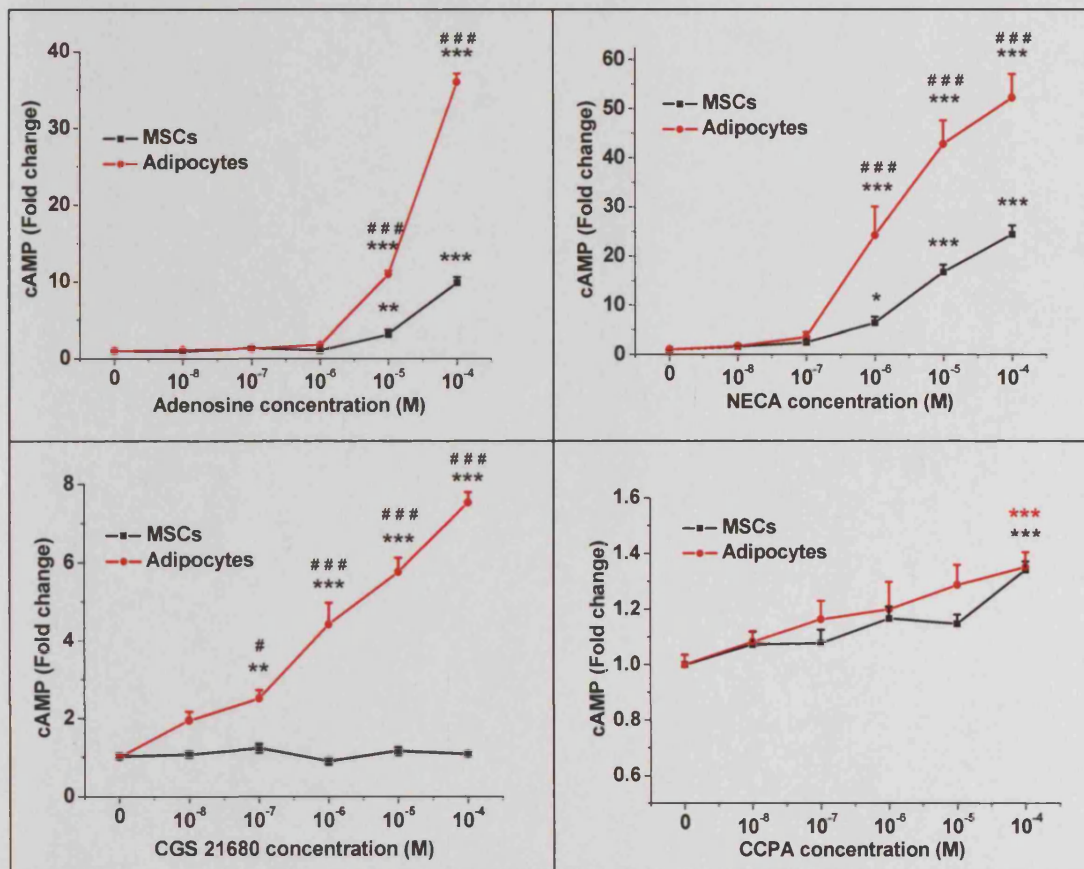
10 and 37 fold (adenosine ( $P < 0.001$ )), 25 and 55 fold (NECA ( $P < 0.001$ )) and 0 and 8 fold (CGS 21680 ( $P < 0.001$ )). These data indicate an overall increase in  $A_2$  receptor function in adipocyte differentiated MSCs compared with undifferentiated MSCs. This increase in cAMP responses indicates that the balance between  $A_1$  and  $A_2$  receptor expression is enhanced in favour of the latter and is consistent with increases in  $A_{2A}$  mRNA and protein expression during MSC differentiation to adipocytes.

Although there was a large increase in  $A_1$  mRNA during MSC differentiation to adipocytes this did not appear to be translated into protein where only a small increase was observed. The  $A_1$  receptor agonist, CCPA failed to have an inhibitory action on forskolin stimulated cAMP levels in either MSCs or adipocyte differentiated MSCs; this also suggests low expression of the  $A_1$  receptor particularly in context of increased  $A_{2A}$  receptor expression. At a concentration of  $10^{-4}$  M, CCPA did have an apparent stimulatory effect on cAMP in both cell types; this was probably due to a lack of specificity and high concentrations of CCPA binding to  $A_2$  receptors.

#### **4.4.2 Role of adenosine signal pathways on adipogenesis**

##### **4.4.2.1 Effects of adenosine and NECA on lipid accumulation**

Changes in adenosine receptor expression as MSCs are induced to differentiate to adipocytes suggest that adenosine itself may have a role in the differentiation process. The effects of adenosine and NECA on lipid accumulation were thus investigated. As shown in Figure 4.4.8, treatment with NECA ( $10^{-6}$  M to  $10^{-4}$  M) increases the number of cells that contain lipid when

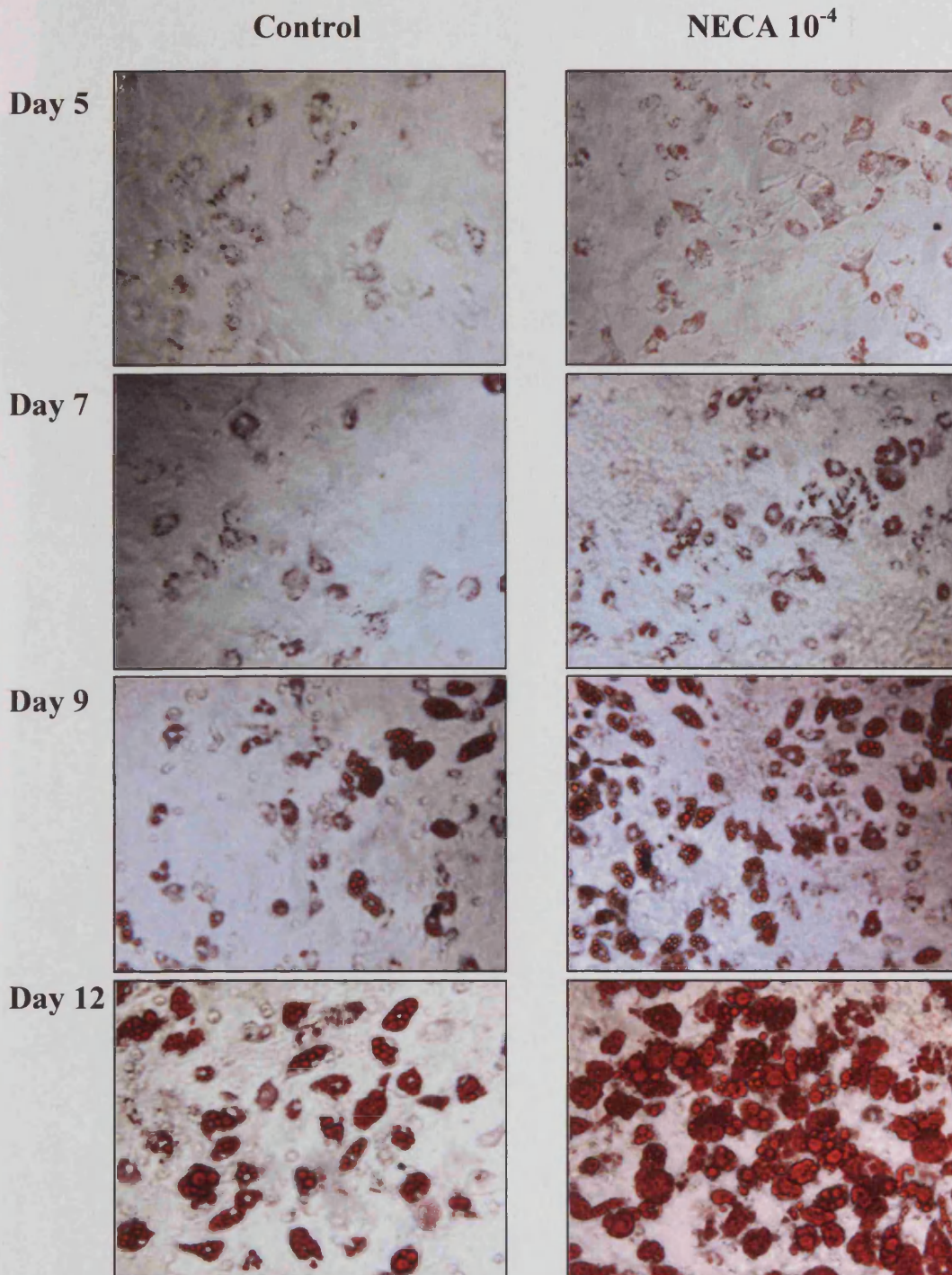


**Figure 4.4.7** Effects of adenosine receptor agonists on intracellular cAMP production in adipocytes derived from MSCs. Cells were cultured for 9 days in adipogenic differentiation medium. At the end of incubation period cells were treated with the indicated concentrations of (A) adenosine, (B) NECA, (C) CGS 21680 and (D) CCPA for 15 minutes in the presence of RO 20-1724 ( $10^{-4}$ M) and cAMP was measured using RIA ( $n=3$ ; total of 12 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with untreated cells; ### $P < 0.001$  when compared with MSCs stimulated with the same concentration of agonist.

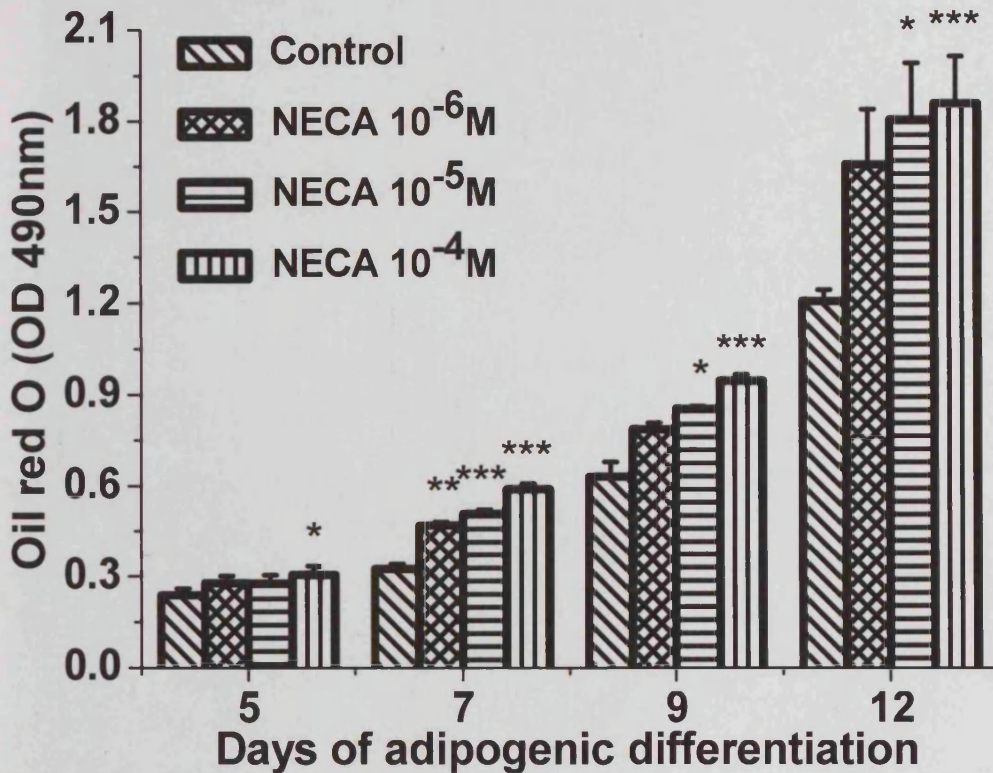
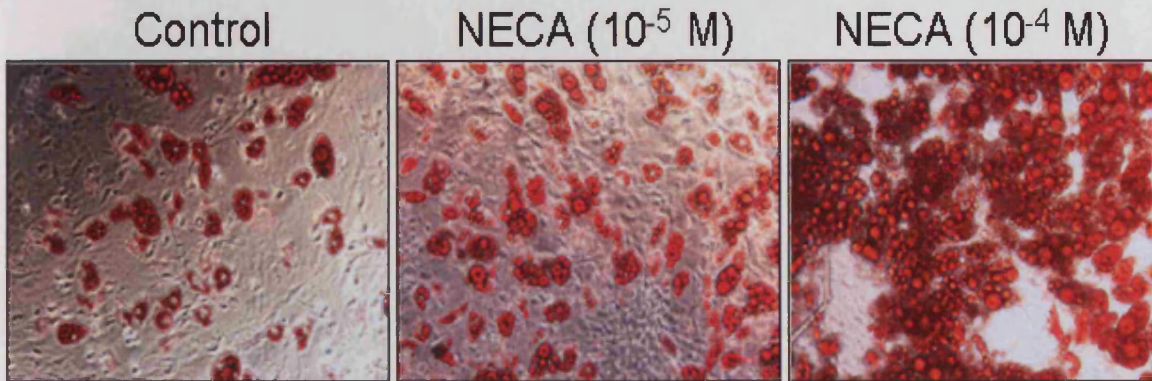
compared with vehicle control. The size and number of adipocytes also increased with time of exposure to the agonist and figures 4.4.9 and 10 showed that the observed effects were dose dependent. The effect was statistically significant at all time points during the differentiation process and  $10^{-6}$  M was the lowest concentration that caused a significant ( $P < 0.01$ ) response. At  $10^{-4}$  M NECA there was over 50% increase ( $P < 0.001$ ) in lipid accumulation when compared with cells incubated in media containing vehicle alone. Adenosine itself had less of an effect in comparison with NECA and probably reflects its short half-life. Treatment with  $10^{-4}$  M adenosine significantly ( $P < 0.01$ ) increased adipogenesis from day 7 with the maximum effect at day 12 (figure 4.4.10).

#### **4.4.2.2 Effects of NECA on adipocyte cell number**

Although Oil Red O staining analysis was used to demonstrate the induction of adipogenesis in NECA treated and untreated cells, this method only distinguishes the differences in total lipid content of the cells and could not identify the number of adipocytes in the population. Therefore, to achieve a quantitative measure of adipocyte number, Nile red staining with flow cytometry analysis was performed; this showed the number of adipocytes increased by up to 100% ( $P < 0.001$ ) in the presence of  $10^{-4}$  M NECA (figure 4.4.11). In addition, mean Nile red fluorescence per unit cell was also upregulated suggesting that the adipocytes also contained, on average, more intracellular lipid. These results are consistent with the data obtained when Oil Red O was used.

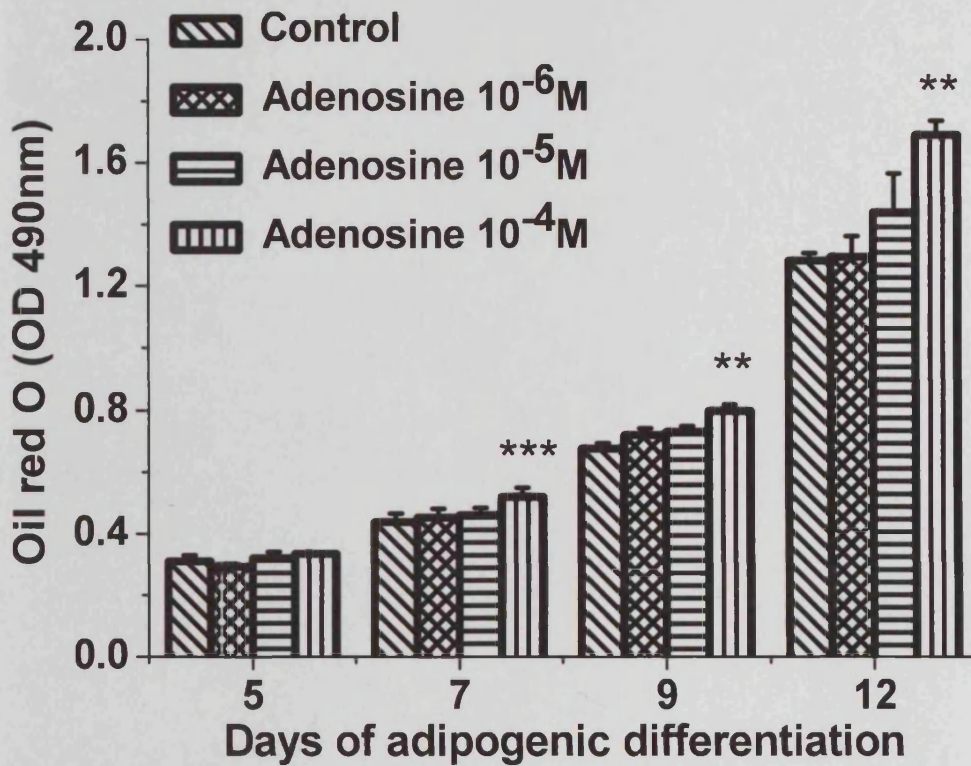
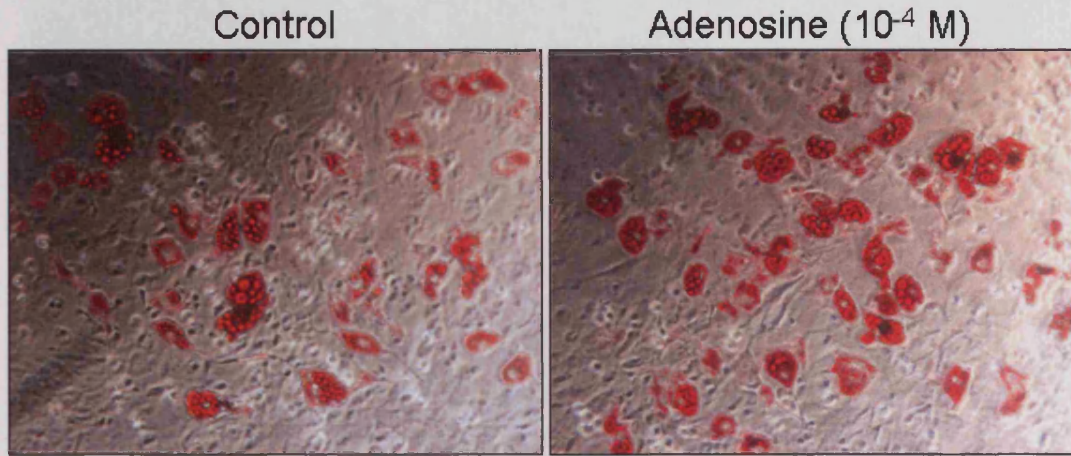


**Figure 4.4.8** Light microscope examination of the effects of NECA on adipogenic differentiation of MSCs. MSCs were incubated with adipogenic differentiation medium in the presence of NECA ( $10^{-4}$  M) or DMSO (vehicle) for 5, 7, 9 and 12 days. Accumulation of lipids in treated and untreated populations was visualised by staining with Oil Red O (x 200).

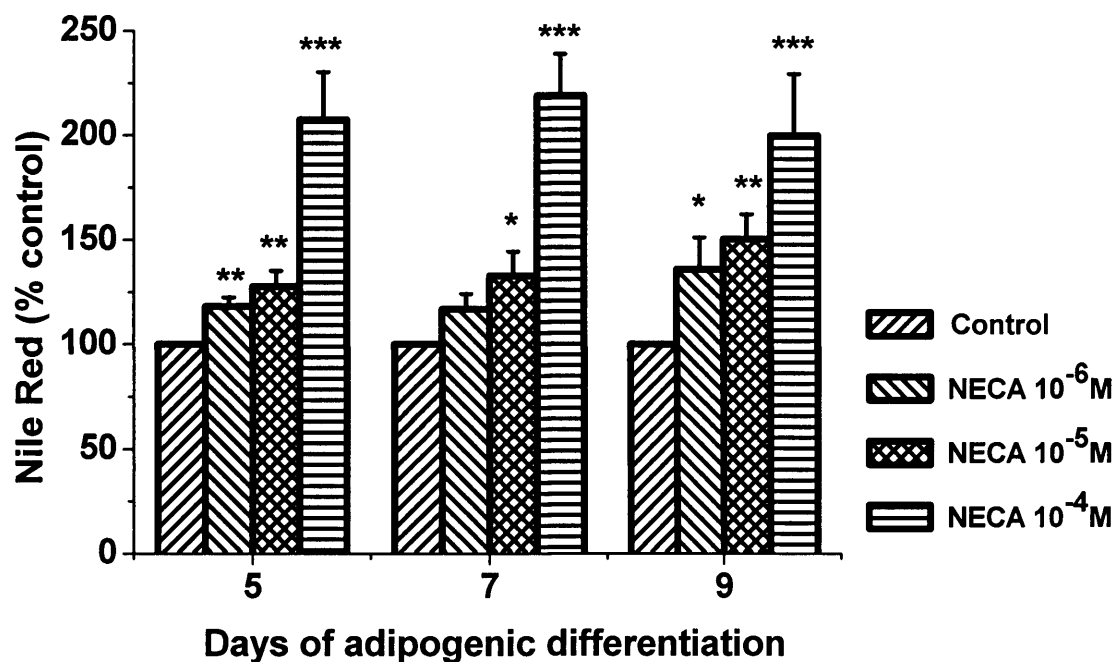


**Figure 4.4.9** Dose and time dependent effects of NECA on adipogenesis of MSCs as measured by Oil Red O staining. MSCs were incubated with adipogenic differentiation medium in the presence of NECA or DMSO for 5, 7, 9 and 12 days. Lipid accumulation was (A) visualised by staining with Oil Red O (x 200) and (B) quantified by measuring the absorbance at 490 nm (n=3; total of 9 replicates). \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 when compared with the control at the same time point.





**Figure 4.4.10 Dose and time dependent effects of adenosine on adipogenesis of MSCs as measured by Oil Red O staining.** MSCs were incubated with adipogenic differentiation medium in the presence of adenosine or PBS for 5, 7, 9 and 12 days. Lipid accumulation was (A) visualised by staining with Oil Red O (x 200) and (B) quantified by measuring the absorbance at 490 nm (n=3; total of 9 replicates). \*\*P < 0.01 and \*\*\*P < 0.001 when compared with the control at the same time point.



**Figure 4.4.11 Dose and time dependent effects of NECA on MSC differentiated adipocyte cell number, as determined by Nile red staining.** MSCs were incubated with adipogenic differentiation medium in the presence of NECA ( $10^{-4}$  to  $10^{-6}$  M) or DMSO for 5, 7 and 9 days. Cells were stained with Nile red and the % of positive cells was quantified with flow cytometry analysis ( $n=4$ ; total of 8 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with control at the same time point.

#### **4.4.2.3 Effect of NECA on the expression of mRNA for adipocytic genes.**

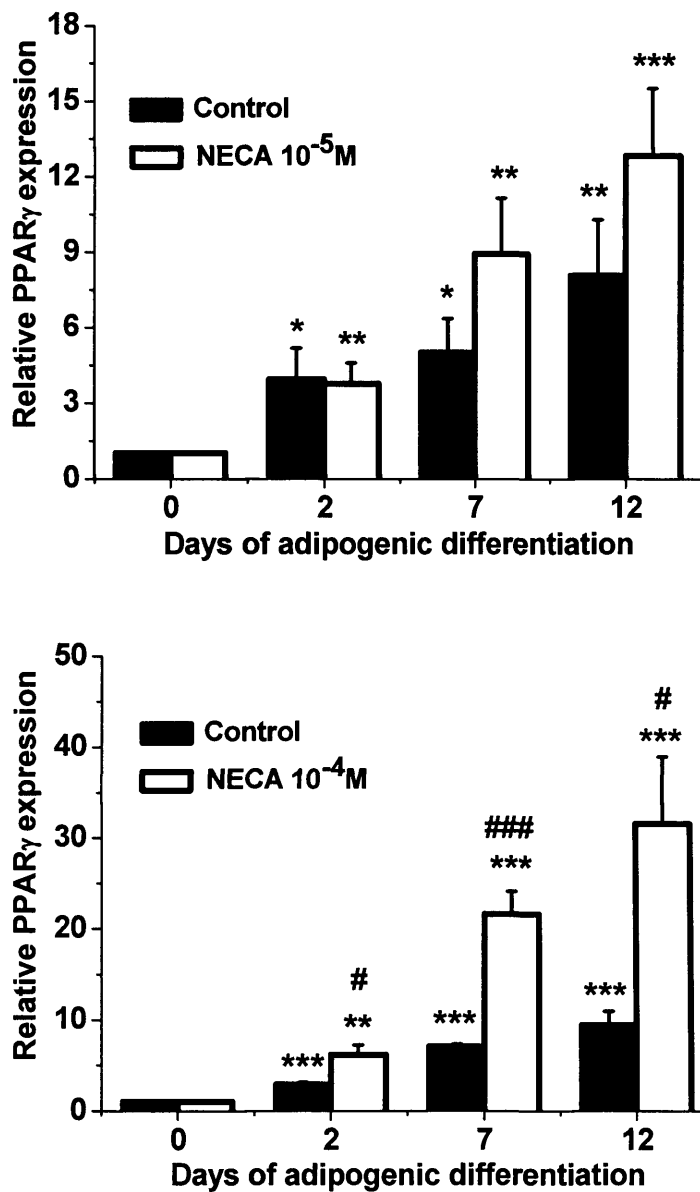
The previous results showed that adenosine and NECA, in the presence of adipogenic differentiation media, can further enhance adipogenesis as determined by lipid accumulation. Adipogenesis was also demonstrated by comparing the expression of adipogenic markers after NECA treatment. mRNA levels were normalised to  $\beta$ -actin and compared at different time points. Adipogenic differentiation caused a gradual increase in the expression of PPAR $\gamma$ , C/EBP $\alpha$  and LPL mRNA, which was further enhanced by the addition of NECA (figures 4.4.12 - 14). PPAR $\gamma$  mRNA expression was induced significantly from 3, 7 and 10 fold in untreated MSCs to 6 ( $P < 0.05$ ), 21 ( $P < 0.001$ ) and 31 ( $P < 0.01$ ) fold in the presence of  $10^{-4}$  M NECA for 2, 7 and 12 days respectively. Treatment with  $10^{-5}$  M NECA also tended to have a similar stimulatory effect, although statistical significant was not reached. NECA also induced similar, but more pronounced, changes in C/EBP $\alpha$  and LPL mRNA expression.

#### **4.4.2.4 Effect of selective adenosine receptor agonists and antagonists on lipid accumulation**

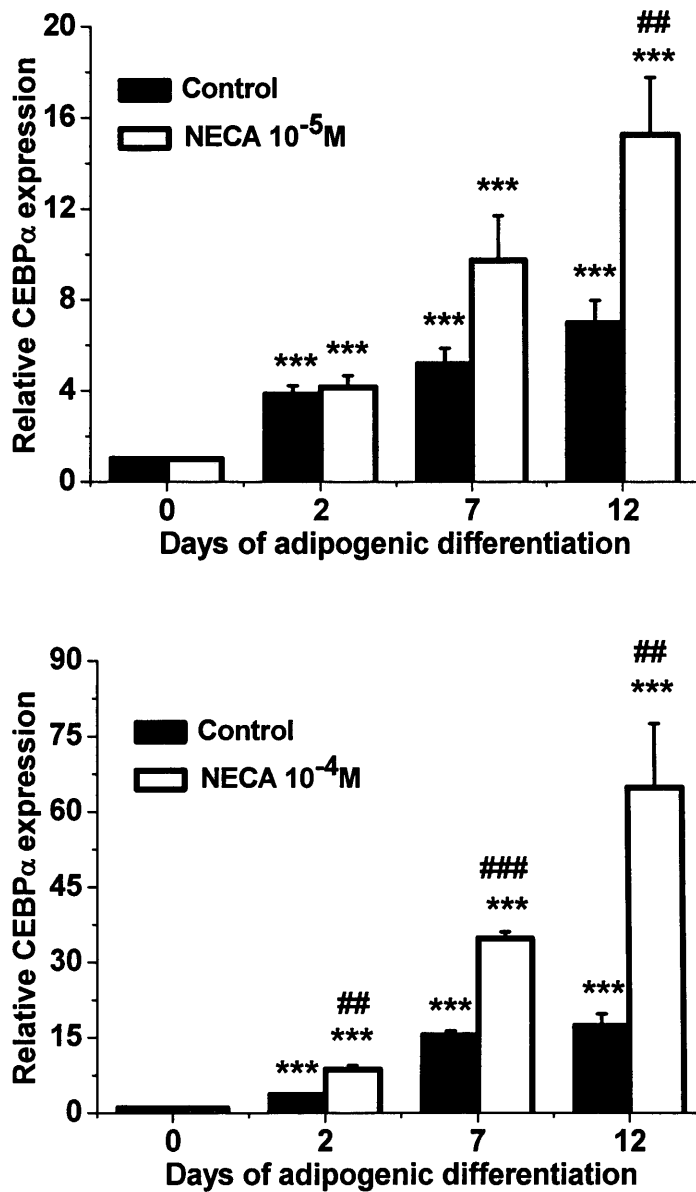
The mRNA, protein analysis and cAMP data suggests that the differentiation of MSCs to adipocytes is associated with changes in expression and activation of A $_{2A}$  and A $_1$  receptors. The effects of CCPA and CGS 21680, in the presence of adipogenic differentiation medium, on Oil Red O staining were thus investigated. Both compounds stimulated adipogenesis in a time and dose related fashion (figures 4.4.15 and 16). There were also increases in adipogenesis in the presence of CCPA at 5, 7 and 9 days but only at the

higher concentrations of  $10^{-5}$  M and  $10^{-6}$  M. Similar concentrations of CGS 21680 had a much more pronounced effect on adipogenesis. The effects of CGS 21680 were comparable to that observed when NECA was used (figure 4.4.9). These results indicate an involvement of  $A_1$  and  $A_{2A}$  receptors in the adipogenic differentiation of MSCs.

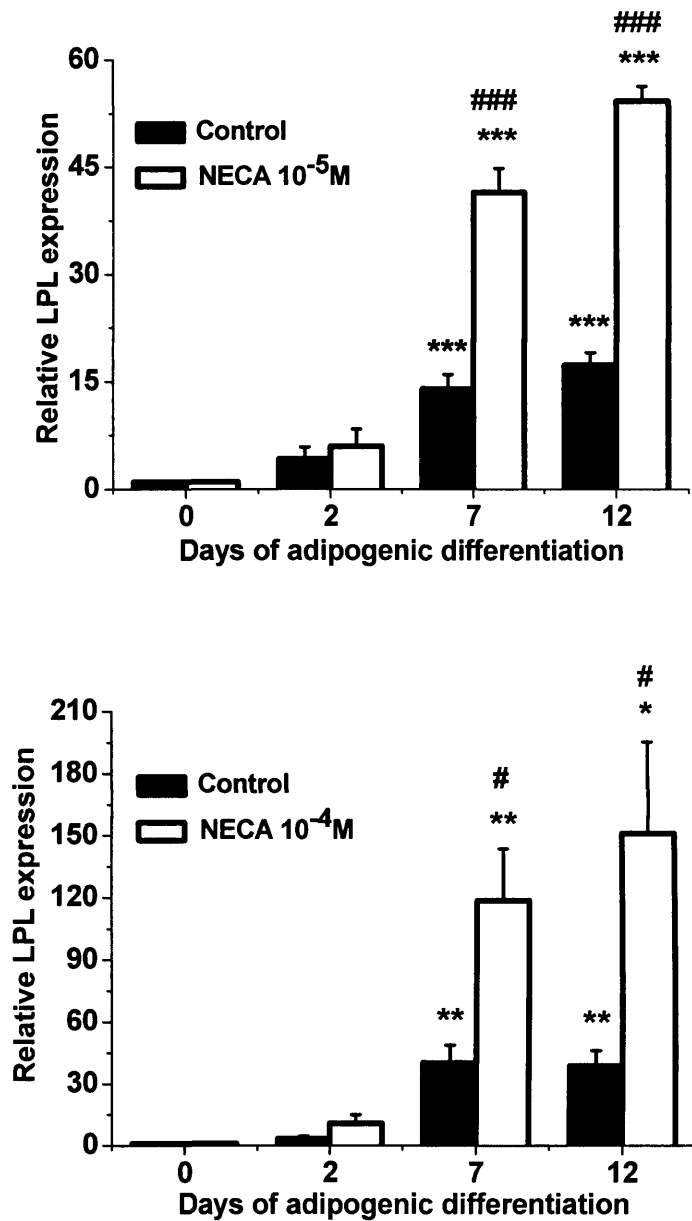
To confirm the involvement of the  $A_1$  and  $A_{2A}$  receptors as MSCs differentiate to adipocytes, the selective antagonists PSB 36 ( $A_1$ ) and SCH 442416 ( $A_{2A}$ ) were incubated together with NECA (figure 4.4.17). The concentrations of antagonist used were  $\leq 10^{-6}$  M, as higher concentrations caused a reduction in cell number. Addition of PSB 36 or SCH 442416 alone at  $10^{-6}$  M and  $10^{-7}$  M had no effect on lipid accumulation. PSB 36, however had no significant effect on the stimulation of adipogenesis by NECA suggesting that  $A_1$  receptors may not have an important role in adenosine mediated adipogenesis. On the other hand, SCH 442416 at  $10^{-6}$  M and  $10^{-7}$  M inhibited NECA stimulation of lipid accumulation by 80% ( $P < 0.001$ ) and 75% ( $P < 0.01$ ) respectively after 12 days of treatment. This inhibitory action of SCH 442416 was however not seen at 7 days.



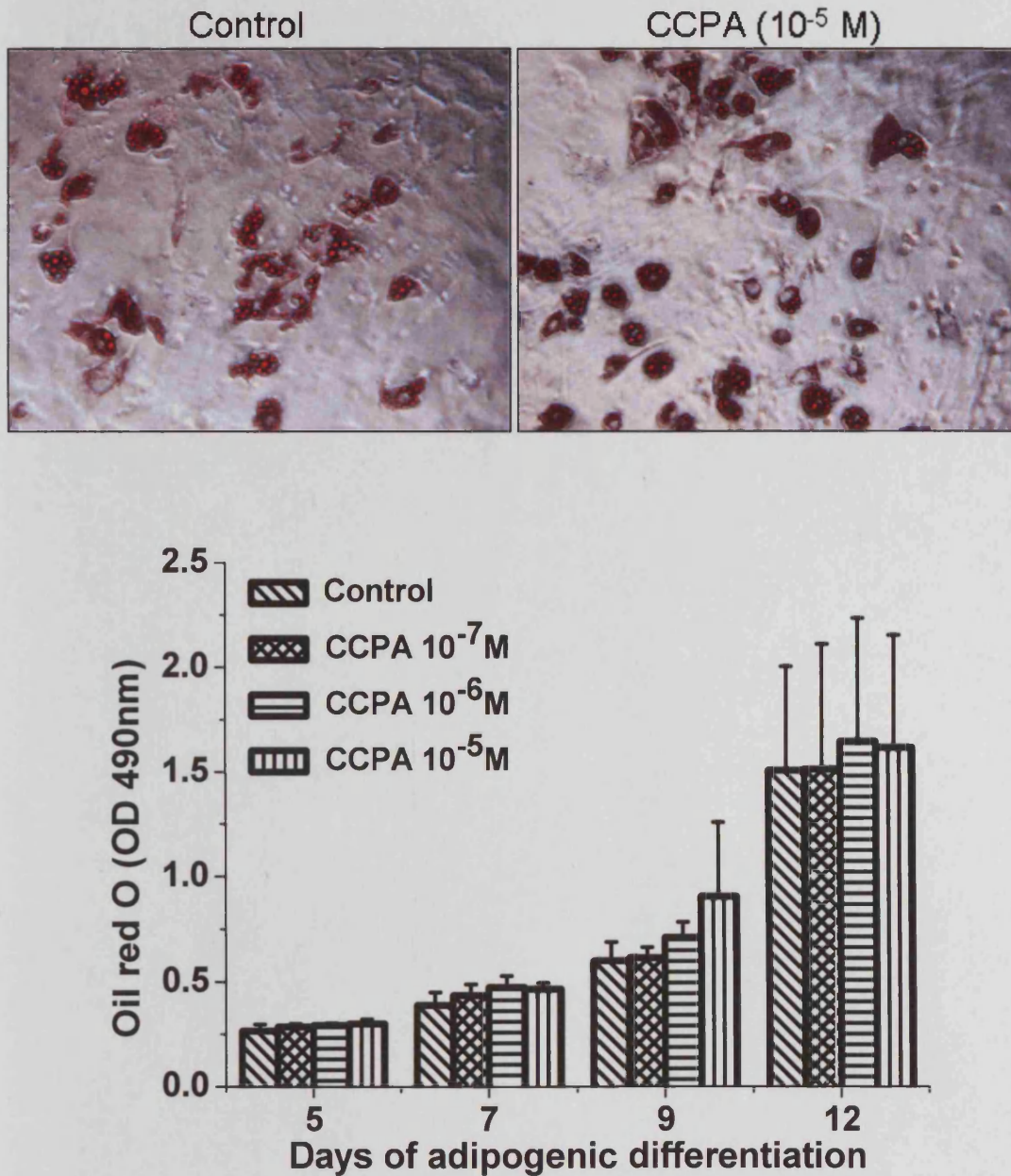
**Figure 4.4.12 Effect of NECA on PPAR<sub>γ</sub> mRNA expression during MSC differentiation to adipocytes.** MSCs were treated with NECA (10<sup>-5</sup> and 10<sup>-4</sup> M) for the indicated times during differentiation; PPAR<sub>γ</sub> mRNA, normalised for β-actin, was quantified by q-RT-PCR (n=3; total of 6 replicates). \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 when compared with day 0 (assigned a value of 1). #P < 0.05 and ###P < 0.001 when compared with vehicle at the same time point.



**Figure 4.4.13 Effect of NECA on C/EBP $\alpha$  mRNA expression during MSC differentiation to adipocytes.** MSCs were treated with NECA ( $10^{-5}$  and  $10^{-4}$  M) for the indicated times during differentiation; C/EBP $\alpha$  mRNA, normalised for  $\beta$ -actin, was quantified by q-RT-PCR ( $n=3$ ; total of 6 replicates). \*\*\* $P < 0.001$  when compared with day 0 (assigned a value of 1). ## $P < 0.01$  and ### $P < 0.001$  when compared with vehicle at the same time point.

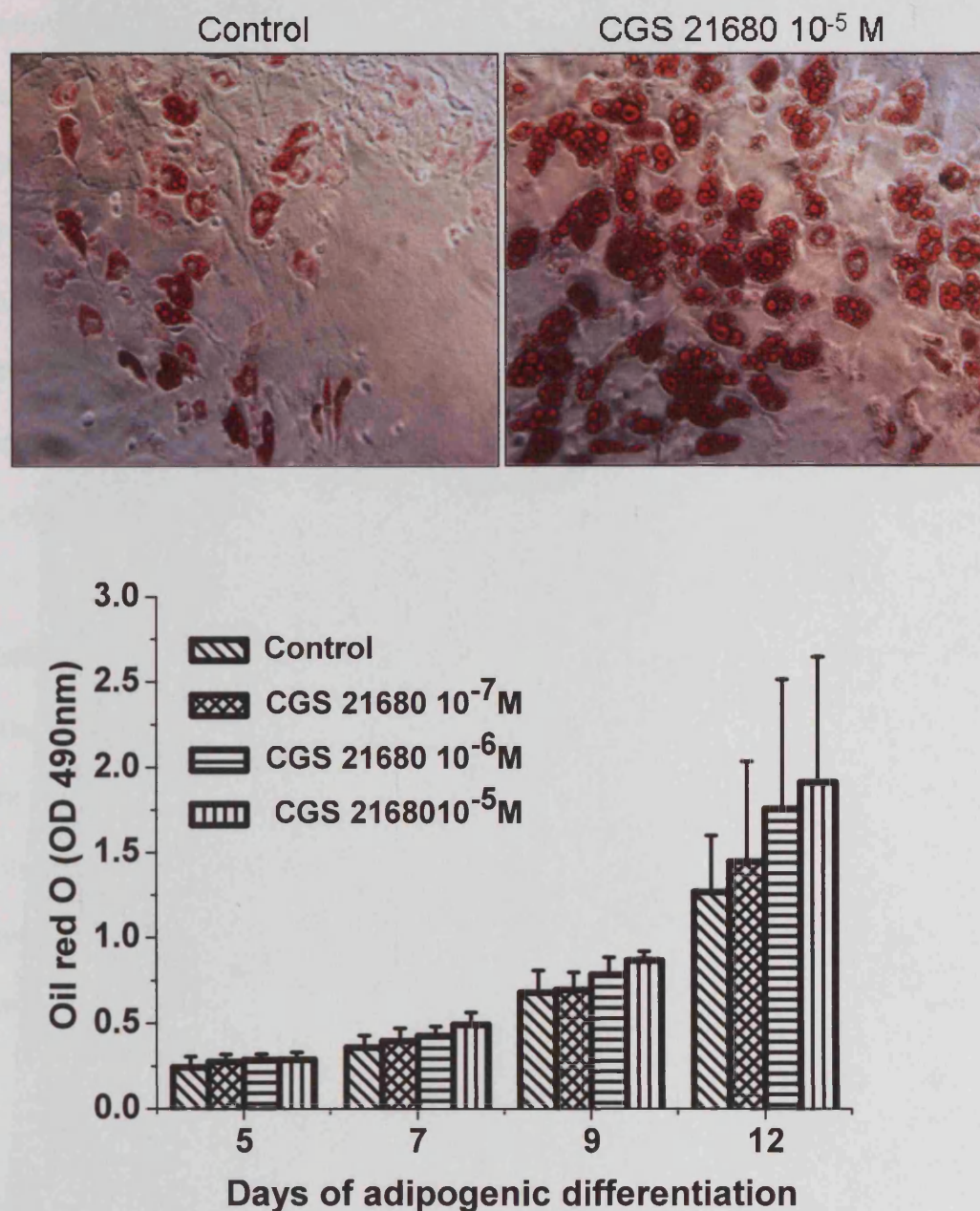


**Figure 4.4.14 Effect of NECA on LPL mRNA expression during MSC differentiation to adipocytes.** MSCs were treated with NECA ( $10^{-5}$  and  $10^{-4}$  M) for the indicated times during differentiation; LPL mRNA, normalised for  $\beta$ -actin, was quantified by q-RT-PCR ( $n=3$ ; total of 6 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with day 0 (assigned a value of 1). # $P < 0.05$  and ### $P < 0.001$  when compared with vehicle at the same time point.



**Figure 4.4.15 Dose and time dependent effects of CCPA on adipogenesis of MSCs.** MSCs were incubated with adipogenic differentiation medium in the presence of CCPA or vehicle for 5, 7, 9 and 12 days. Lipid accumulation was (A) visualised by light microscopy of Oil Red O staining (x 200) and (B) quantified by measuring the absorbance at 490 nm (n=2; total of 6 replicates). Data are expressed as mean  $\pm$  SDM.





**Figure 4.4.16 Dose and time dependent effects of CGS 21680 on adipogenesis of MSCs.** MSCs were incubated with adipogenic differentiation medium in the presence of CGS 21680 or vehicle for 5, 7, 9 and 12 days. Lipid accumulation was (A) visualised by light microscopy of Oil Red O staining ( $\times 200$ ) and (B) quantified by measuring the absorbance at 490 nm ( $n=2$ ; total of 6 replicates). Data are expressed as mean  $\pm$  SDM.

#### 4.5 Discussion

Induction of adipogenesis *in vitro* using Dex, indomethacin and insulin is well documented (Novakofski, 2004; Ookura et al., 2007; Pittenger et al., 1999; Yang et al., 2008). In my experiments, about 15% of the cells contained lipid droplets as determined by flow cytometry analysis of Nile red stained cells. Such cells also showed a change in morphology, from a fibroblast-like spindle shape for MSCs to a more spherical shape for adipocytes. The process of adipogenesis was supported by marked increases in expression of PPAR $\gamma$ , C/EBP $\alpha$  and LPL.

Differentiation of preadipocytes to mature adipocytes has been previously shown to be associated with alterations in adenosine receptor expression. A $_1$  receptor mRNA expression was reported to increase as the preadipocyte cell line Ob1771 underwent adipogenesis (Børghlum et al., 1996). This was supported by decreased cAMP levels in differentiated Ob1771 preadipocytes and in mature adipocytes derived from mouse, rat and human in response to A $_1$  receptor agonists (Børghlum et al., 1996; Fathollahi et al., 2006; Johansson et al., 2008; Vassaux et al., 1993). These data are in agreement with the dramatic up regulation of A $_1$  receptor mRNA expression seen in the experiments described here during the differentiation of MSCs into adipocytes. Changes in A $_1$  receptor protein expression was however less marked possibly due to a low level of translation of mRNA into protein. In addition, as with undifferentiated MSCs, inhibition of forskolin induced cAMP levels using the selective A $_1$  receptor agonist CCPA could not be demonstrated in MSC adipocytes. In fact, CCPA at 10 $^{-4}$  M resulted in an

increase, rather than a decrease in cAMP accumulation, which could be due to activation of  $A_{2B}$  receptors as previously described in chapter 3 and by other groups (Klotz et al., 1997; Lyngge et al., 2003; Rees et al., 2002). These findings were perhaps not unexpected as only a small proportion (15%) of the cells could be designated as adipocytes after differentiation and  $A_2$  receptors are dominant in MSCs and preadipocytes as shown in chapter 3 and by Borglum et al (1996).

Furthermore the increase in  $A_1$  receptor expression during adipogenesis was unexpectedly accompanied by increases in  $A_{2A}$  receptor expression at both the mRNA and protein level. It is likely that in these cultures  $A_2$  receptors are dominant (increase in cAMP accumulation) and thus  $A_1$  receptor inhibition of cAMP is masked by a greater stimulation of cAMP through activation of  $A_2$  receptors. Dominance of  $A_2$  over  $A_1$  receptors has also been previously reported in anterior pituitary cells where the the inhibitory effect of CCPA on cAMP was also masked (Rees et al., 2002). An alternative explanation could be alterations in sub-cellular localisation of the receptors that result in diminished signal transduction of the expressed receptor proteins. For instance,  $A_1$  receptor is reported to be present at high density throughout the somatodendritic cytoplasm of the cortical neurons and caveolin membrane of rat ventricular myocytes. This may prevent the expressed receptor proteins from interacting with functional G-proteins or agonists (Lasley and Smart, 2001; Ochiishi et al., 1999; Zeng et al., 2003).

The lack of an inhibitory effect on cAMP may not necessarily mean the lack of functional  $A_1$  receptors.  $A_1$  receptors could act via other signal transduction

pathways including PKC, PI3K and MAPK as well as activation of K<sup>+</sup> channels and inhibition of Q-, P- and N-type Ca<sup>2+</sup> channels (Jacobson and Gao, 2006). In fact, cAMP independent action of A<sub>1</sub> receptors has been demonstrated in isolated white adipocytes, where adenosine acting through the PLC-PKC pathway is able to regulate the insulin stimulated release of leptin (Cheng et al., 2000). Nevertheless the data in the experiments presented here shows that the increases in A<sub>1</sub> and A<sub>2A</sub> receptor expression correlate in time with increases in lipid accumulation and expression of adipocyte marker genes. These findings suggest a potential role for both receptors in the adipocyte differentiation process or in the functional activities of adipocytes.

The increased expression of A<sub>2</sub> receptors in my experiments is particularly surprising as Borglum and colleagues reported a loss of A<sub>2</sub> receptor expression and function during adipogenesis of Ob1771 (Børglum et al., 1996). Other researchers failed to detect A<sub>2</sub> receptor expression in primary mature adipocytes (Vassaux et al., 1993). Increases in A<sub>2</sub> receptor mRNA and protein expression on induced adipogenesis was supported by increases in cAMP stimulation in response to adenosine NECA and the A<sub>2A</sub> receptor agonist CGS 21680. CGS 21680 was ineffective on cAMP stimulation in MSCs. Although, adenosine and NECA induced cAMP accumulation was also increased in adipocyte differentiated MSCs, both of these compounds are universal agonists and thus can stimulate all adenosine receptors. However these changes in cAMP responses are likely due to activation of A<sub>2A</sub> receptors as there was no demonstrable change in A<sub>2B</sub> receptor expression. The potency of CGS 21680 to induce cAMP was almost equivalent to that of

NECA, but NECA was more efficacious than CGS 21680. This pharmacological profile is in agreement with the functional expression of  $A_{2A}$  receptors.

The increased expression of  $A_1$  and  $A_{2A}$  receptors as MSCs were induced to differentiate to adipocytes suggest that activation of these receptors will also regulate adipogenesis. In this context, adenosine has been shown to regulate various adipocyte functions including lipolysis, lipogenesis, insulin activity, glucose transport and leptin secretion (Cheng et al., 2000; Dhalla et al., 2007b; Johansson et al., 2008; Rice et al., 2000; Sollevi and Fredholm, 1981; Vannucci et al., 1989). The results presented herein clearly show that adenosine is also involved in the differentiation of MSCs to adipocytes. The degree and rate of adipogenic differentiation was increased in the presence of both adenosine and NECA, which elicited profound stimulatory effects on the accumulation of lipid and the number of adipocytes. NECA also stimulated the expression of mRNAs for  $PPAR\gamma$  and  $C/EBP\alpha$  throughout the duration of treatment, and LPL expression at the later stages of the differentiation process. These findings are consistent with those of Børghlum and co-workers, who reported that NECA enhanced corticosterone-induced adipogenic differentiation of a human preadipocyte cell line (Børghlum et al., 1996).

The action of adenosine on adipocyte function has been mainly attributed to signalling via  $A_1$  receptors and negative regulation of cAMP production (Fatholahi et al., 2006; Johansson et al., 2008; Sollevi and Fredholm, 1981; Vannucci et al., 1989). Although  $A_1$  receptor coupling to an inhibition of

forskolin induced cAMP was not demonstrated in my experiments the  $A_1$  receptor agonist CCPA did stimulate adipogenesis as shown by increased lipid accumulation. On the other hand the selective  $A_1$  receptor antagonist PSB 36 failed to have a significant effect on NECA induced adipogenesis. These results suggest that other adenosine receptors/mechanisms are also involved in adipogenesis. Increased expression of  $A_{2A}$  receptors and a stimulation of Oil Red O staining by CGS 21680 clearly indicate an involvement of  $A_{2A}$  receptor signalling in adipogenesis. Furthermore the adipogenic stimulatory effect of NECA was also inhibited by the  $A_{2A}$  receptor antagonist SCH 442416. These results are in agreement with the hypothesis by Vassuex and colleagues who suggested a bimodal role for adenosine in adipose tissue; the  $A_2$  receptor is expressed in preadipocytes and involved in adipocyte cell differentiation whereas the  $A_1$  receptor is expressed in mature adipocytes and involved in cell function (Vassaux et al., 1993). The observed induced adipogenesis by CCPA could be attributed to increased lipogenic activity of adipocytes as well as a stimulation of differentiation.

The comparable potencies of CGS 21680 and NECA on adipogenesis suggest that cAMP may be an important signalling molecule for regulating adipogenesis. The cAMP pathway has been previously reported, in murine preadipocytes 3T3-L1 and in human MSCs, to stimulate adipogenesis and expression of adipocytic genes (Petersen et al., 2008; Reusch et al., 2000; Watanabe et al., 2003; Yang et al., 2008). In addition, activation of cAMP in preadipocytes has been associated with suppression of *Wnt10b* and *Sp1*, both of which are negative regulators of adipogenesis (Bennett et al., 2002;

Tang et al., 1999). Furthermore *in vitro* studies show that IBMX and forskolin, molecules that upregulate cAMP levels, are important for inducing adipogenesis (Farmer, 2006).

In the context of adipocyte differentiation, two mechanisms have been described for the action of cAMP. The most important and well documented is through activation of PKA, phosphorylation of CREB on serine 133 and subsequent activation of transcription factors. cAMP has also recently been reported to induce adipogenesis via activation of an Epac1/Rap1-dependent pathway, which induces ERK1/2-dependent phosphorylation and activation of CREB. The Epac1/Rap1-dependent pathway that also induces changes in cytoskeletal organisation and extracellular matrix has been implicated in IBMX induced adipogenesis (Petersen et al., 2008). It is not known whether NECA can activate the Epac1/Rap1-dependent pathway and my experiments appear to suggest that NECA has little effect on ERK1/2 activation in adipocytic differentiated MSCs.

In, summary, I have shown that adenosine receptor expression and function triggers a cascade of transcriptional events (C/EBP $\alpha$ , PPAR $\gamma$  and LPL) that leads to the expression of factors associated with a mature adipocyte phenotype. The adenosine receptors involved in adipogenesis of MSCs are primarily the A<sub>2A</sub> and A<sub>1</sub> receptors. The signal molecules involved in this process are likely to be cAMP/PKA/CREB. However other pathways such as MAPK, PLC, or PKC that are known to be activated by adenosine receptors might also be important. Experiments using additional specific receptor or

pathway inhibitors or gene inhibition using siRNA are needed to confirm the current findings. These procedures have been used for analysing the effects of cAMP/PKA/CREB on adipogenesis (Pekkinen et al., 2008; Reusch et al., 2000; Yang and Gerstenfeld, 1996).

Adenosine has also been shown to be involved in the release of hormones, growth factors and cytokines, such as leptin and IL-6, from MSCs and adipocytes and such molecules are known to be involved in differentiation and function (Cheng et al., 2000; Hamrick et al., 2005; Hasko et al., 2008; Rice et al., 2000). Adenosine induced adipogenesis could thus also be regulated through the secretion of autocrine and paracrine factors and this hypothesis needs to be explored.



## **Chapter 5**

# **Adenosine receptor expression and function in osteoblasts and during their transdifferentiation to adipocytes**

## 5.1 Introduction

### 5.1.1 Transdifferentiation

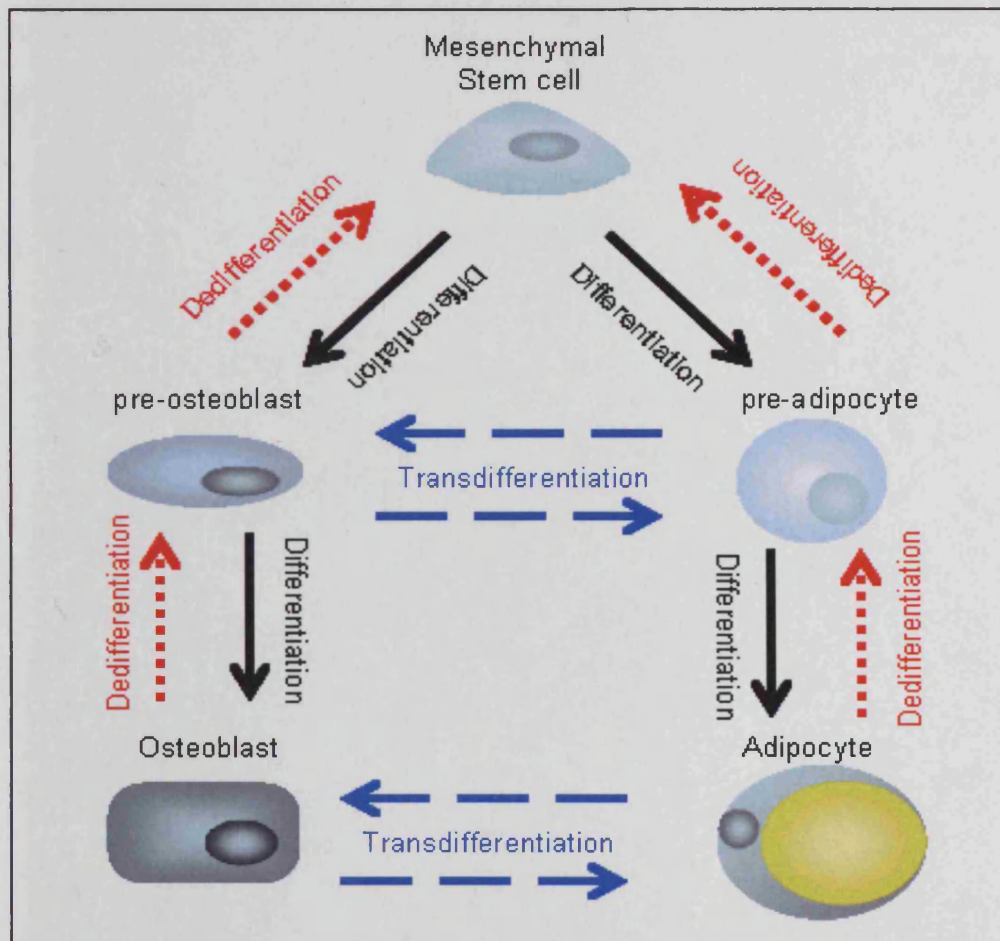
Transdifferentiation is a process whereby one type of differentiated cell is converted to another (Eguchi, 1995; Tosh and Slack, 2002). It belongs to a larger class of cell-type conversions known as metaplasias, which include transformation of stem cells of one lineage to another e.g. haematopoietic progenitor cells to astrocytes or to hepatocytes (Alison et al., 2000; Eglitis and Mezey, 1997; Slack, 2007; Tosh and Slack, 2002). Transdifferentiation is thought to occur as a result of changes in the expression of key transcription factors, either due to somatic mutation or by changes in the cellular environment (Thowfeequ et al., 2007). Whatever the underlying mechanism, it eventually results in a change in gene expression with a loss of some genes and a gain in other genes. Transdifferentiation may be accompanied by a dedifferentiation step, whereby a differentiated cell type is first reversed to an immature stem or progenitor cell stage (dedifferentiation) and then differentiated to another distinct cell type. In this case, cells lose their lineage specific genotypic and phenotypic properties before gaining others. However if transdifferentiation happens rapidly then the dedifferentiation stage may not occur and genotypic properties of both cell types may coexist (Liu and Rao, 2003; Slack and Tosh, 2001). Transdifferentiation can only be considered to have taken place when a cellular phenotype with new characteristics is stably established (Slack and Tosh, 2001). Numerous examples of transdifferentiation exist within the literature including that of pancreatic cells to hepatocytes and myoblasts to adipocytes (Hu et al., 1995; Shen et al., 2003).

### 5.1.2 Transdifferentiation of osteoblasts to adipocytes and *vice versa*

In recent years several research groups have demonstrated possible phenotype switches between committed or differentiated osteoblast and adipocyte lineages *in vitro*. An early study by Nuttall and co-workers demonstrated that fully differentiated osteoblasts, as shown by ALP activity and osteocalcin synthesis, were able to undergo adipogenic differentiation after treatment with adipogenic inducing medium containing Dex and IBMX (Nuttall et al., 1998). Cells derived from collagenase-treated adult human trabecular bone fragments, when treated with Dex, indomethacin, IBMX and insulin transdifferentiated into adipocytes (Nöth et al., 2002).

Overexpression of PPAR $\gamma$  alone or in combination with C/EBP $\alpha$  in the mouse osteoblastic MC3T3-E1 cells in the presence of appropriate stimuli resulted in marked transdifferentiation to mature adipocytes expressing molecular markers of adipogenesis (Kim et al., 2005). In addition, osteoblasts were shown to undergo transdifferentiation towards an adipogenic phenotype after 3 days of 18- $\alpha$ -glycyrrhetic acid (AGRA) or oleamide-mediated inhibition of Gap-junctional communication (GJC) (Schiller et al., 2001). Moreover, single cell MSC derived clones were able to differentiate into osteoblasts and adipocytes and then transdifferentiate into other mesenchymal lineages by dedifferentiating back to the primitive stem cell stage, and subsequently differentiating into mesenchymal lineages (Song and Tuan, 2004; Song et al., 2006). Adipocytes cloned from human bone marrow were shown to dedifferentiate into fibroblast-like cells, and subsequently to differentiate into osteoblasts and adipocytes under appropriate culture conditions (Park et al.,

1999). Human subcutaneous extramedullary adipocytes were also shown to dedifferentiate prior to differentiation to either osteoblasts or adipocytes (Justesen et al., 2004). Evidence for dedifferentiation and/or transdifferentiation between osteoblasts and adipocytes has also been reported by other groups (Schilling et al., 2008; Schilling et al., 2007; Wang et al., 2007). Figure 5.1.1 shows possible mechanism of switching between osteoblasts and adipocytes.



**Figure 5.1.1** Postulated mechanisms in mesenchymal lineage switching.

Although there is little information available, switching between osteoblasts and adipocytes might also occur *in vivo* and could partly account for bone loss seen in osteoporosis or osteopenia which are accompanied by increased

adipocytes and a decreased osteoblasts in the bone marrow. Therefore, understanding the signalling molecules and the mechanisms behind the transdifferentiation process between osteoblasts and adipocytes could be important for the development of therapeutic strategies for the retention of osteoblasts or to prevent their transdifferentiation to adipocytes.

## **5.2 Aims**

This chapter describes the expression and function of adenosine receptors in a murine osteoblast cell line (7F2) and in primary human osteoblasts (HOBS) and as they are induced to transdifferentiate to adipocytes.

Specifically the aims are:

- To investigate the expression and function of adenosine receptors in 7F2 and HOB cells.
- To induce differentiation of 7F2 and HOB cells towards an adipocyte lineage and to investigate related changes in the expression and function of adenosine receptors.
- To investigate the role of exogenous adenosine in the regulation of adipocyte differentiation of 7F2 and HOB cells.

### **5.3 Methods**

#### **5.3.1 Analysis of expression and function of adenosine receptors in 7F2 cells and as they transdifferentiate into adipocytes**

7F2 cells were seeded in to multiwell plates, as appropriate, at a density of  $4 \times 10^3$  cells per  $\text{cm}^2$  in normal growth medium for 24 hours. Transdifferentiation into adipocytes was induced by addition of 50  $\mu\text{g/ml}$  AA,  $10^{-7}$  M Dex and 50  $\mu\text{M}$  indomethacin. The cells were either left untreated in differentiation medium or were treated with vehicle or the indicated concentrations of adenosine receptor agonist and antagonists for the indicated times with the medium being changed every 2 - 3 days. Changes in adenosine receptor, adipocyte and osteoblast marker mRNA and protein expression were assessed by q-RTPCR and Western blotting, as appropriate and cAMP accumulation determined by RIA as described in chapters 2 and 3. Adipogenesis in treated and untreated adipocyte populations was visualised by staining with Oil Red O and accumulation of lipids was quantified spectrophotometrically following extraction of the dye retained by the cells. The number of adipocytes in the sample was also determined by flow cytometry analysis of Nile red stained cells as described in chapter 2.

In some experiments ALP activity was assessed in 96 well plates following treatment with adenosine receptor agonists and antagonists or vehicle for 2 and 5 days. The results were corrected for changes in cell number, determined on the same plate by the MTS assay and expressed as percentage of control.

### **5.3.2 Analysis of expression and function of adenosine receptors in HOB cells and as they transdifferentiate into adipocytes**

HOB cells were seeded in to multiwell plates at a density of  $1.05 \times 10^4$  cells per  $\text{cm}^2$  and cultured in normal growth medium supplemented with 50  $\mu\text{g/ml}$  AA for 24 hours. Transdifferentiation of HOB cells into adipocytes was induced using adipogenic differentiation medium consisting of the normal growth medium supplemented with  $10^{-8}$  M Dex, 60  $\mu\text{M}$  indomethacin and 10  $\mu\text{g/ml}$  insulin. The cells were either left untreated in differentiation medium or were treated with vehicle or NECA at  $10^{-5}$  and  $10^{-4}$  M for 2, 7, 12 and 17 days with the medium being changed every 2 - 3 days. Adipogenesis was visualised by staining with Oil Red O. Changes in adenosine receptor, adipocyte and osteoblast marker mRNA and protein expression, as appropriate, was assessed by q-RTPCR and Western blotting as described in chapter 2. ALP activity was also assessed as above.

In some experiments the effect of adenosine receptor agonists on mineralisation of 7F2 and HOB cells was also carried out. Mineralisation of 7F2 cells was induced by addition of 50  $\mu\text{g/ml}$  AA,  $10^{-7}$  M Dex and 6 mM  $\beta$ -GP for 10 - 12 days and HOBs by addition of 2 mM  $\beta$ -GP for 20 days. Mineralisation was assessed by Alizarin Red S staining.

### **5.3.2 Analysis of ERK and CREB phosphorylation**

Cells were cultured in either normal growth medium until 80% confluent or in differentiation medium for the indicated times in 6 well multidishes. After 17 hours serum starvation, cells were exposed to adenosine receptor agonists in

the presence or absence of adenosine receptor antagonists. Antagonists were given 30 minutes prior to stimulation. Time course responses to 0 - 60 minutes exposure to NECA  $10^{-6}$  M and dose response (NECA at  $10^{-8}$  M to  $10^{-5}$  M) experiments were performed. Cell lysates were prepared and equal amounts of protein were separated on 10% SDS-PAGE; total and phosphorylated ERK1/2 and CREB were detected by probing with specific antibodies. Blots were then stripped and re-probed with unphosphorylated ERK1/2 or CREB antibodies. The ratio of phospho protein (ERK or CREB) to total protein (ERK or CREB) was calculated from densitometry of Western blots and compared to the unstimulated control. The detailed methodology for protein extraction and Western blotting is described in chapter 2.



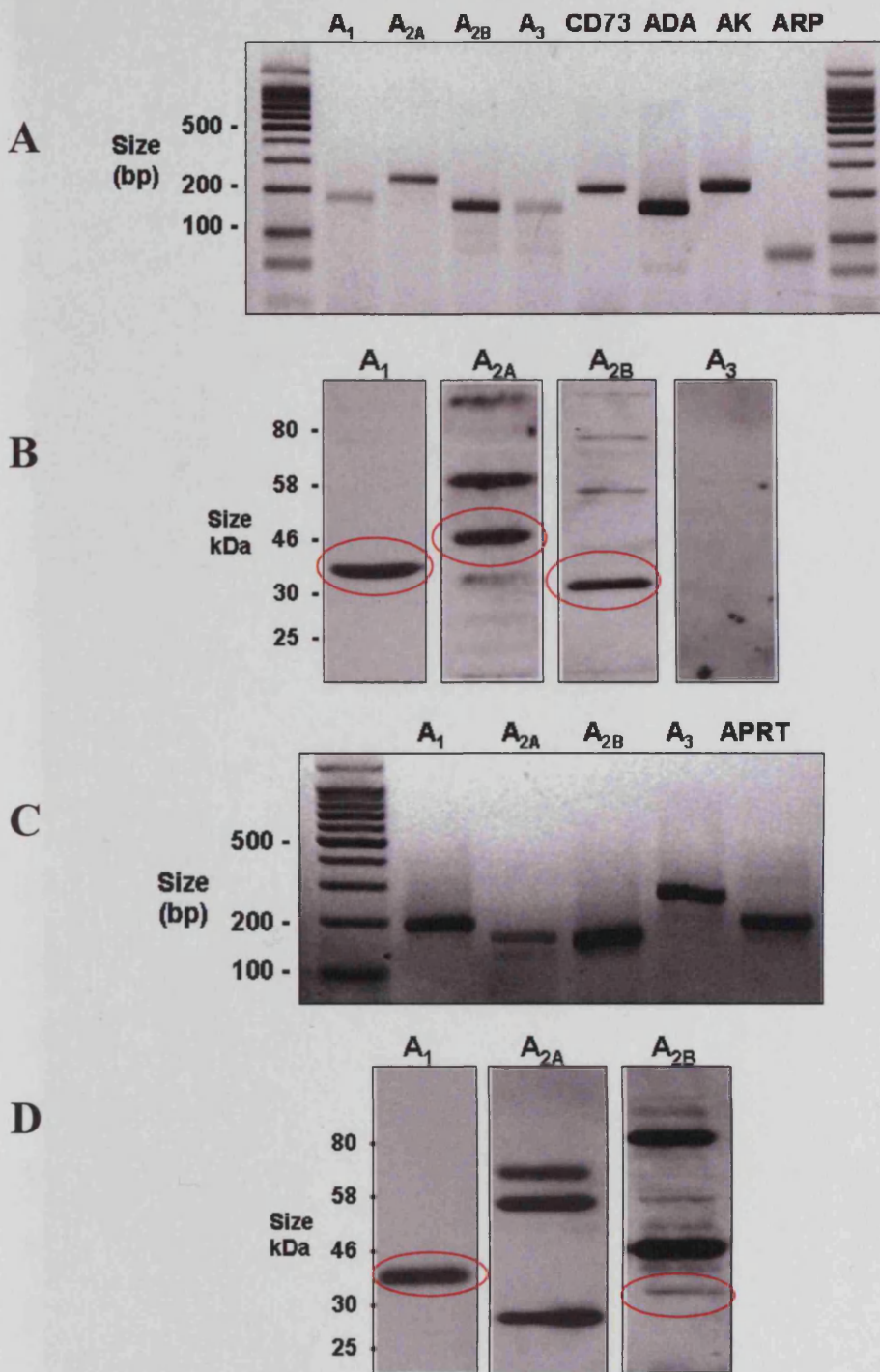
## 5.4 Results

### 5.4.1 Adenosine receptor expression and function in 7F2 and HOB cells

#### 5.4.1.1 mRNA and protein expression

The expression of adenosine receptors was investigated in the 7F2 cell line and in primary HOB cells. PCR amplification of mRNA to adenosine receptors and adenosine metabolic enzymes gave rise to sharp bands of the appropriate size in mouse ( $A_1$ : 160bp,  $A_{2A}$ : 228bp,  $A_{2B}$ : 158bp,  $A_3$ : 151bp, CD73: 220bp, ADA: 165bp, AK: 225bp, ARP: 72bp) and human ( $A_1$ : 186bp,  $A_{2A}$ : 156bp,  $A_{2B}$ : 152bp,  $A_3$ : 360bp, APRT: 247bp) osteoblasts when visualised on an agarose gel stained with ethidium bromide (figure 5.4.1).

The presence of adenosine receptors was also checked at the protein level using Western blotting analysis. For 7F2 cells, antibodies against adenosine  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptors recognised bands with the expected molecular weights of around 38, 46 and 36 kDa respectively (figure 5.4.1 B). With the  $A_{2A}$  antibody, bands of apparent molecular masses, 58 and >100 kDa were also seen, the identity of which are unknown but are likely to be non-specific. There was no detectable band for the  $A_3$  receptor although the gene was detectable by PCR. For HOB cells, Western blotting showed a strong band for the  $A_1$  receptor and the  $A_{2B}$  receptor antibody recognised a faint band of the expected size (36 KDa) but two strong bands of a much higher molecular weight (figure 5.4.1 D). The  $A_{2A}$  antibody also picked up bands of unexpected sizes (25, 58 and 70 kDa) in HOB cells, the 46 kDa band, as found in 7F2 cells was however not detectable. The presence of adenosine receptors in 7F2 cells were further investigated using cAMP measurements.

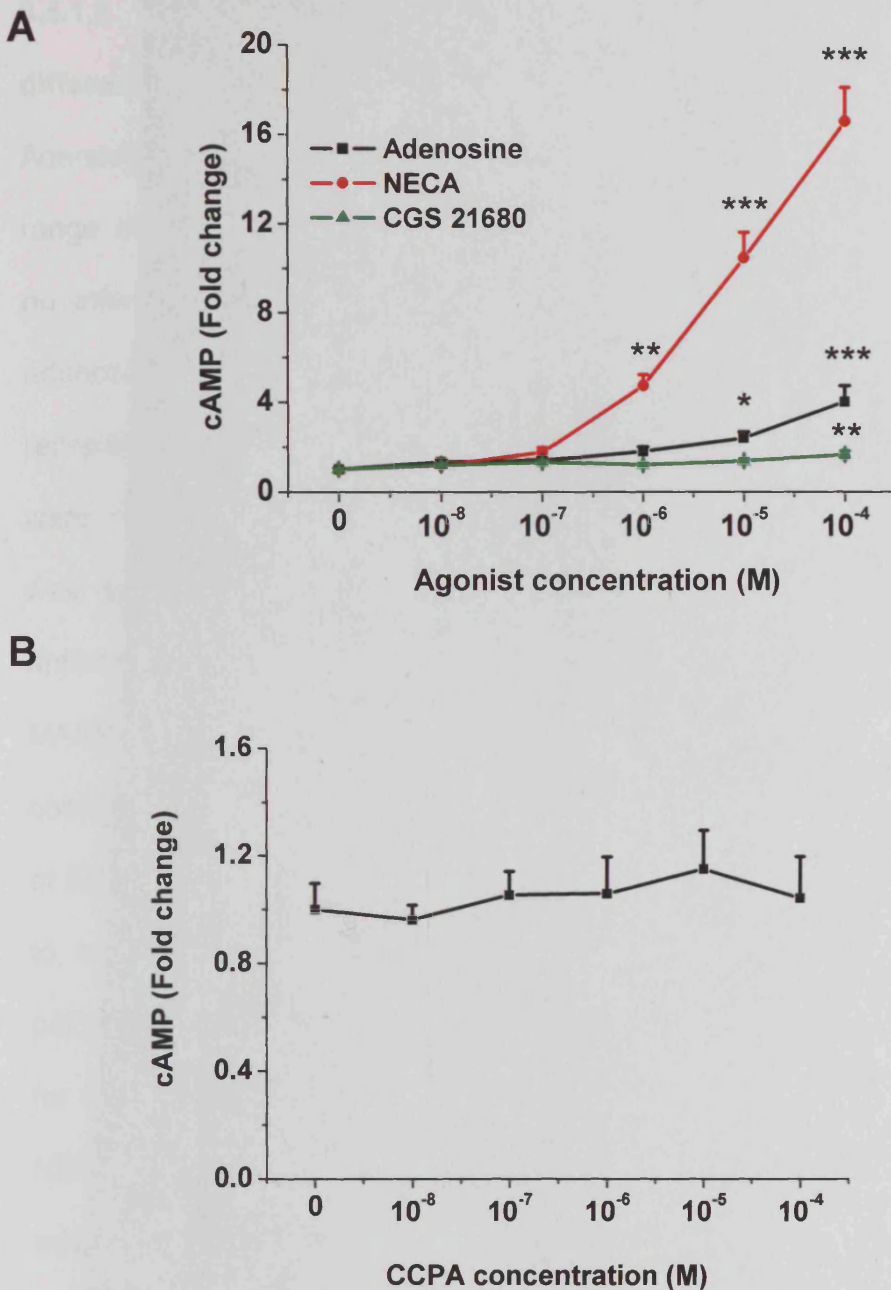


**Figure 5.4.1** Expression of adenosine receptors and enzymes on 7F2 and HOB cells. RT-PCR analysis of 7F2 (A) and HOB cells (C) for A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, A<sub>3</sub> receptors and for (A) the enzymes CD73, ADA and AK. PCR products were separated on a 2% agarose gel and detected by ethidium bromide staining. Figures on the left show sizes (bp) of DNA ladder. (B and D) Western blot analysis of adenosine receptor protein expression in 7F2 (B) and in HOB cells (D). Equal amount of protein lysates were separated on 10% SDS-PAGE and the expression of adenosine receptors were detected by probing with specific primary polyclonal antibodies. Figures on the left show the sizes (kDa) of protein markers.

#### 5.4.1.2 Effect of adenosine on cAMP accumulation in 7F2 cells

The functional dominance of the  $A_{2B}$  receptor during osteoblastogenesis of MSCs suggests that this receptor might also be dominant in 7F2 osteoblastic cells; the action of adenosine receptor agonists on cAMP activation was thus investigated. Adenosine and NECA produced a dose dependent accumulation of cAMP, suggesting the dominance of  $A_2$  receptors. At  $10^{-4}$  M adenosine and NECA the cAMP levels were respectively 4 and 16 fold of basal ( $P < 0.001$ ). In contrast, CGS 21680 did not produce a dose dependent response and only increased cAMP levels by 1 - 2 fold at  $10^{-4}$  M (figure 5.4.2 A).

These findings indicate a rank order of potency, NECA > adenosine > CGS 21680, which is consistent with the presence of functional  $A_{2B}$  receptors (Feoktistov and Biaggioni, 1998). Although an overall stimulation of cAMP, rather than an inhibition, by NECA and adenosine indicates the predominance of  $A_2$  receptors on 7F2 cells, co-expression of functional  $A_1$  receptors is also possible particularly as the mRNA and protein were detectable. CCPA ( $10^{-8}$  M to  $10^{-4}$  M) however failed to inhibit forskolin induced cAMP accumulation, suggesting a lack of significant  $A_1$  receptor coupling to adenylate cyclase and the cAMP pathway (figure 5.4.2 B).

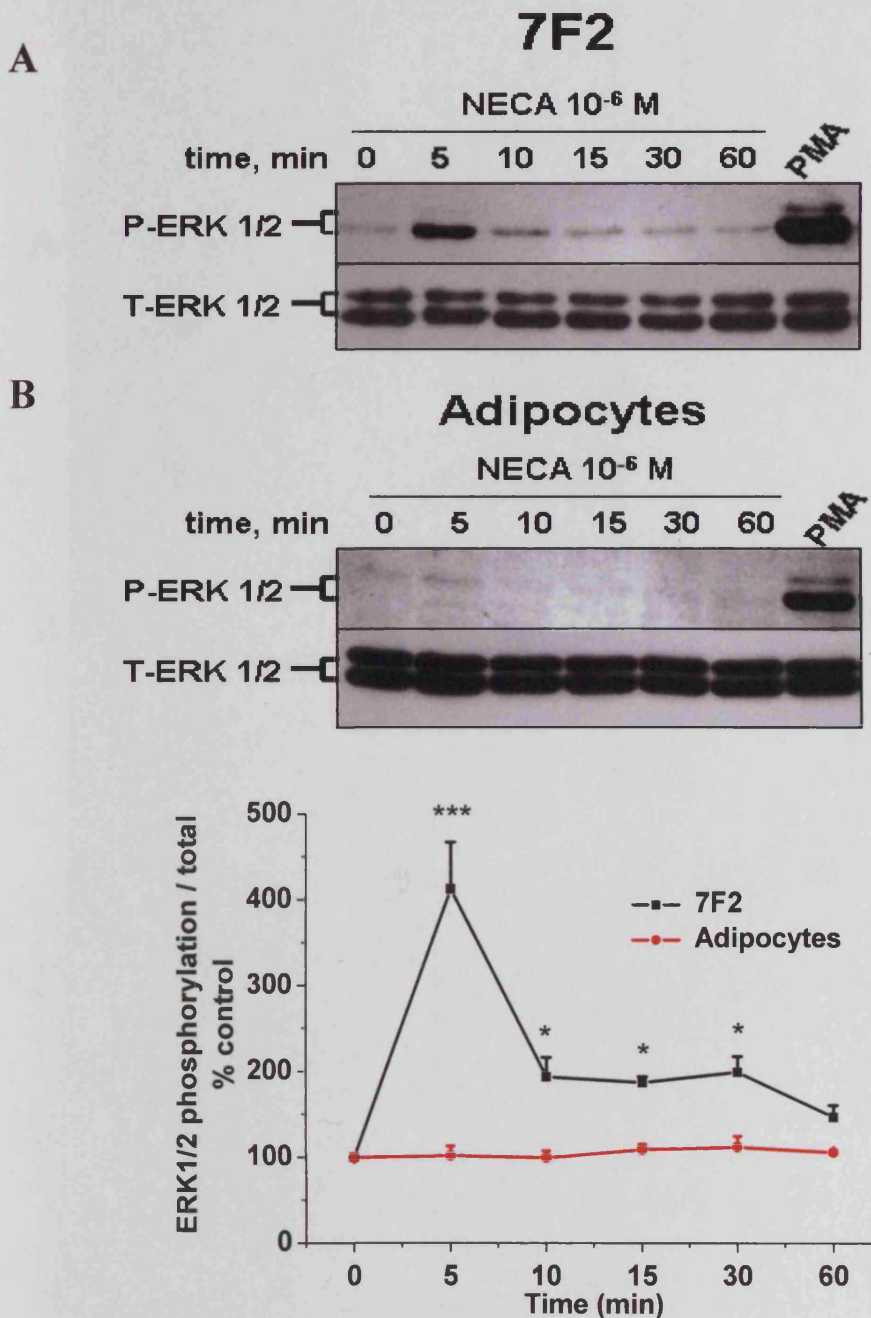


**Figure 5.4.2 Effect of adenosine receptor agonists on intracellular cAMP production in 7F2 cells. (A)** Cells were incubated with the indicated concentrations of adenosine, NECA and CGS 21680 for 15 minutes in the presence of RO 20-1724 ( $10^{-4}$ M) and cAMP was measured by an in-house RIA ( $n=3$ ; total of 12 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with zero (basal values). **(B)** To assess the presence of  $A_1$  receptors, cells were pre-incubated for 15 minutes with CCPA ( $10^{-8}$  M to  $10^{-4}$  M) in the presence of 10  $\mu$ M RO 20-1724 at 37°C. The cells were then incubated in the same medium for a further 5 minutes with forskolin ( $0.5 \times 10^{-5}$  M) (to stimulate cAMP production) and cAMP was measured ( $n=2$ ; total of 8 replicates). Data are expressed as mean  $\pm$  SDM.

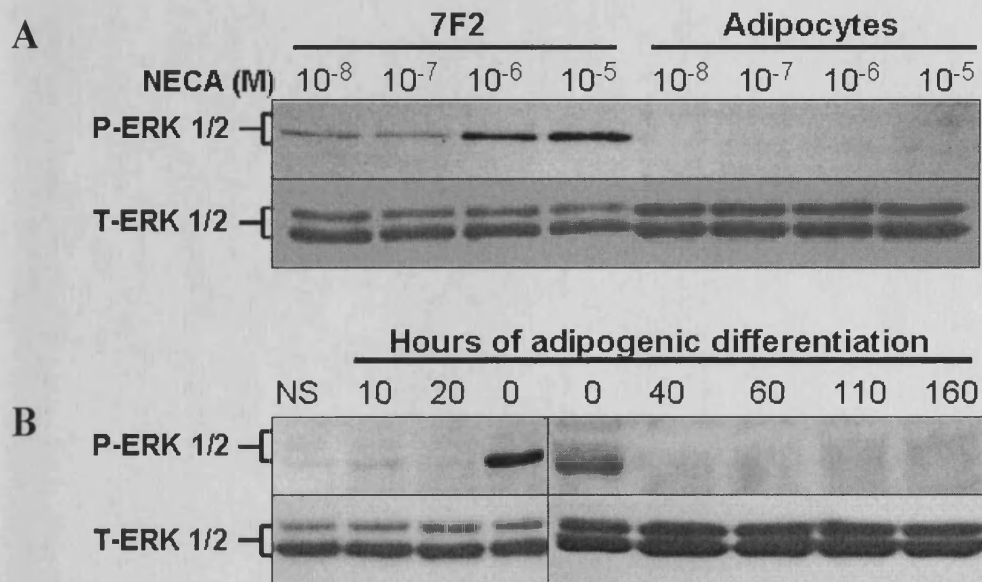
### 5.4.1.3 Effect of NECA on ERK activation in 7F2 cells and 7F2 differentiated adipocytes

Adenosine receptors have been shown to induce ERK activation in a wide range of tissues and culture cells (Schulte and Fredholm, 2003b). However, no information is available regarding the regulation of ERK signalling by adenosine receptors in osteoblasts. Therefore, to determine if adenosine receptors couple to ERK signalling in osteoblasts, serum starved 7F2 cells were incubated for 5 to 60 minutes with NECA at  $10^{-6}$  M and ERK activation was determined by Western blotting analysis using phospho specific ERK antibodies recognising the phosphorylated (Thr202/Tyr204) forms of p42/p44 MAPK. Phorbol 12-myristate 13-acetate (PMA) at  $10^{-6}$  M was used as positive control. As shown in figure 5.4.3 A, NECA and PMA induced phosphorylation of ERK; the effect of NECA was transient, peaking at 5 minutes and returning to near basal levels by 60 minutes. Total non-phosphorylated forms of p42/p44 remain unchanged. Comparing band intensities and after normalising for total ERK there was a 4 fold increase in phosphorylation at 5 minutes. NECA had little or no effect on ERK phosphorylation in 7F2 differentiated adipocytes (figure 5.4.3 B).

The dose response effect of NECA on ERK phosphorylation is shown in figure 5.4.4 A. At  $10^{-7}$ ,  $10^{-6}$  and  $10^{-5}$  M the increases in ERK phosphorylation were 2, 4 and 7 fold when compared with  $10^{-8}$  M NECA. The loss of NECA stimulated ERK phosphorylation in 7F2 adipocytes occurred within 10 hours of inducing differentiation (figure 5.4.4 B).



**Figure 5.4.3** Time course of NECA-stimulated ERK1/2 activation in 7F2 cells and after their differentiation into adipocytes. Serum starved (16 hours) 7F2 osteoblasts (A) or adipocytes (B) were treated with NECA ( $10^{-6}$  M) for 0, 5, 10, 15, 30 and 60 minutes. Cells were lysed in RIPA buffer and analysed for phospho-ERK1/2 by Western blotting. Data were normalised to total ERK and compared with unstimulated controls. Representative blots and densitometric values from at least three separate experiments are shown above. \* $P < 0.05$  and \*\*\* $P < 0.001$  when compared with zero (basal values).



**Figure 5.4.4 Dose response of NECA-stimulated ERK1/2 activation in 7F2 cells and after their differentiation into adipocytes. (A)** Dose dependent ERK1/2 activation in response to NECA. Serum starved (16 hours) 7F2 osteoblasts or adipocytes were treated for 5 minutes with various concentrations of NECA ( $10^{-5}$  to  $10^{-8}$  M) and the ERK1/2 phosphorylation was analysed by Western blotting. **(B)** ERK1/2 activation in response to NECA during adipogenesis. 7F2 cells were differentiated to adipocytes for the indicated times, serum starved and treated for 5 minutes with NECA ( $10^{-6}$  M) (NS = not stimulated). Blots were stripped and re-probed for unphosphorylated ERK1/2. Representative blots from at least two separate experiments are shown above.

#### **5.4.1.4 Effect of adenosine receptor agonists and antagonists on ERK activation**

Previous data suggests that  $A_{2B}$  receptors are predominant in 7F2 cells. To determine if the  $A_{2B}$  receptor was also responsible for stimulation of ERK phosphorylation, various adenosine receptor agonists and antagonists were also used. As shown in figure 5.4.5, adenosine and NECA induced phosphorylation of ERK, whereas CCPA and CGS 21680 were ineffective and IB-MECA was only weakly effective. These findings and complete antagonism by MRS 1706 but only weakly by PSB 36 and SCH 442416 (figure 5.4.5) indicate that the  $A_{2B}$  receptor is primarily the mediator of ERK phosphorylation.

#### **5.4.1.5 Transduction signals upstream of NECA stimulated ERK activation**

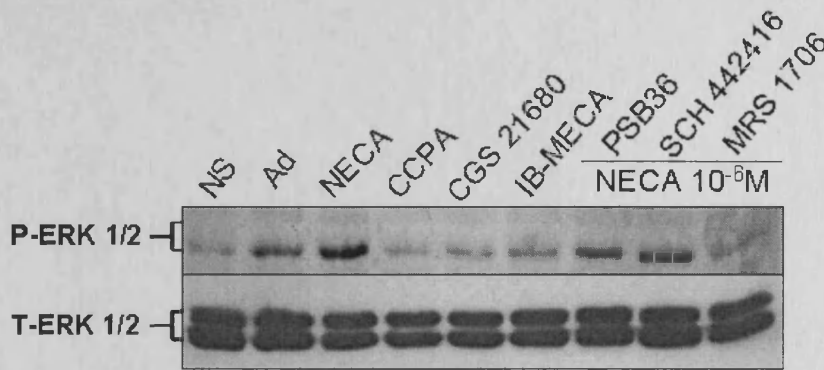
Adenosine receptor activation of ERK is shown to be coupled to major signalling pathways such as PKC, PKA and PI3K in a number of cell types (Schulte and Fredholm, 2003b); various pathway inhibitors were used to investigate which occurred in 7F2 cells. Serum starved 7F2 cells were pre-treated with inhibitors for 30 minutes prior to stimulation with NECA for 5 minutes and ERK activation analysed. As shown in figure 5.4.6, NECA induced ERK activation was completely abolished by pre-treatment with PD 98059, an inhibitor of MAPK/ERK kinase (MEK) 1 and 2. MEKs are part of the MAPK cascade that leads from the Raf kinases and directly causes phosphorylation of ERK1/2 on Thr and Tyr residues (Alessi et al., 1995). Upstream regulators of MEK1/2 were also investigated. Pre-treatment with Wortmannin, a specific inhibitor of PI3K or the PKC inhibitor, RO-32-0432,



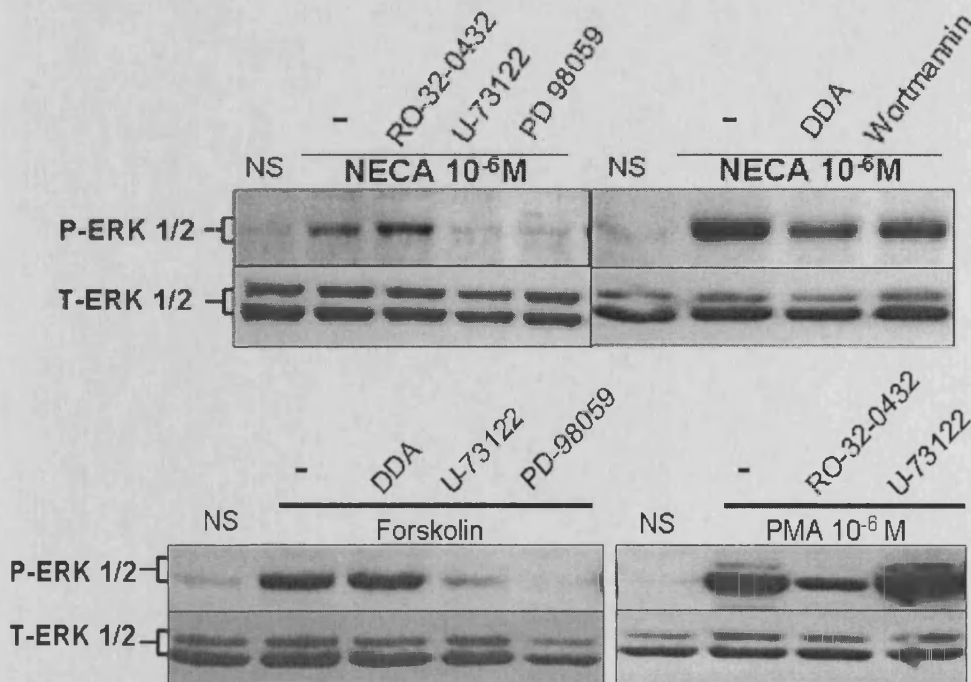
had little effect on NECA stimulated ERK phosphorylation. As shown in figure 5.4.6, 20  $\mu\text{M}$  RO-32-0432 did however cause a dramatic reduction in PMA (PKC activator) induced ERK phosphorylation. These data suggest that PKC is not involved in NECA induced ERK activation. In contrast, pre-treatment with the specific PLC inhibitor, U-73122, completely abolished the NECA induced effects on ERK phosphorylation whereas it had no effect on the activity of PMA. To investigate if cAMP levels were responsible for ERK phosphorylation, an inhibitor of adenylate cyclase (DDA) was used. Pre-treatment with 50  $\mu\text{M}$  DDA partially blocked NECA stimulated ERK activation. These results suggest that cAMP accumulation is only partly responsible for NECA stimulated ERK activation.

#### **5.4.1.6 Effect of NECA on CREB activation in 7F2 cells**

The effect of signalling pathways involving cAMP and MAPK on CREB activation have been studied intensively. Since  $A_{2B}$  receptors are positively coupled to both cAMP and ERK signalling, experiments were set out to determine if  $A_{2B}$  receptors were also involved in CREB phosphorylation. Serum starved 7F2 cells were incubated for 5 to 60 minutes with NECA ( $10^{-6}$  M) and CREB phosphorylation was analysed using a CREB phospho specific antibody. Forskolin was used as a positive control. As shown in figure 5.4.7 A NECA induced CREB phosphorylation in a time dependent manner; the effect was evident after 5 minutes, peaked at 10 minutes (with a 2 - 3 fold increase) and a return to basal levels by 30 minutes. As expected NECA failed to induce CREB phosphorylation in 7F2 differentiated adipocytes (figure 5.4.7 B)



**Figure 5.4.5 Effect of adenosine receptor agonists and antagonists on ERK1/2 phosphorylation in 7F2 cells.** Serum-starved 7F2 cells were stimulated for 5 minutes with 10<sup>-4</sup> M adenosine or 10<sup>-6</sup> M NECA, CCPA, CGS 21680 and IB-MECA and in some experiments were pre-treated with selective adenosine receptor antagonists 10<sup>-6</sup> M PSB 36, SCH 442416 or MRS 1706 for 30 minutes prior to stimulation for 5 minutes with 10<sup>-6</sup> M NECA. ERK phosphorylation was then assessed by Western blotting. Blots were stripped and re-probed for unphosphorylated ERK1/2. Representative blots from at least two separate experiments are shown.



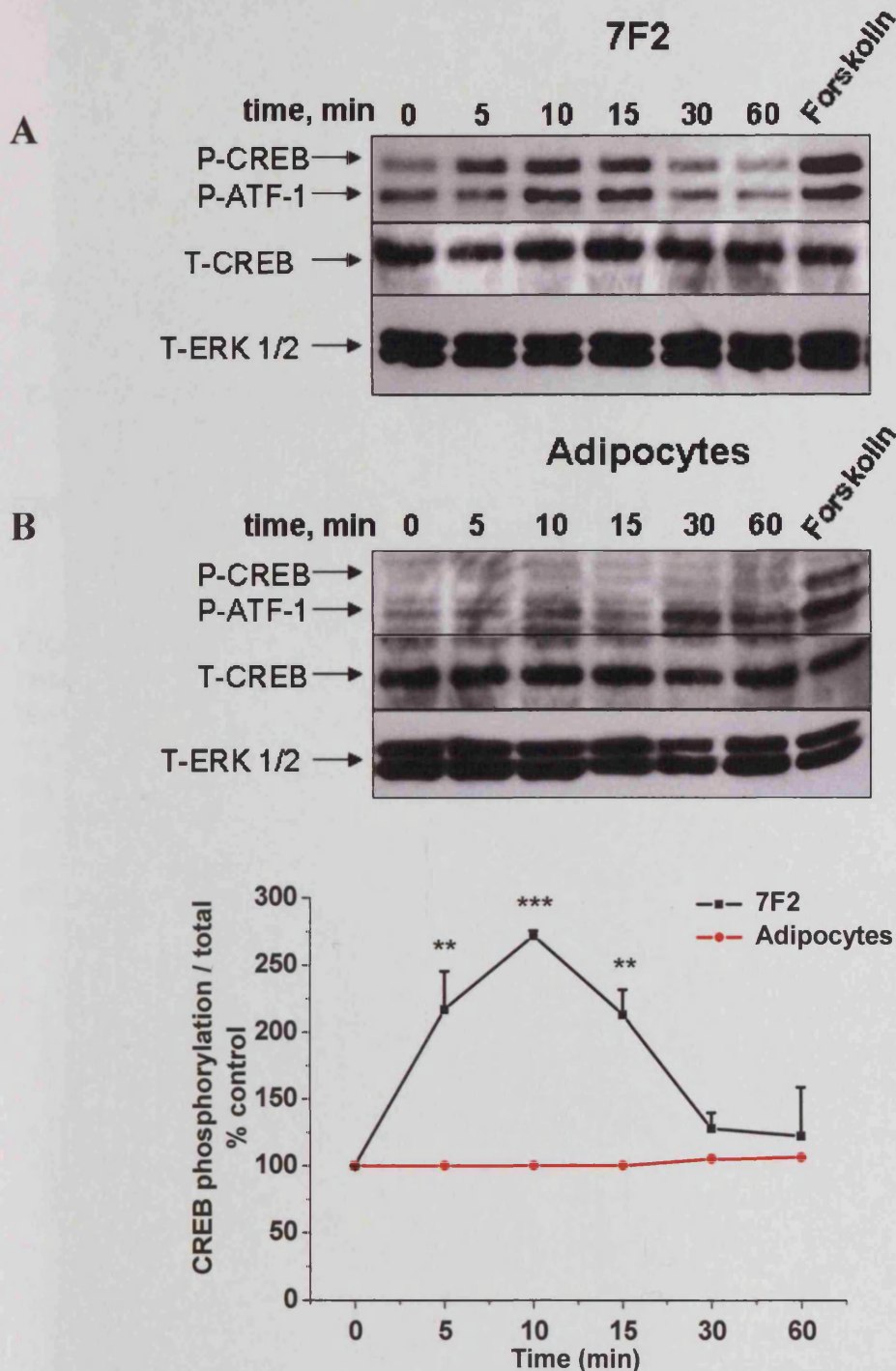
**Figure 5.4.6 Involvement of signal pathways upstream of ERK1/2 phosphorylation in 7F2 cells.** Serum-starved 7F2 cells were pre-treated at 37°C for 30 minutes with 20 μM RO-32-0432, 4 μM U-73122, 10 μM PD 98059, 0.2 μM Wortmannin or 50 μM 2',5'-dideoxyadenosine (DDA) and compared with NS. Subsequently, the cells were stimulated with NECA (10<sup>-6</sup> M) for 5 minutes, forskolin (10<sup>-5</sup> M) or PMA (10<sup>-6</sup> M) for 10 minutes and ERK1/2 phosphorylation measured. Representative blots and densitometric values from at least two separate experiments are shown above.

again suggesting a loss of A<sub>2</sub> receptors whereas CREB phosphorylation to forskolin was retained in both types of cells (figure 5.4.7 A&B).

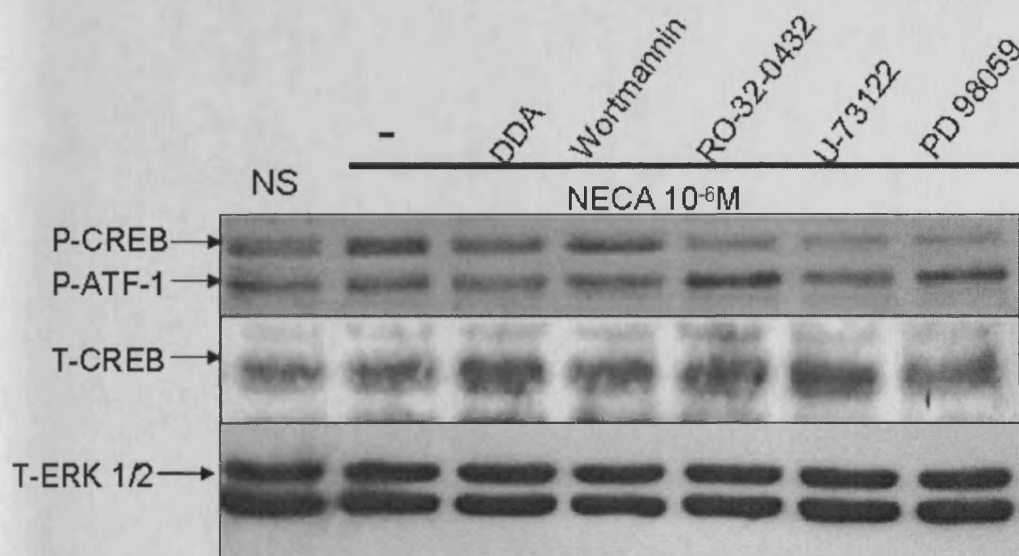
#### **5.4.1.7 Mechanisms of CREB activation by A<sub>2B</sub> receptor**

To investigate the mechanisms of CREB phosphorylation via the A<sub>2B</sub> receptor, serum starved 7F2 cells were pre-treated with inhibitors of upstream components of signal pathways that may be involved in the phosphorylation of CREB. As shown in figure 5.4.8, NECA induced CREB activation was partly reversed by pre-treatment with DDA, suggesting that cAMP accumulation was involved. Pre-treatment with PD 98059 resulted in complete loss of NECA induced CREB phosphorylation, suggesting that ERK activation was an intermediate. Similar results were obtained with U-73122. This was expected as PLC was found to be responsible for ERK phosphorylation in 7F2 cells. Since ERK was responsible for CREB phosphorylation, loss of ERK activation by U-73122 subsequently led to loss of CREB activation.

Surprisingly, pre-treatment with RO-32-0432 also completely abolished the NECA-induced CREB activity, suggesting that PKC may be an intermediate step downstream of ERK and upstream of CREB signalling. Pre-treatment with Wortmannin had no effect on NECA stimulated CREB phosphorylation, suggesting that PI3K was not involved in CREB activation by NECA. Table 5.4.1 shows a summary illustrating the sites of action and the effects of the different inhibitors on NECA induced ERK activation.



**Figure 5.4.7** Time course of NECA stimulated CREB activation in 7F2 cells and after their differentiation into adipocytes. Serum starved (16 hours) 7F2 osteoblasts (A) or adipocytes (B) were treated with NECA ( $10^{-6}$  M) for 0, 5, 10, 15, 30 and 60 minutes. Cells were lysed in RIPA buffer and analysed for phospho-CREB by Western blotting. Data were normalised to total CREB and compared with the unstimulated controls. Representative blots and densitometric values from at least three separate experiments are shown above. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  when compared with zero (basal values).



**Figure 5.4.8 Involvement of signal pathways downstream of adenosine receptors on CREB phosphorylation in 7F2 cells.** Serum-starved 7F2 cells were pre-treated at 37°C for 30 minutes with 20  $\mu$ M RO-32-0432, 4  $\mu$ M U-73122, 10  $\mu$ M PD 98059, 0.2  $\mu$ M Wortmannin or 50  $\mu$ M DDA. Subsequently, the cells were stimulated with NECA ( $10^{-6}$  M) for 5 minutes and CREB phosphorylation was determined by Western analysis. Representative blots and densitometric values from at least two separate experiments are shown above.

Inhibitors	Site of action	ERK	CREB
RO-32-0432	PKC	-	↓
U-73122	PLC	↓↓	↓↓
PD 98059	MEK1/2	↓↓	↓↓
Wortmannin	PI3K	-	-
DDA	Adenylate cyclase	↓	↓

**Table 5.4.1 Summary illustrating the sites of action and the effects of the different inhibitors on NECA induced ERK or CREB activation.**

↓↓ = High inhibition of ERK or CREB; ↓ = Low inhibition of ERK or CREB.

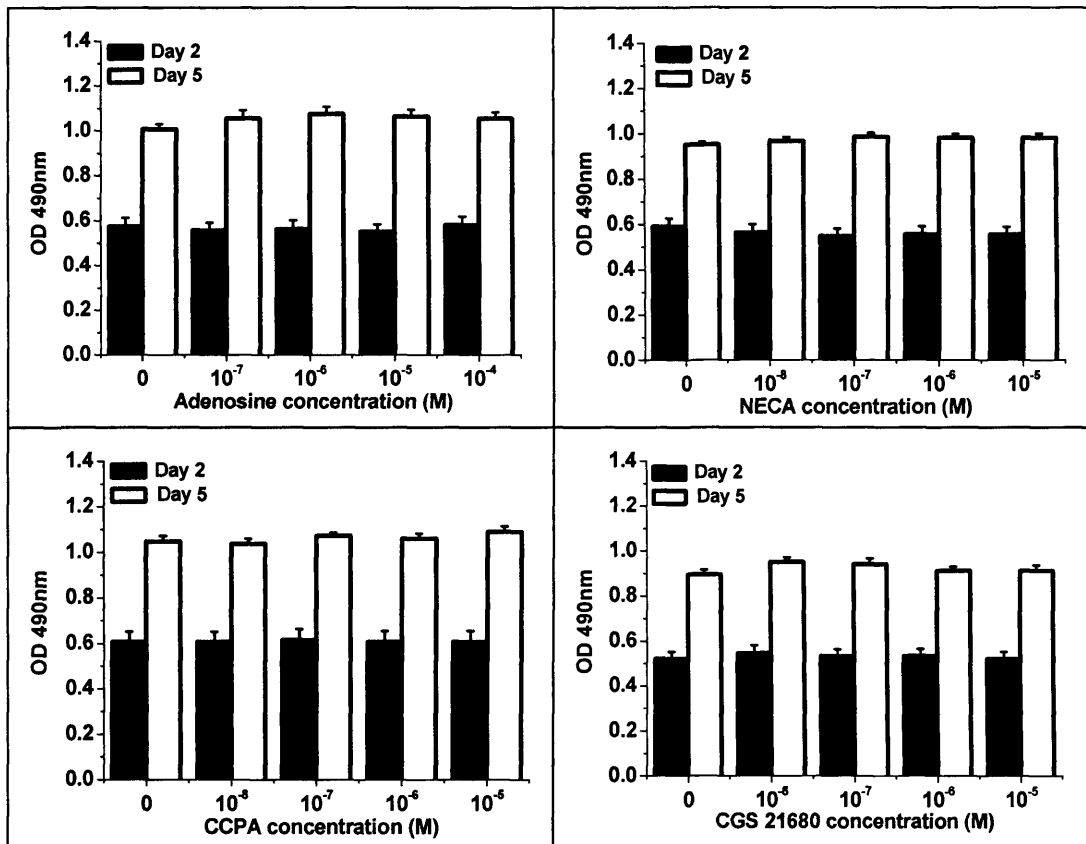
## **5.4.2 Role of adenosine receptors on 7F2 cell proliferation and maturation**

### **5.4.2.1 Effects of adenosine receptors agonists on cell number and ALP activity in 7F2 cells.**

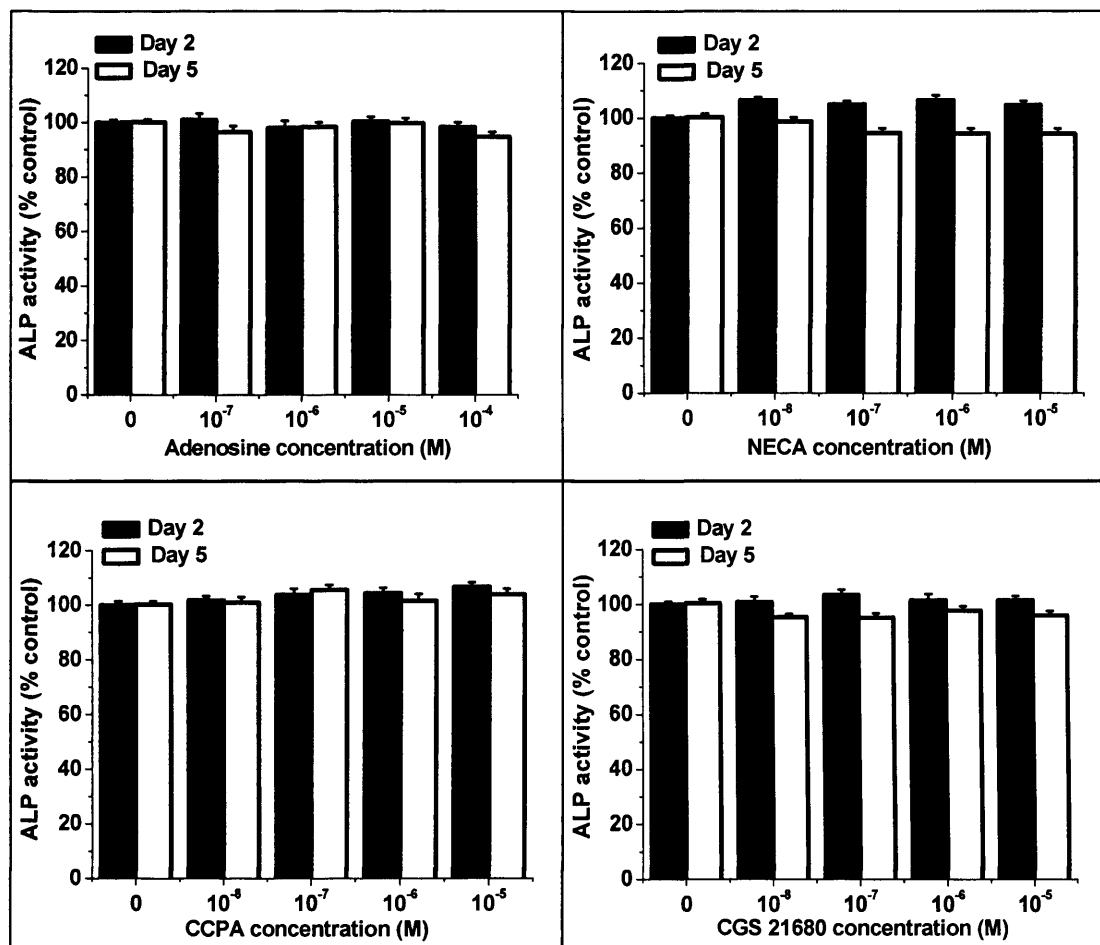
Expression of adenosine receptors on 7F2 cells suggested that adenosine might play a physiological role in these cells. Dose response and time course experiments using the adenosine receptor agonists (NECA, CCPA and CGS 21680) were investigated in 5% FCS containing medium. As shown in figures 5.4.9 and 10, treatment with adenosine receptor agonists for 2 and 5 days had no effects on cell number or ALP activity.

### **5.4.2.2 Effects of adenosine receptors agonists on mineralisation of 7F2 cells.**

7F2 cells are considered as differentiated osteoblasts as they express osteoblast markers and have the capacity to mineralise. Optimal  $\beta$ -GP concentration for mineralisation were determined by comparing the effects of 2 to 8 mM  $\beta$ -GP on mineral deposition. As shown in figure 5.4.11 A, culturing with 2 - 4 mM  $\beta$ -GP resulted in minor mineral matrix deposition, whereas 8 mM  $\beta$ -GP induced widespread deposition. Sub-maximal (6 mM)  $\beta$ -GP was used in all subsequent experiments. Treatment with adenosine receptor agonists for 10 days, however did not modify the mineral matrix deposition figure 5.4.11 B&C.

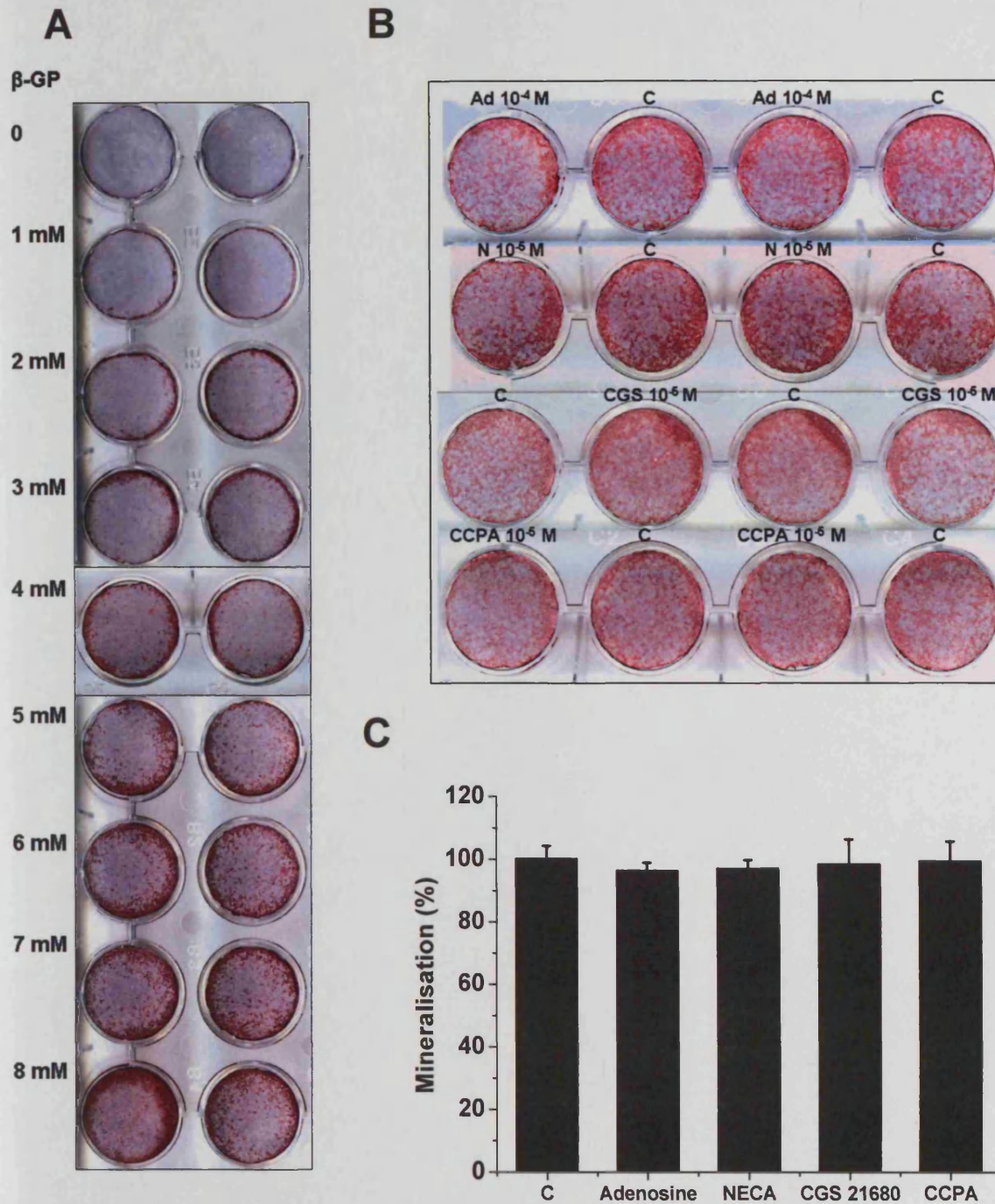


**Figure 5.4.9 Effects of adenosine receptor agonists on 7F2 cell number.** 7F2 cell numbers following 2 and 5 days of treatment with the indicated concentrations of adenosine, NECA, CCPA and CGS 21680 in 5% FCS containing medium (n=2; total of 24 replicates).



**Figure 5.4.10** Effects of adenosine receptor agonists on ALP activity in 7F2 cells. 7F2 cells treated with the indicated concentrations of adenosine, NECA, CCPA and CGS 21680. ALP activity was measured after 2 and 5 days (n=2; total of 24 replicates).





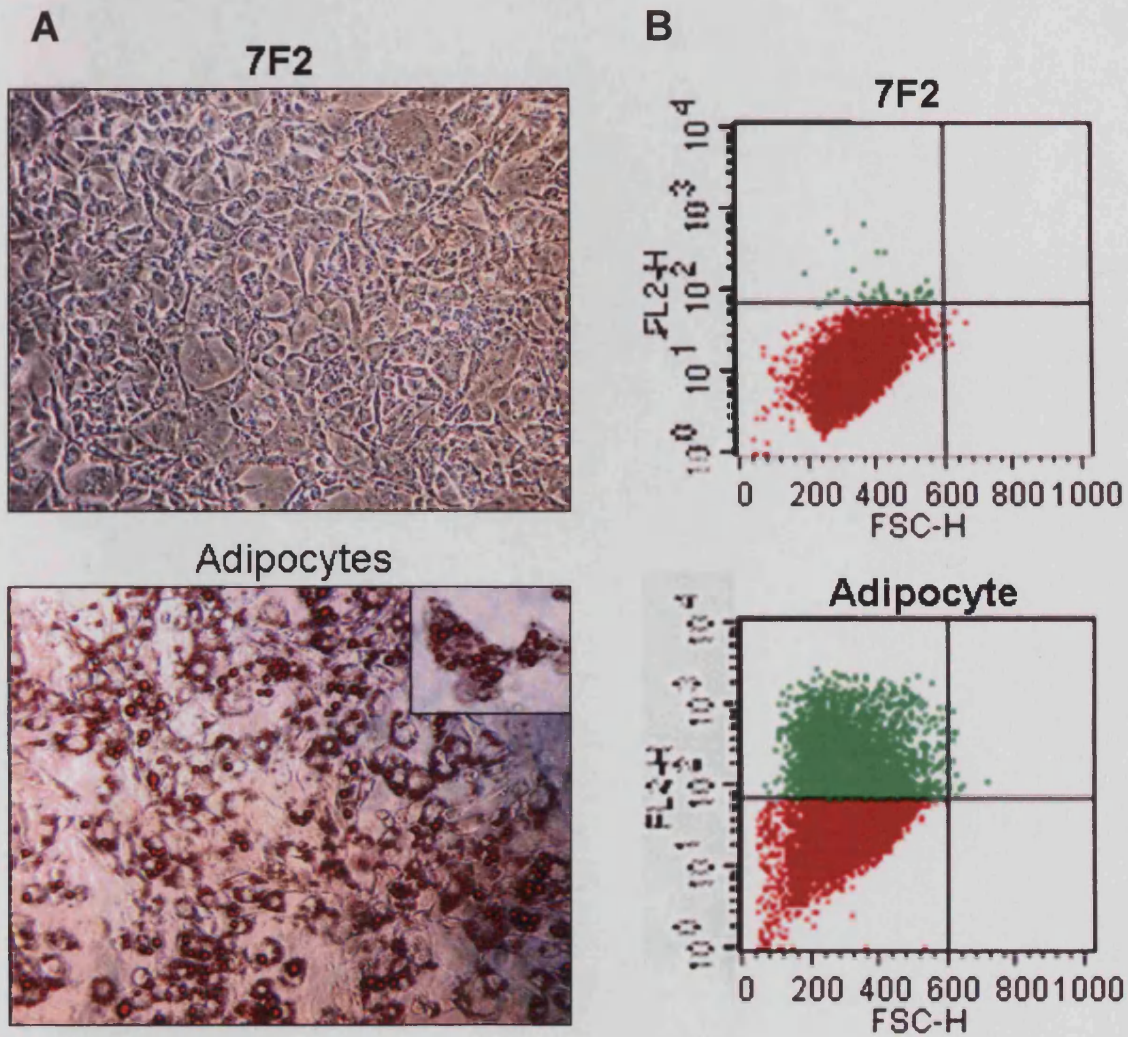
**Figure 5.4.11 Effect of adenosine receptor agonists on mineralisation of 7F2 osteoblasts.** (A) 7F2 cells were incubated with mineralisation medium containing varying concentration of  $\beta$ -GP for 10 days and calcified extracellular matrix was visualised by Alizarin Red S staining. (B and C) Effect of adenosine receptor agonists at  $10^{-5}$  M after 12 days of treatment on mineralisation. (C) Mineralisation was quantified by extracting the dye and measured at 490 nm ( $n=3$ ; total of 12 replicates).

### 5.4.3 Transdifferentiation of 7F2 osteoblast cells to adipocytes

#### 5.4.3.1 Evaluation of adipogenesis

7F2 cells were induced to transdifferentiate into adipocytes using a cocktail of culture media additives (Dex, indomethacin, and AA) as described previously (Thompson et al., 1998). Under these conditions, adipocytic cells containing intracellular lipid droplets were detectable microscopically after 3 days and the number of adipocytes and the size of their lipid vesicles increased progressively up to day 7 post differentiation. Figures 5.4.12 A shows Oil Red O staining of 7F2 cells and after 7 days of adipocyte differentiation. Beyond 7 days some adipocytes became detached and floating cells with large intracellular lipid vacuoles were visible. Flow cytometry analysis demonstrated that up to 50% of the cells stained positive for Nile red and following 7 days of adipocyte transdifferentiation Figures 5.4.12 B.

Expression of adipocyte (PPAR $\gamma$ , C/EBP $\beta$ , LPL and GPDH) and osteoblast (osteocalcin) lineage markers were also assessed in 7F2 adipocyte differentiated cultures. Consistent with the increased adipocyte morphology after differentiation all adipocyte markers, as determined by q-RTPCR, showed an increase up to 7 days before falling back but still remained above that of undifferentiated 7F2 cells after 9 and 12 days (figure 5.4.17). Adipocyte differentiated 7F2 cells also showed marked reduction in osteocalcin expression within 1 day of adipocyte differentiation (figure 5.4.19).



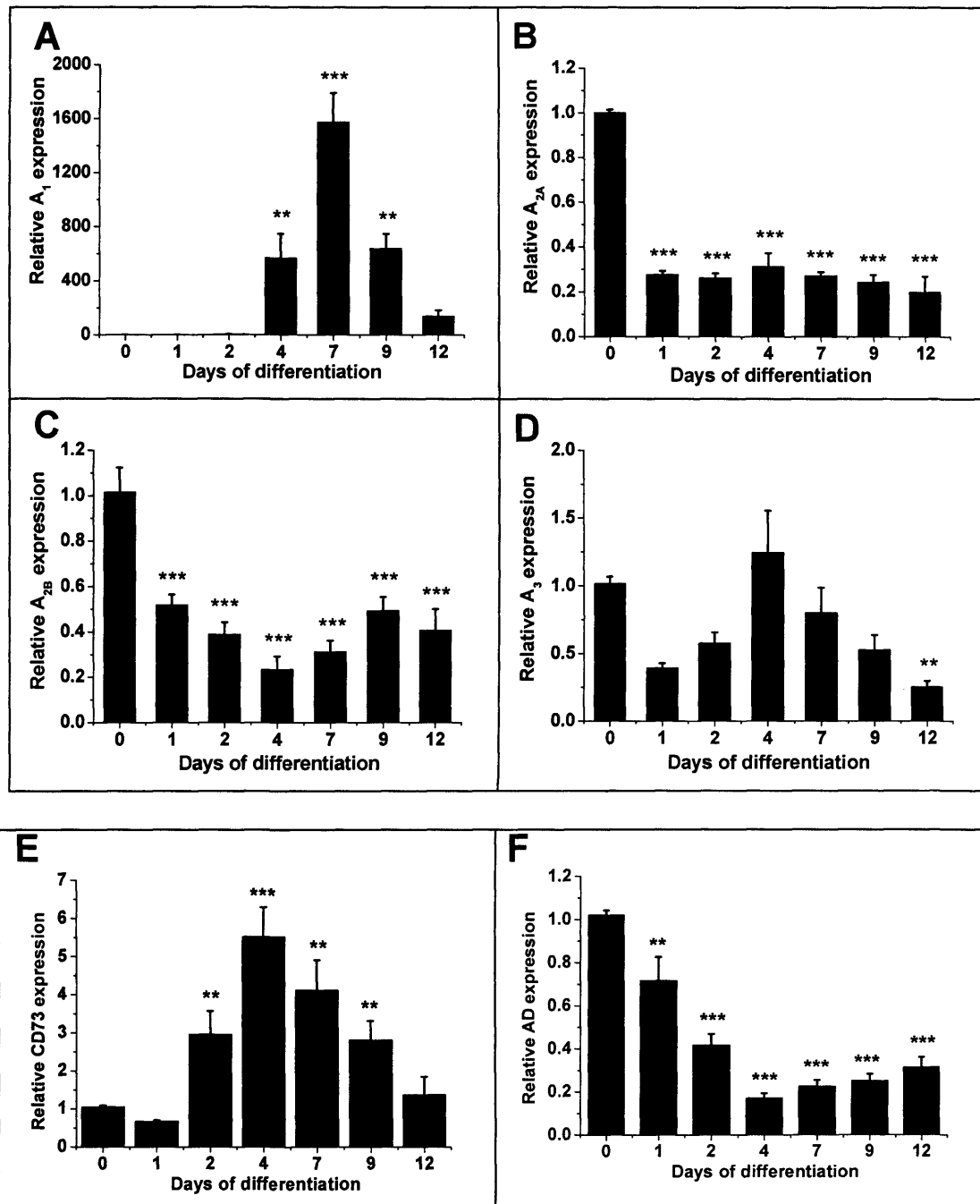
**Figure 5.4.12 Transdifferentiation of 7F2 cells into adipocytes.** 7F2 cells and adipocytes (7 days of differentiation) were stained with Oil Red O (A) and subjected to flow cytometry analysis following Nile red staining (B). Flow cytometry data analysis is presented using the FL2 (fluorescent emission) versus FSC (size of the cells) plot. The plot was divided into quadrants using the undifferentiated cells (lower quadrants) as the base line of fluorescent emission. The lower quadrants (red dots) therefore display events that are negative for Nile red fluorescent emission and represent undifferentiated cells. The upper quadrants (green dots) on the other hand contain events that are positive for Nile red fluorescent emission and therefore represent the adipocyte population.

### 5.4.3.2 Adenosine receptor expression during transdifferentiation of 7F2 cells into adipocytes

Previous investigations examining the expression and function of adenosine receptors during differentiation of MSCs into adipocytes demonstrated upregulation and involvement of  $A_1$  and  $A_{2A}$  receptors. q-RTPCR was used to determine whether these changes also occurred during transdifferentiation of 7F2 cells into adipocytes, mRNA expression in individual samples post differentiation, was normalised to a housekeeping gene (ARP) and compared to that in undifferentiated 7F2 cells. As shown in figure 5.4.13 A, the  $A_1$  receptor was dramatically increased during differentiation and its pattern of expression mirrored exactly the adipogenic marker genes (figure 5.4.17).  $A_1$  receptor mRNA expression peaked at day 7 with >1500 ( $P < 0.001$ ) fold increase above that in undifferentiated 7F2 cells.

In contrast to that seen during adipocyte differentiation of MSC, expression of the  $A_{2A}$  receptor was diminished significantly by about 75% ( $P < 0.001$ ) as early as 24 hours after adipogenic induction and stayed at more or less the same level throughout the differentiation process (figure 5.4.13 B). A similar inhibition of expression of up to 80% ( $P < 0.001$ ) was also seen with the  $A_{2B}$  receptor and the  $A_3$  receptor only showed inhibition 12 days after differentiation (figure 5.4.13 C&D).

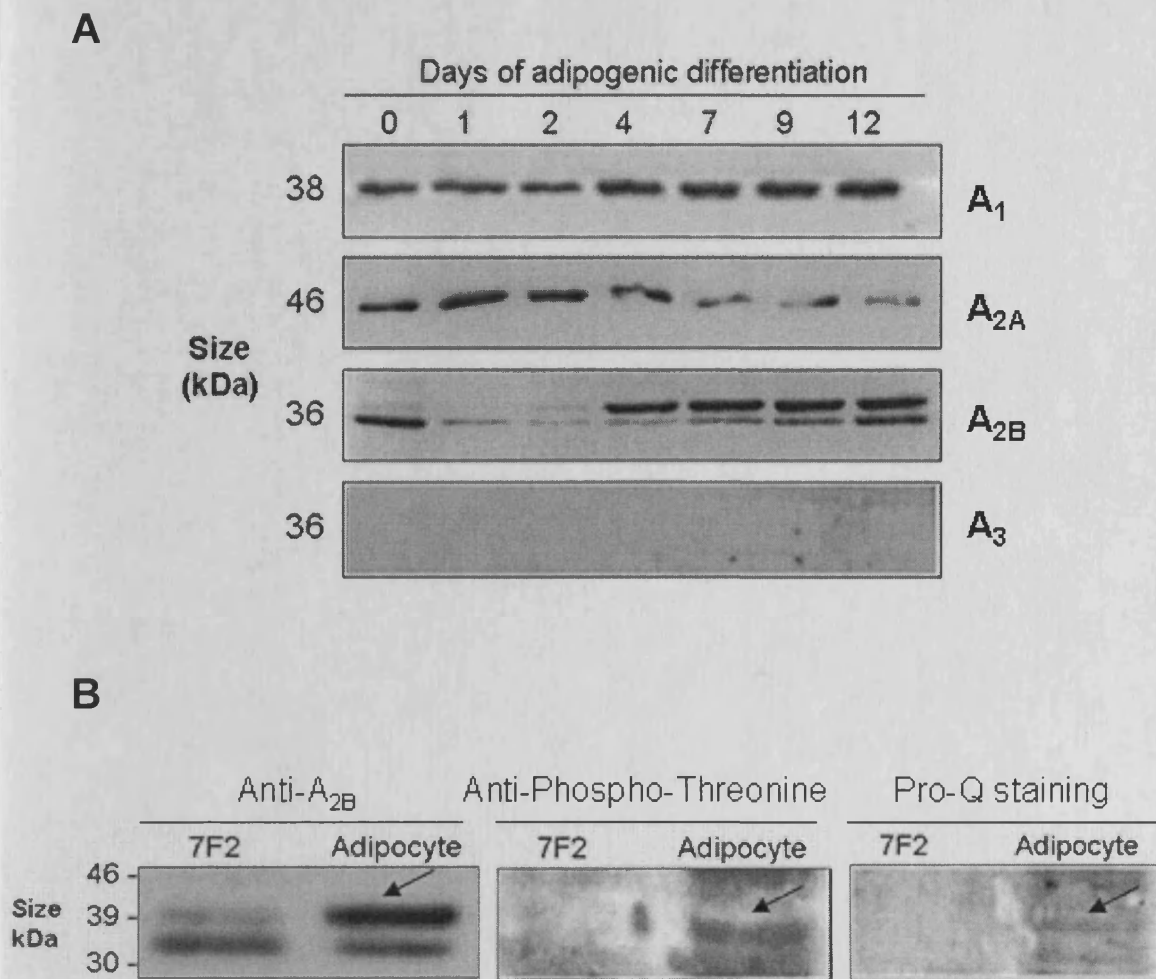
The expression of CD73 and ADA during adipocyte differentiation was also investigated, these enzymes are particularly important for regulating extracellular levels of adenosine. Changes in CD73 expression followed that



**Figure 5.4.13 Adenosine receptor and adenosine metabolic enzyme mRNA expression during the transdifferentiation of 7F2 into adipocytes.** Cells were cultured in adipogenic medium for the indicated times and q-RT-PCR used to determine gene expression. Expression of A<sub>1</sub> (A), A<sub>2A</sub> (B), A<sub>2B</sub> (C) and A<sub>3</sub> (D) receptors and (E) CD73 and (F) ADA were normalised to ARP and compared with undifferentiated cells (day 0); the day 0 was assigned a value of 1 (n=3; total of 6 replicates). \*\*P < 0.01 and \*\*\*P < 0.001 when compared with day 0.

of the adipocyte marker genes and the  $A_1$  receptor with an increase of up to 6 fold ( $P < 0.001$ ) at day 4 of differentiation (figure 5.4.13 E). ADA, on the other hand, showed a marked reduction of more than 80% at a similar time point (day 4) ( $P < 0.001$ ) (figure 5.4.13 F). These data suggest that there is a net accumulation of adenosine in the extracellular space during adipocyte differentiation of 7F2 cells.

Changes in adenosine receptors during differentiation were also assessed at the protein level. Representative Western blots figure 5.4.14 A showed that the  $A_1$  receptor increased while  $A_{2A}$  receptor expression decreased during differentiation. The relative level of protein change for the  $A_1$  receptor was much smaller when compared with the observed changes in mRNA. Western blotting for the  $A_{2B}$  receptor showed two bands, the smaller size band is of a much higher intensity. On differentiation the expression of this band decreased with concomitant increase in expression of the higher molecular weight band. This higher molecular weight band may represent a phosphorylated form of the  $A_{2B}$  receptor and thus its phosphorylation status was investigated. Labelling with Pro-Q or detection using a phospho-threonine antibody showed bands which co-eluted with the higher molecular weight band. These bands were not detectable in the 7F2 undifferentiated cells but only after adipocyte differentiation (figure 5.4.14 B). The  $A_3$  receptor protein was not detectable in 7F2 cells or after adipocyte differentiation.

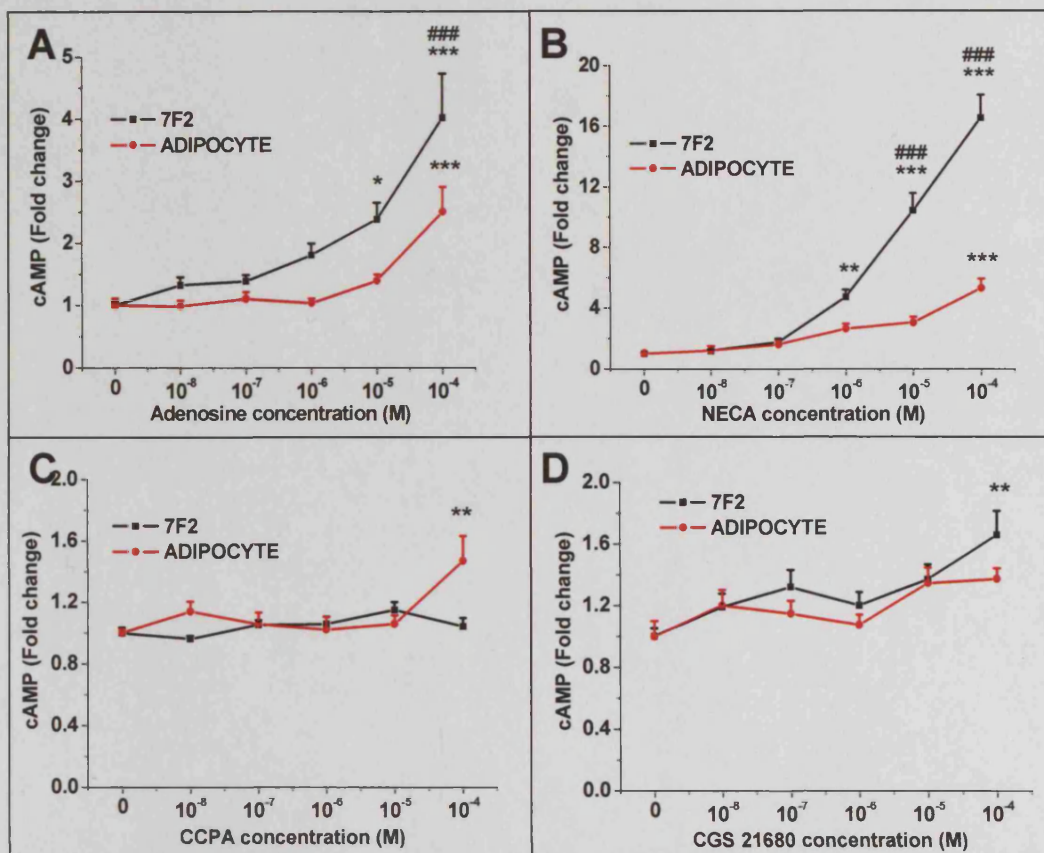


**Figure 5.4.14 Adenosine receptor protein expression during the transdifferentiation of 7F2 cells into adipocytes.** 7F2 cells were cultured for the indicated times in adipogenic medium. Cells were lysed in RIPA buffer and equal amount of protein lysates were separated on 10% SDS-PAGE. **(A)** The expression of adenosine receptors were detected by probing with specific antibody for each adenosine receptor. Representative blots are shown above. **(B)** A representative Western blot analysis of A<sub>2B</sub> receptor, phospho-threonine and Pro-Q staining in 7F2 cells and after 12 days of adipocyte differentiation. Figures on the left indicate sizes (kDa) of protein molecular weight markers.

### 5.4.3.3 Adenosine mediated cAMP expression in 7F2 cells and after adipocyte differentiation

Studies of adenosine receptor mRNA and protein expression during the differentiation of 7F2 cells into adipocytes indicate an increase in  $A_1$ , a decrease in  $A_{2A}$  and an inactivation of  $A_{2B}$  receptors. To assess if the alterations in adenosine receptor expression during differentiation reflect changes in adenosine receptor mediated cAMP expression cultures before and after differentiation (7 days) were treated with different concentrations of adenosine receptor agonists. When compared to undifferentiated 7F2 cells stimulation of cAMP accumulation by adenosine and NECA was markedly reduced in the adipocyte cultures (figure 5.4.15 A&B). Following adipogenesis cAMP accumulation by adenosine and NECA ( $10^{-4}$  M) was significantly reduced respectively from 4 and 16 fold to 2.5 ( $P < 0.001$ ) and 5 fold ( $P < 0.001$ ). CGS 21680 was only a weak stimulator of cAMP in both 7F2 cells and 7F2 adipocyte cultures (figure 5.4.15 D). The  $A_1$  receptor agonist CCPA (figure 5.4.15 C) failed to inhibit forskolin stimulated cAMP levels in 7F2 and adipocyte cultures and at  $10^{-4}$  M it caused an apparent stimulation. These findings overall indicate a loss of adenosine receptor mediated cAMP signalling as 7F2 cells are induced to differentiate to adipocytes. Such data is consistent with the loss of  $A_2$  receptors (both mRNA and protein) as previously described. The weak or lack of effect of CCPA suggests that the  $A_1$  receptor is lowly expressed in 7F2 and adipocyte cultures when compared with levels of  $A_2$  and in particular the  $A_{2B}$  receptor. It is, of course, possible that the large increase in  $A_1$  receptor mRNA on differentiation is not translated to protein.





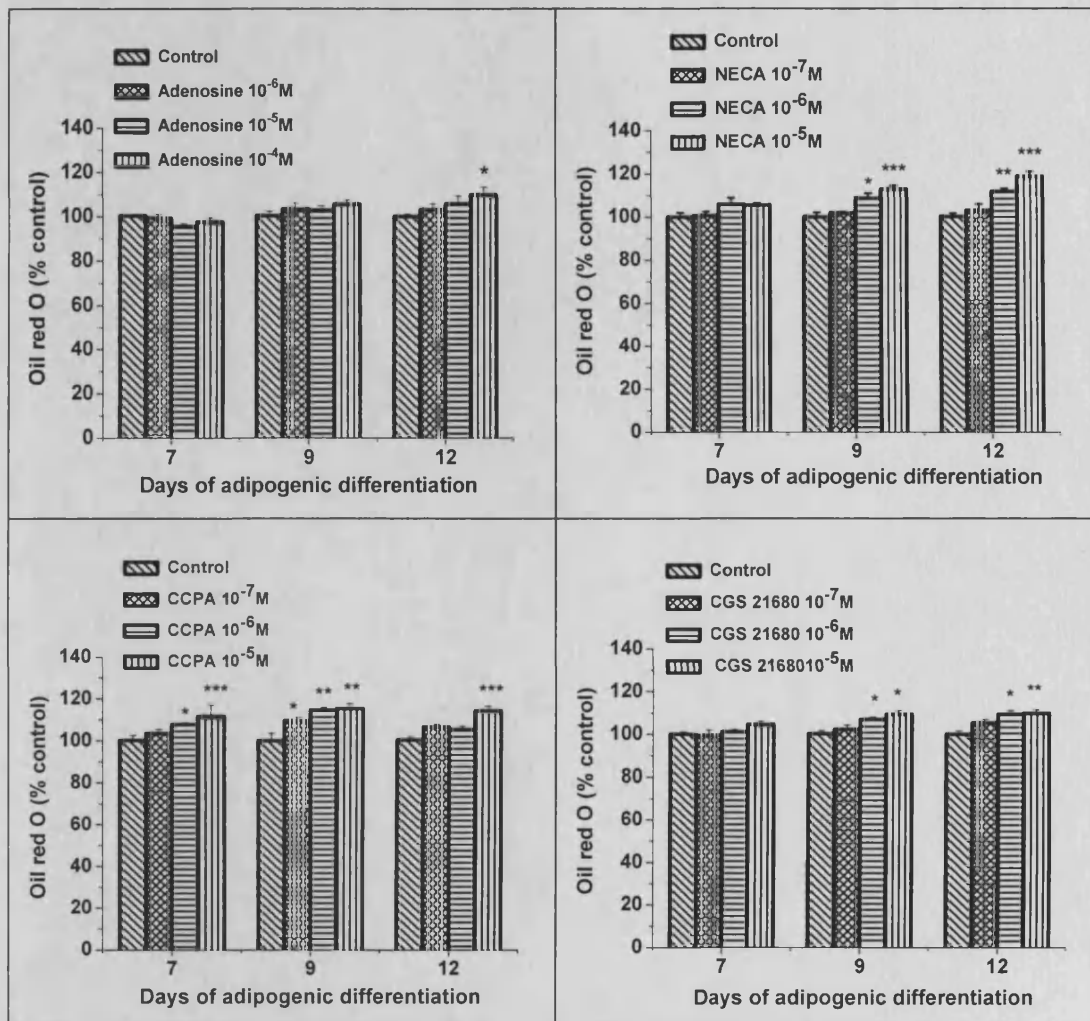
**Figure 5.4.15** Effect of adenosine receptor agonists on the accumulation of cAMP in 7F2 cells and after transdifferentiation into adipocytes. Cells were cultured for 9 days in the adipogenic differentiation medium. At the end of the incubation period cells were treated with the indicated concentrations of (A) adenosine, (B) NECA, (C) CCPA and (D) CGS 21680 for 15 minutes in the presence of RO 20-1724 ( $10^{-4}$ M) and cAMP was measured using RIA ( $n=3$ ; total of 12 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with untreated cells; ### $P < 0.001$  when compared with adipocytes treated with agonist at the same concentration.

#### **5.4.3.4 Effects of adenosine receptor signalling on transdifferentiation of 7F2 cells**

The increase in  $A_1$  receptor expression on adipocyte differentiation suggests that adenosine itself may be important in the differentiation or function of adipocytes. Adenosine (after 12 days of exposure) had only a weak stimulatory effect on lipid accumulation and this may reflect its short half-life and stability. When the stable agonist, NECA was used stimulated lipid accumulation was observed at a much lower concentration of agonist ( $10^{-6}$  M for NECA compared with  $10^{-4}$  M for adenosine) and after 9 days of treatment ( $P < 0.01$ ) (figure 5.4.16). Surprisingly, CGS 21680 also induced adipogenesis; this was rather unexpected since it was only a weak stimulator of cAMP in both 7F2 and adipocyte cultures. The  $A_1$  receptor agonist CCPA also resulted in a dose dependent increase in lipid accumulation after 7 days of differentiation ( $P < 0.001$ ), yet its antagonist, PSB 36, failed to inhibit adipogenesis when added to the differentiation media alone (figure 5.4.18 A).

#### **5.4.3.5 Effect of adenosine receptor signalling on the expression of adipocyte marker genes during the transdifferentiation of 7F2 cells**

As previously described (section 5.4.3.1) the adipogenic marker genes,  $PPAR\gamma$ ,  $C/EBP\beta$ , LPL and GPDH were upregulated in 7F2 cells as they were induced to differentiate to adipocytes. Whether these markers could be influenced further by adenosine receptor activation was also investigated particularly since a stimulatory effect on lipid accumulation was observed. Surprisingly, NECA failed to have effects on any of the adipocyte markers above any increases seen with differentiation media alone figure 5.4.17.



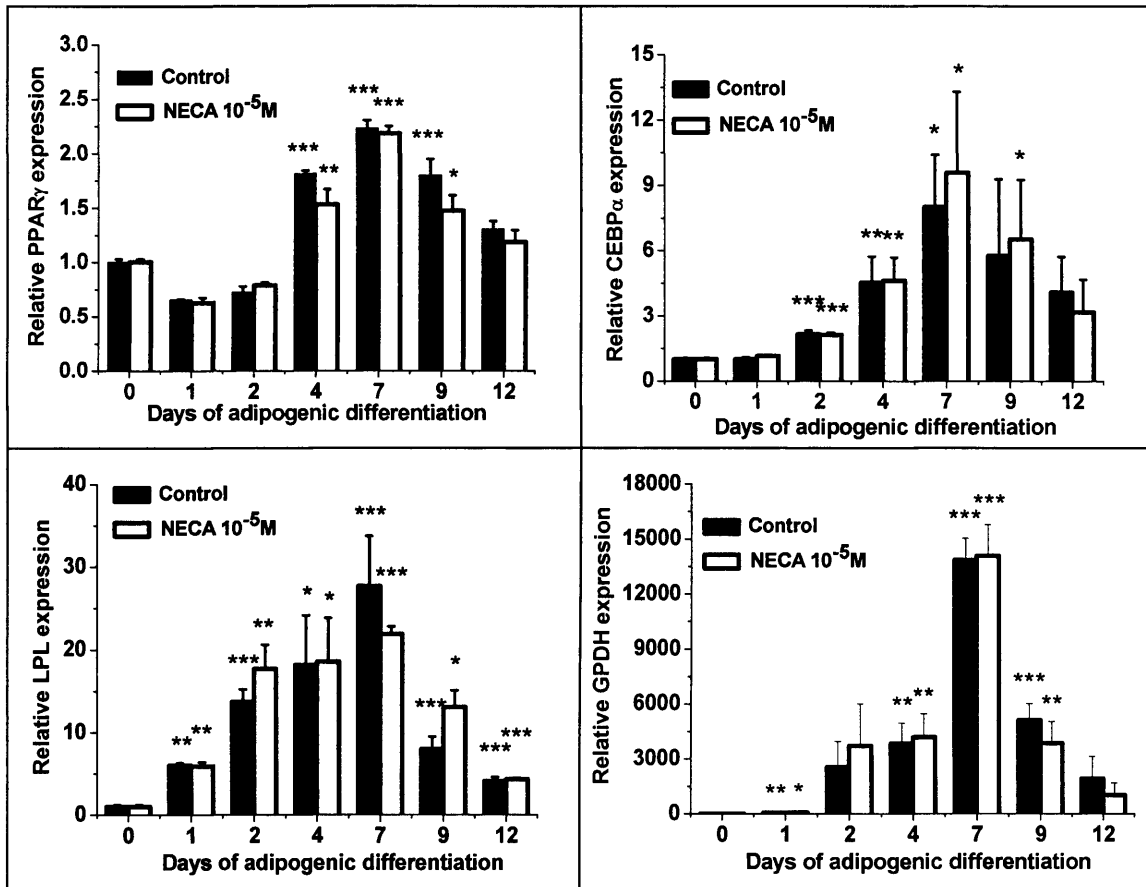
**Figure 5.4.16 Effect of adenosine receptor agonists on adipogenesis of 7F2.** 7F2 cells were incubated with adipogenic differentiation medium in the presence of the indicated concentrations of adenosine, NECA, CCPA and CGS 21680 for 7, 9 and 12 days. Extracted Oil Red O was measured at 490 nm ( $n=3$ ; total of 9 replicates). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  when compared with the control at the same time point.

A lack of effect was also seen with the  $A_1$  receptor agonist (CCPA) and its antagonist (PSB 36) (figure 5.4.18 B). Adipogenic differentiation of 7F2 cells also resulted in reduced osteocalcin mRNA expression, again addition of NECA for up to 12 days had no further effect (figure 5.4.19).

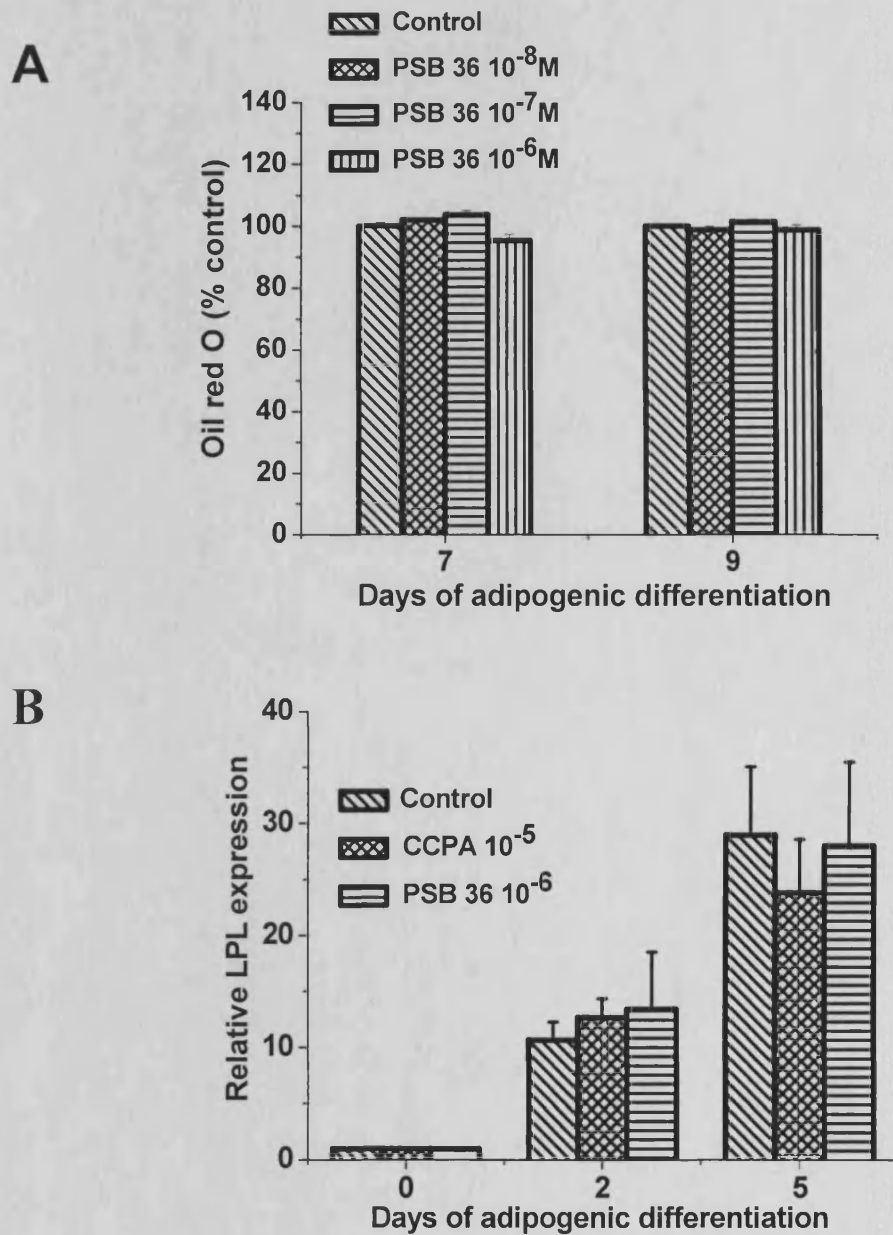
#### 5.4.3.6 Stable transfection of $A_1$ and $A_{2B}$ receptors in 7F2 cells

My data indicates that the  $A_1$  receptor is important in adipogenesis and the  $A_{2B}$  receptor is important for osteoblastogenesis. These receptors were thus over expressed in 7F2 cells by stable transfection, subsequently named 7F2 $A_1$  and 7F2 $A_{2B}$ . Expression of adipocyte and osteoblast markers were compared in these cells with cells transfected with only plasmid (pcDNA3.1) (7F2c). 7F2 $A_1$  cells showed increased LPL and GPDH mRNA expression ( $P < 0.01$ ) (figure 5.4.20 B&C) and reduced ALP enzyme activity ( $P < 0.001$ ) when compared to 7F2c cells (figure 5.4.21 A). PPAR $\gamma$  expression in these cells, also tended to increase, although statistical significance was not reached (figure 5.4.20 A). Surprisingly, enhanced  $A_1$  receptor expression also induced the expression of osteocalcin ( $P < 0.01$ ) (figure 5.4.21 B). Overexpression of the  $A_{2B}$  receptor on the other hand, did not modify LPL, GPDH or PPAR $\gamma$  expression (figure 5.4.20 A - C), but did significantly increase ALP activity ( $P < 0.05$ ) and osteocalcin expression ( $P < 0.001$ ) (figure 5.4.21 A&B).

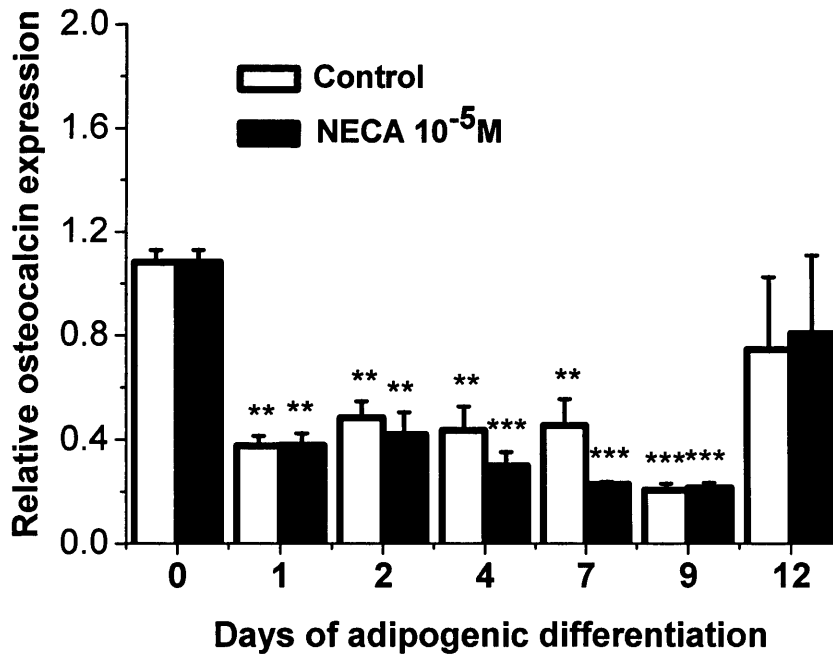
Morphologically, 7F2 $A_1$  and 7F2 $A_{2B}$  cells showed respectively increased and decreased adipogenesis when compared with 7F2c cells (figure 5.4.22 A). Overexpressing the  $A_1$  receptor also induced adipogenesis in relation to both



**Figure 5.4.17 Adipogenic gene expression in the presence or absence of NECA during the differentiation of 7F2 cells into adipocytes.** Cells were treated with NECA ( $10^{-5}$  M) for the indicated times in adipogenic media. PPAR $\gamma$ , C/EBP $\alpha$ , LPL and GPDH mRNA were determined by q-RT-PCR and compared with undifferentiated cells after normalisation to ARP (n=3; total of 6 replicates). \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 when compared with day 0.



**Figure 5.4.18** Effect of an  $A_1$  receptor agonist and antagonist on adipogenesis of 7F2 cells. Cells were treated with CCPA or PSB 36 for the indicated times in adipogenic medium. **(A)** Lipid accumulation quantified by Oil Red O ( $n=4$ ; total of 8 replicates) and **(B)** LPL mRNA expression determined by q-RT-PCR and compared with undifferentiated cells after normalisation to ARP ( $n=3$ ; total of 6 replicates).



**Figure 5.4.19 Osteocalcin mRNA expression in the presence or absence of NECA during differentiation of 7F2 cells into adipocytes.** Cells were treated with NECA ( $10^{-5}$  M) for the indicated times in adipogenic media. Osteocalcin mRNA expression determined by q-RTPCR and compared with undifferentiated cells after normalisation to ARP ( $n=3$ ; total of 6 replicates). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  when compared with day 0.

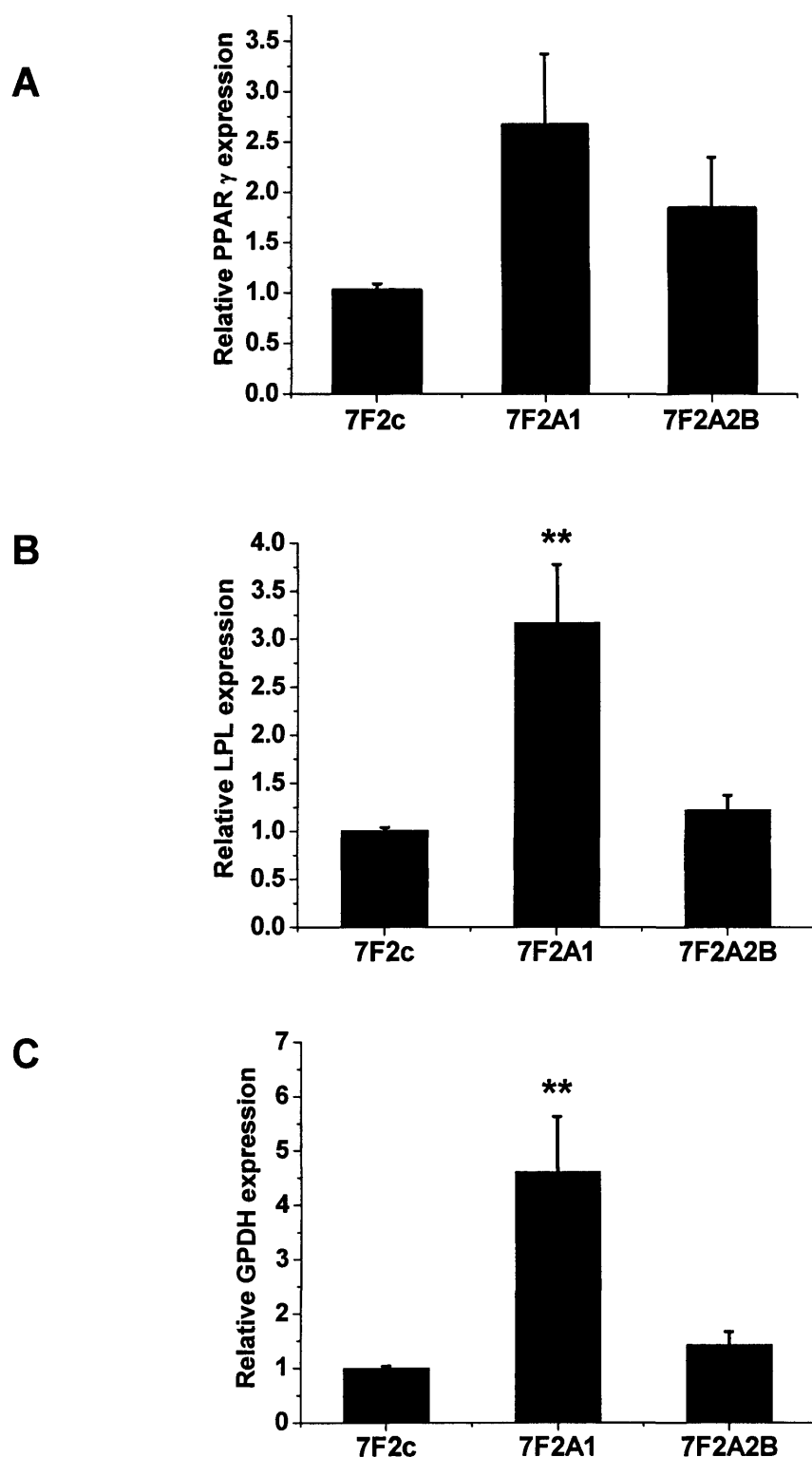
an increase in the number and size of lipid droplets and the number of individual adipocytes in the culture. In contrast, cells overexpressing the A<sub>2B</sub> receptor showed reduced adipocyte numbers when assessed microscopically. Quantitative analysis of the Oil Red O dye extract showed a significant reduction in 7F2A<sub>2B</sub> cells and a significant increase ( $P < 0.001$ ) in 7F2A<sub>1</sub> cells at 5 and 7 day of adipogenic differentiation (figure 5.4.22 B). Similar findings were obtained when flow cytometry analysis of Nile red stained cells (figure 5.4.23 A) was carried out. In addition to an increase in number of adipocytes, the mean Nile red fluorescence per unit cell was also upregulated in 7F2A<sub>1</sub> cultures, suggesting that these cells also contained on average more intracellular lipid. Conversely, 7F2A<sub>2B</sub> cells showed a decrease in Nile red fluorescence emission and thus a reduction in the number of adipocytes. The amount of lipid accumulated per cell was unaltered (figure 5.4.23 A&B).

#### **5.4.4 Transdifferentiation of HOB cells to adipocytes**

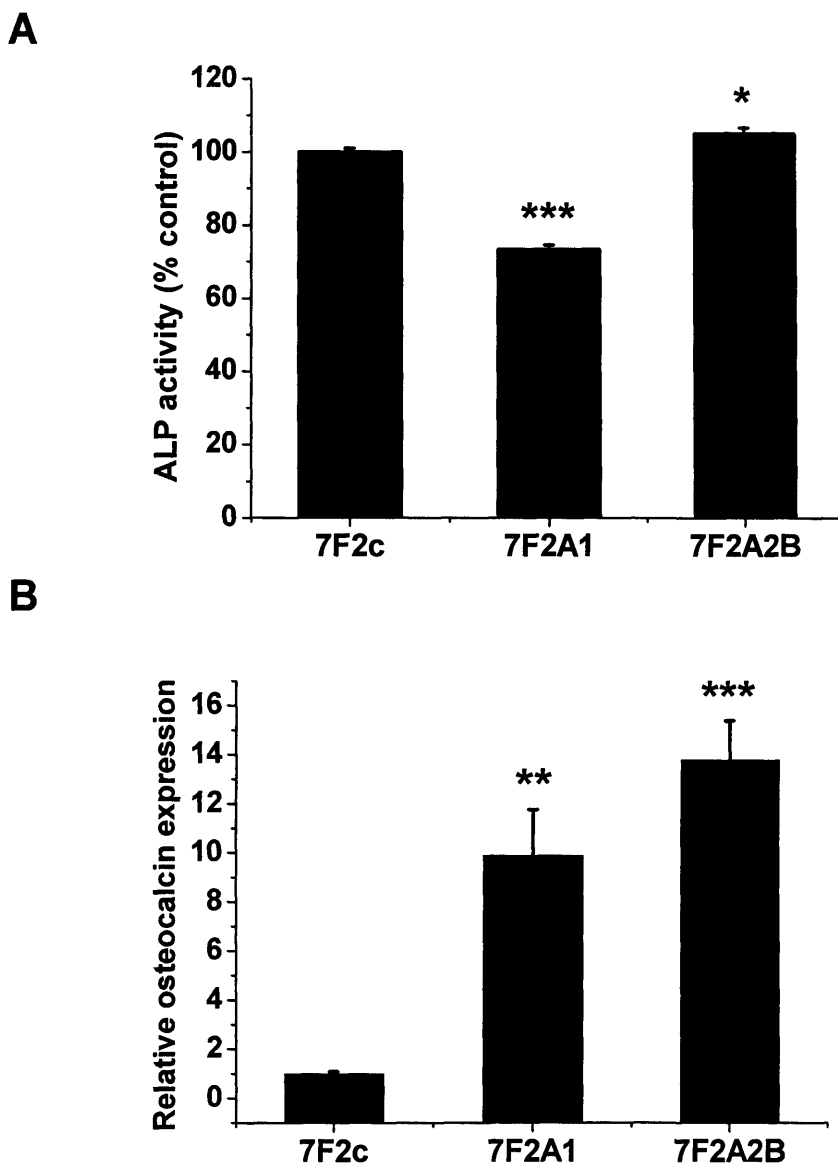
##### **5.4.4.1 Evaluation of adipogenesis**

HOB cells were induced to differentiate towards the adipocyte lineage using insulin, Dex and indomethacin. During 17 days of differentiation, only a small number of cells (about 1 - 2%) acquired morphological characteristics of adipocytes (figure 5.4.24 B). Lipid droplets were detectable after 14 days and increased in size upon longer incubation. HOB cells in basal medium expressed high levels of osteocalcin mRNA, however within 2 days of inducing adipocyte differentiation expression was reduced by 80% and remained at this level thereafter (figure 5.4.25 C).

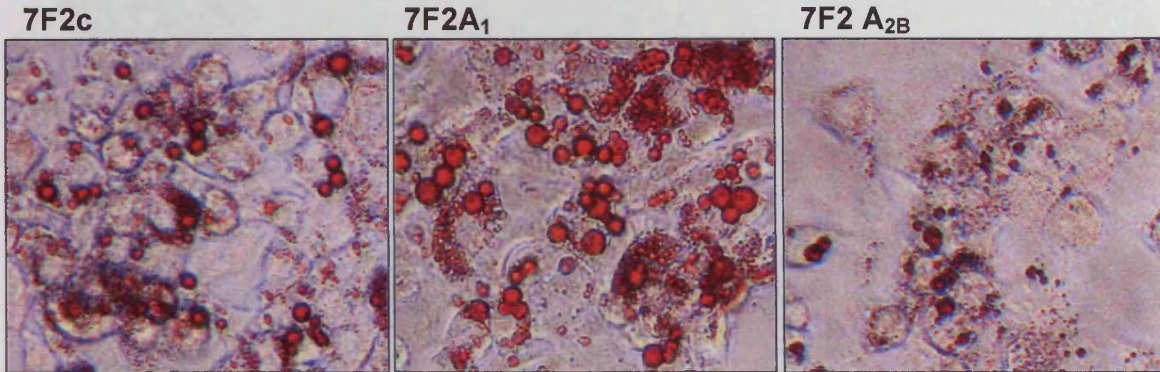
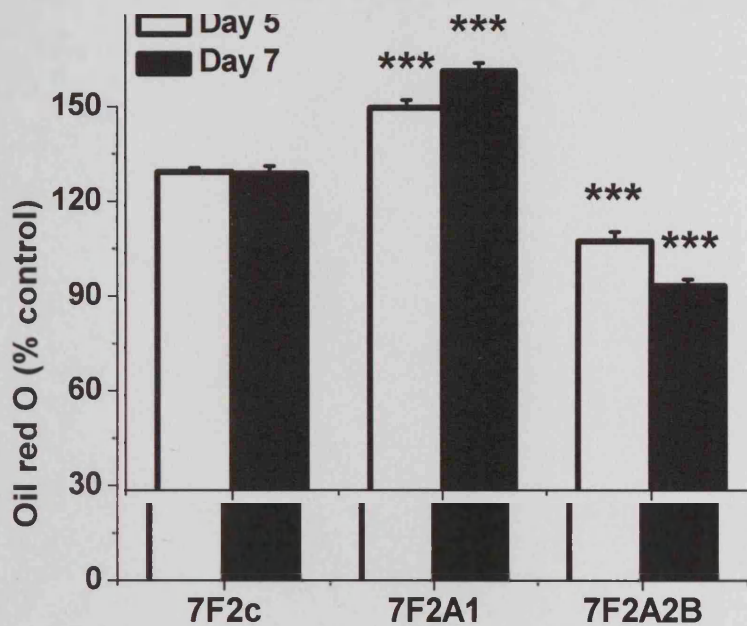




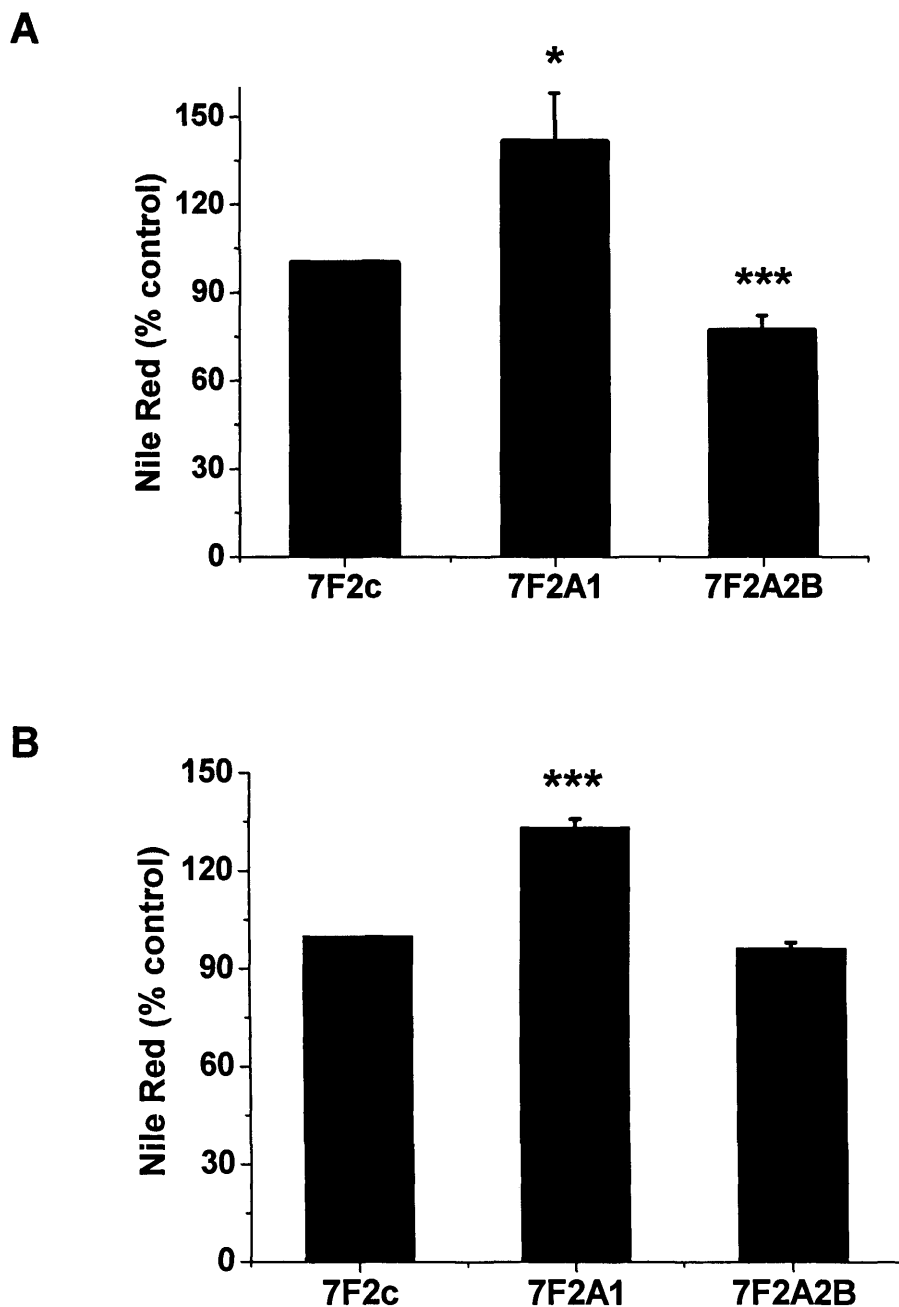
**Figure 5.4.20** Expression of adipogenic markers in 7F2 cells stably transfected with A<sub>1</sub> and A<sub>2B</sub> receptors. (A) PPAR $\gamma$ , (B) LPL and (C) GPDH mRNA expression was determined by q-RTPCR and compared with pcDNA3.1 alone (7F2c) cells after normalisation to ARP (n=3; total of 6 replicates). \*\*P < 0.01 when compared with 7F2c.



**Figure 5.4.21 ALP enzyme activity and osteocalcin mRNA expression in 7F2 cells stably transfected with A<sub>1</sub> and A<sub>2B</sub> receptors. (A)** ALP activity was measured and normalised to cell number (n=3; total of 36 replicates). **(B)** Osteocalcin was determined by q-RT-PCR and compared with pcDNA3.1 alone (7F2c) cells after normalisation to ARP (n=3; total of 6 replicates). \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 when compared with 7F2c

**A****B**

**Figure 5.4.22 Adipogenesis as determined by Oil Red O staining in 7F2 cells stably transfected with A<sub>1</sub> and A<sub>2B</sub> receptors.** Cells were incubated with adipogenic medium for 5 and 7 days. Lipid accumulation was (A) visualised after 7 days by staining with Oil Red O and (B) quantified by measuring the amount of retained dye in lipid vacuoles at 490 nm (n=3; total of 9 replicates). \*\*\*P < 0.001 when compared with the 7F2c.



**Figure 5.4.23 Adipogenesis as determined by Nile red staining in 7F2 cells stably transfected with A<sub>1</sub> and A<sub>2B</sub> receptors.** Cells were incubated with adipogenic medium for 5 days. **(A)** Number of Nile red positive cells and **(B)** mean Nile red fluorescence emission was quantified with flow cytometry (n=3; total of 6 replicates). \*P < 0.05 and \*\*\*P < 0.001 when compared with the 7F2c.

HOB cells also expressed PPAR $\gamma$  mRNA, which increased progressively during the transdifferentiation process (15 - 20 fold after 17 days) (figure 5.4.25 A). In contrast, expression of LPL mRNA was undetectable in HOB cells and was only detectable after 17 days of adipocyte differentiation (figure 5.4.25 B).

#### **5.4.4.2 Adenosine receptor expression during adipocytic transdifferentiation of HOB cells**

In contrast to adipogenesis of MSCs and 7F2 cells where there were marked upregulation in expression of A<sub>1</sub> adenosine receptor, adipogenic transdifferentiation of HOB cells was associated with a reduction in A<sub>1</sub> receptor expression. As shown in figure 5.4.24 C, A<sub>1</sub> receptor mRNA was reduced within 2 days and remained low during 17 days of differentiation; there was however, no effect on A<sub>1</sub> receptor protein expression (figure 5.4.24 F). Induced transdifferentiation of HOB cells did not affect A<sub>2A</sub> and A<sub>2B</sub> receptor mRNA or protein expression (figure 5.4.24 D - F).

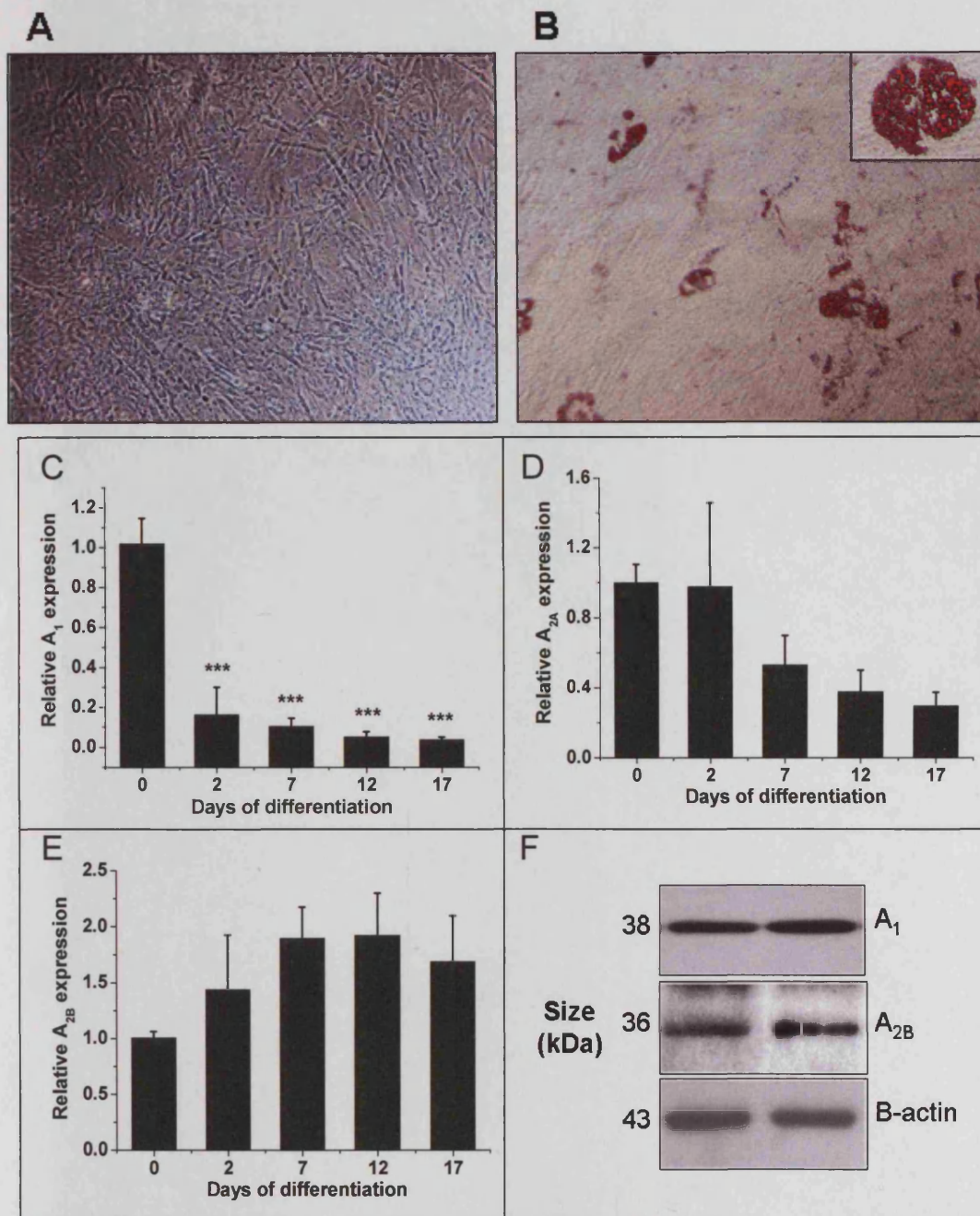
#### **5.4.4.3 Effects of NECA on adipogenic transdifferentiation of HOB cells**

Treatment with NECA stimulated adipocyte formation of HOB cells when compared to untreated controls. This effect was apparent by an increase in the number of lipid containing adipocytes seen microscopically in NECA treated cultures. However, due to a limited number of cells the analysis by Oil Red O staining was not possible. In addition, PPAR $\gamma$  expression tended to increase in NECA treated cells, although statistical significance was not reached (figure 5.4.25 A). Similar to untreated HOB cells, the expression of

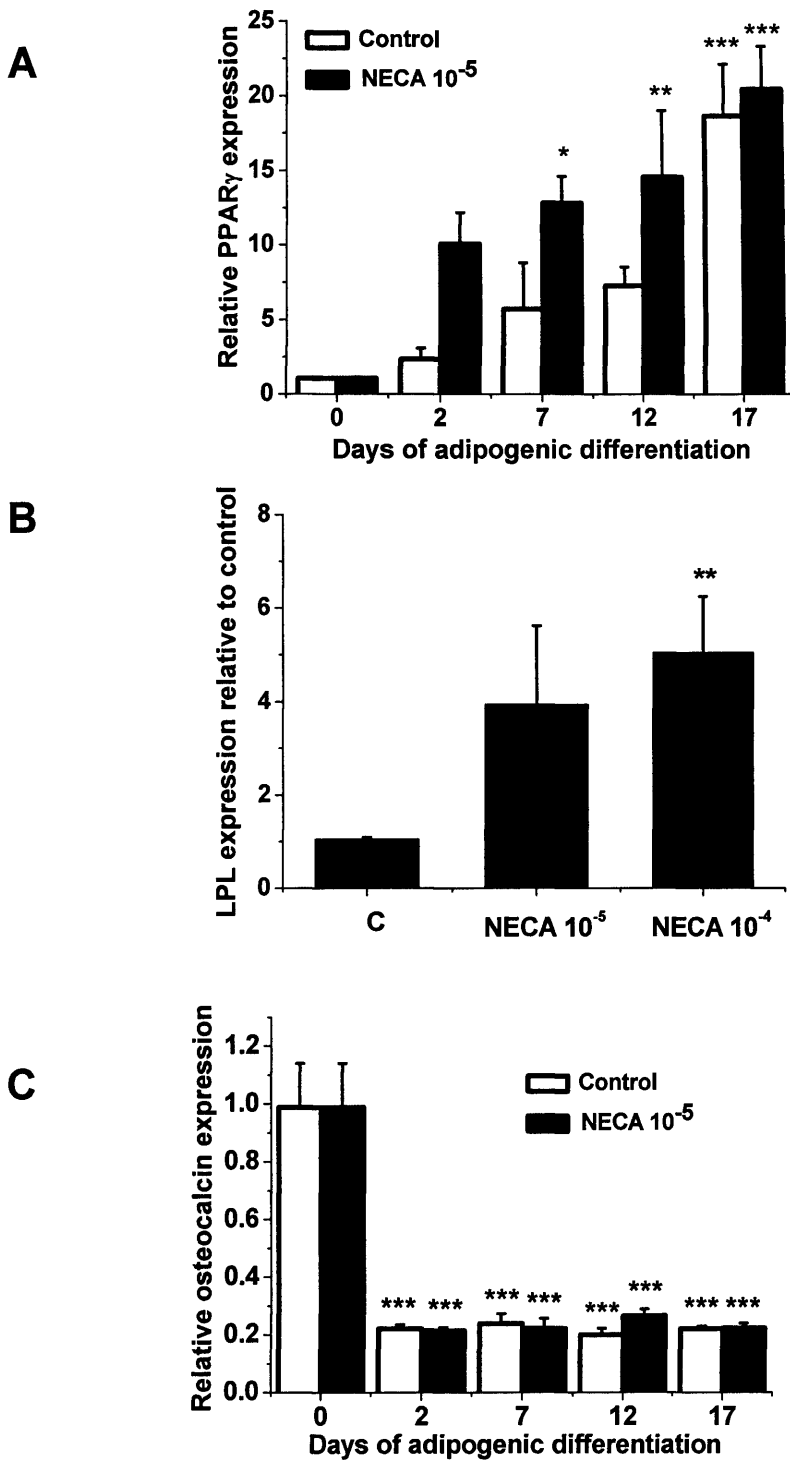
LPL mRNA was undetectable after 12 days of adipocyte differentiation; at day 17 LPL was clearly detectable and significantly induced by NECA (figure 5.4.25 B).

#### **5.4.4.4 Effects of NECA on osteoblast marker expression in HOB cells in the absence of differentiating media**

HOB cells were cultured in the presence or absence of NECA ( $10^{-5}$  M) for the indicated times and ALP and osteocalcin expression was analysed by q-RTPCR. When normalised and compared to untreated cells, the expression of both ALP and osteocalcin was found significantly reduced in NECA treated cells (figure 5.4.26 A and 5.4.27 A respectively). The relative mRNA expression for ALP was reduced by 40% and 45% and for osteocalcin by 14% and 30% after 2 and 7 days treatment with NECA respectively. Similar data were observed when NECA treated cells were analysed for ALP enzyme activity (figure 5.4.26 B). NECA and adenosine also inhibited mineralisation of HOB cells as determined by Alizarin Red S staining (figure 5.4.27 B). Treatment with adenosine ( $10^{-4}$  M) and NECA ( $10^{-5}$  M) respectively also resulted in a reduction in mineralisation in presence of AA and  $\beta$ -GP.

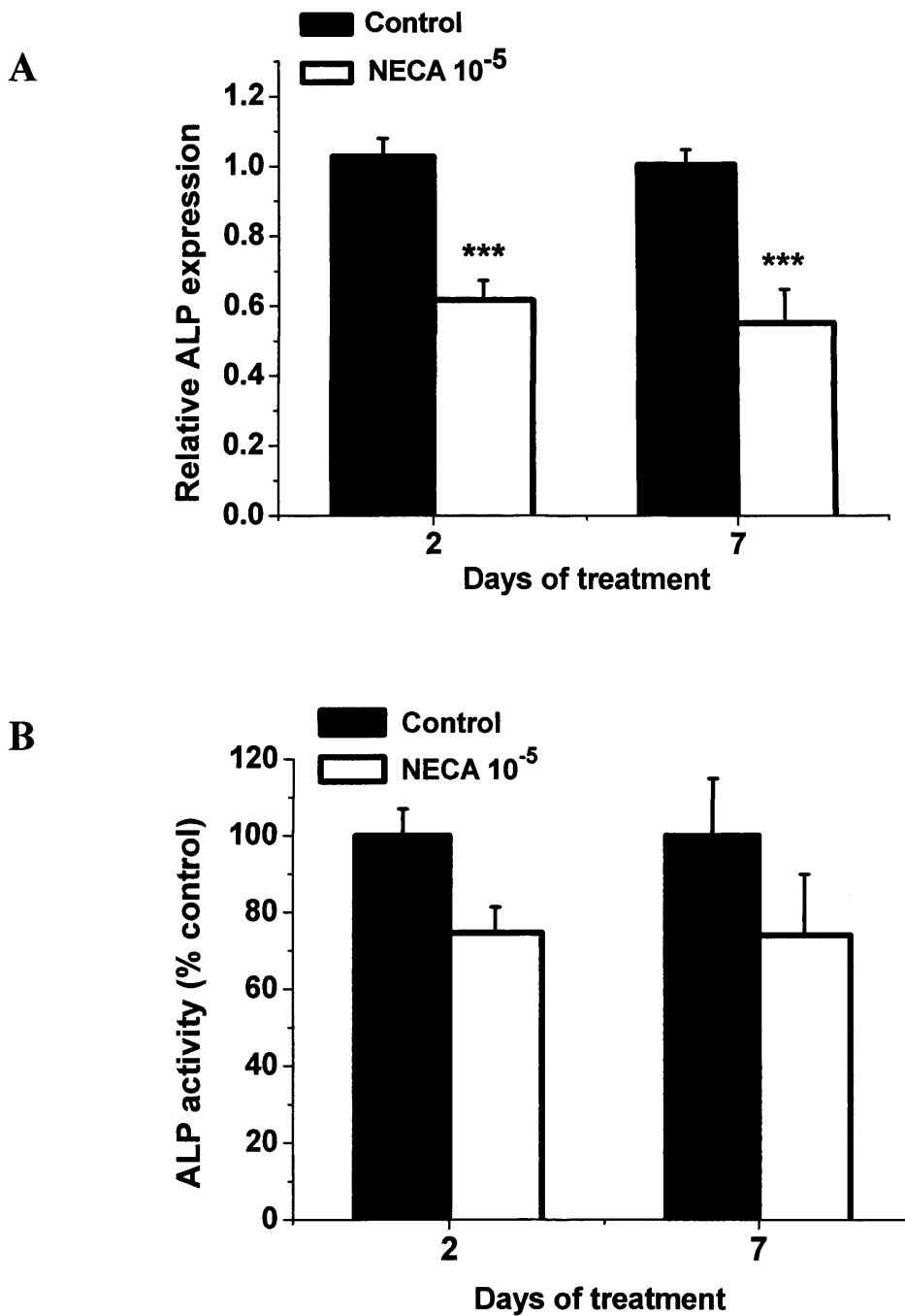


**Figure 5.4.24 Differentiation of HOB cells to adipocytes and expression of adenosine receptors.** Oil Red O staining of (A) HOB cells and after (B) 17 days of adipogenesis (Magnification x100, inset x200). (C - E) q-RTPCR shows adenosine receptor mRNA expression, relative to the housekeeping gene, APRT (n=3; total of 6 replicates). \*\*\*P < 0.001 when compared with day 0. (F) A representative Western blot showing expression of  $A_1$  and  $A_{2B}$  receptor proteins in HOB cells and after 17 days of adipocyte differentiation. The  $A_{2A}$  and  $A_3$  receptors were not detectable. Left margin indicates the sizes (kDa) of protein marker.

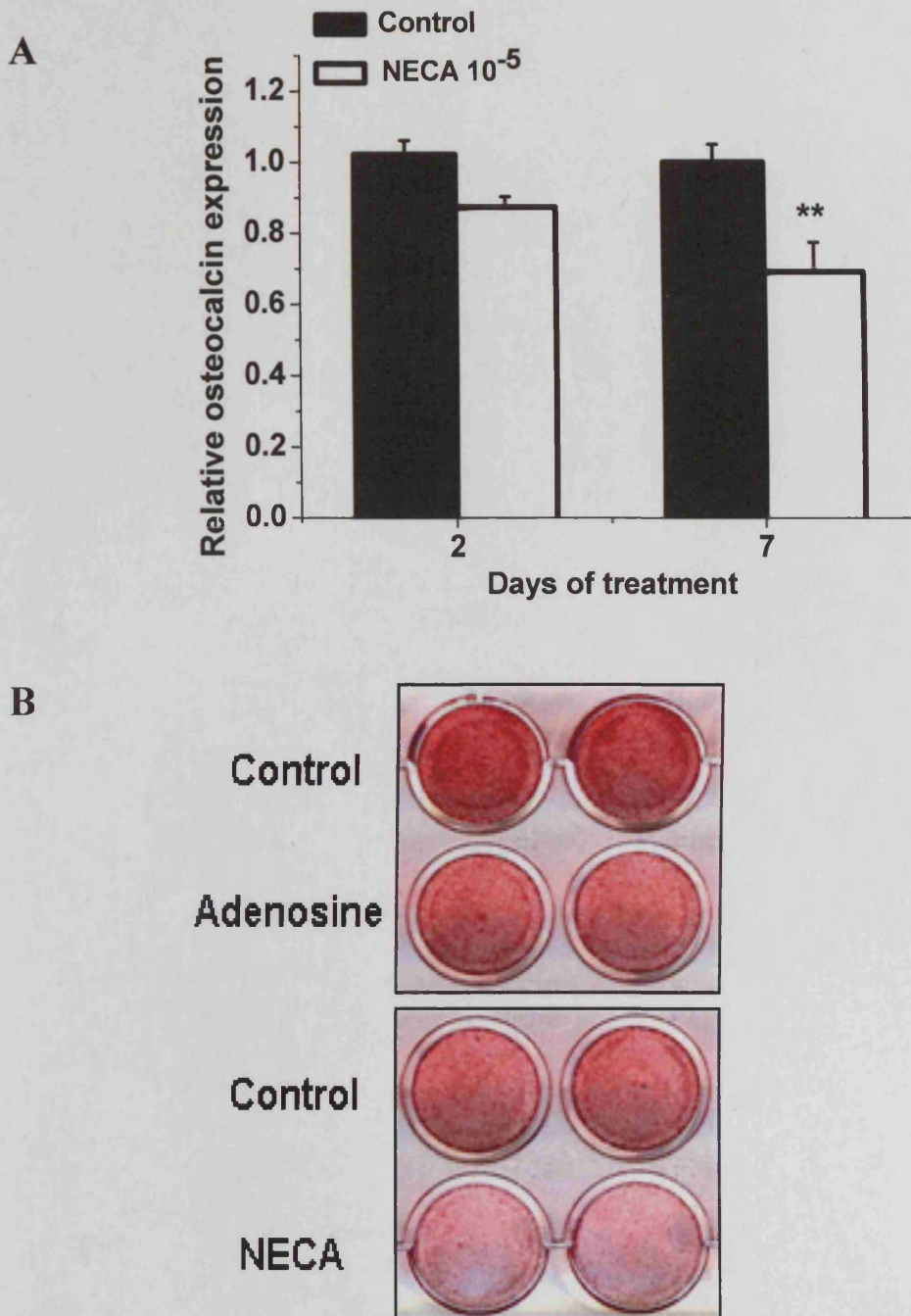


**Figure 5.4.25 Expression of PPAR $\gamma$ , LPL and osteocalcin mRNA in the presence or absence of NECA during the differentiation of HOB cells into adipocytes.** Cells were treated with NECA for the indicated times in adipogenic medium. mRNA expression for (A) PPAR $\gamma$ , (B) LPL (for 17 days) and (C) osteocalcin was determined by q-RT-PCR and presented as the relative mRNA expression level to undifferentiated cells after normalisation to APRT (n=3; total of 6 replicates). \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 when compared with day 0.





**Figure 5.4.26** Effect of NECA on ALP mRNA expression and enzyme activity in HOB cells. Cells were treated with NECA ( $10^{-5}$  M) for 2 and 7 days. **(A)** ALP mRNA was determined by q-RTPCR and presented as relative expression to that of undifferentiated cells after normalisation to APRT ( $n=3$ ; total of 6 replicates). \*\*\* $P < 0.001$  when compared with vehicle control. **(B)** ALP activity was measured and normalised to protein content ( $n=2$ ; total of 24 replicates). Data are expressed as mean  $\pm$  SDM.



**Figure 5.4.27** Effects of adenosine and NECA on osteocalcin mRNA expression and mineralisation in HOB cells. Cells were treated with NECA ( $10^{-5}$  M) for 2 and 7 days and (A) osteocalcin mRNA was determined by q-RTPCR and presented as relative expression to undifferentiated cells after normalisation to APRT ( $n=3$ ; total of 6 replicates). \*\* $P < 0.01$  when compared with vehicle control. (B) Cells were treated with adenosine ( $10^{-4}$  M) and NECA ( $10^{-5}$  M) or appropriate vehicle control in mineralisation medium and calcium deposition was determined.

## 5.5 Discussion

### 5.5.1 Expression of adenosine receptors in 7F2 and HOB cells

In the previous chapters, adenosine receptors were shown to be differentially expressed and functionally involved in the differentiation of MSCs into osteoblasts and adipocytes. The goal of this chapter was to determine the expression profile and functional properties of adenosine receptors in primary HOB and mouse 7F2 osteoblasts and during their transdifferentiation into adipocytes. In agreement with earlier findings in rat MSCs and human osteoprogenitor cells, mRNA expression for all four adenosine receptors was present in both cell types. Similar findings were obtained by Russel et al (2007), however they were unable to detect expression of the  $A_3$  receptor in MG-63 osteoblastic cells. In both 7F2 and HOB cells the  $A_3$  receptor was detectable using qRT-PCR but the protein was not evident, at least on Western blotting analysis.

Protein bands for the  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptors were easily detectable in 7F2 cells. The sizes of the  $A_1$  and  $A_{2A}$  receptors in rat MSC and mouse 7F2 cells were similar but the  $A_{2B}$  receptor appeared as a band with apparent molecular mass of 36 kDa in both 7F2 and HOB cells, in contrast to the 52 kDa observed in rat. This correlates with the predicted molecular mass of  $A_{2B}$  receptors and is consistent with those reported by other groups in many cell types including a human osteoblast cell lines (Linden et al., 1999; Russell et al., 2007). The larger mass for the  $A_{2B}$  receptor in rat MSCs could be attributed to tissue or species specific differences as shown by other investigators (Puffinbarger et al., 1995).  $A_1$  but not  $A_{2A}$  receptor proteins were

also detectable in HOB cells. The lack of A<sub>2A</sub> receptor expression is consistent with the low level of mRNA expression observed in HOB cells.

The cAMP data obtained from 7F2 cells indicates a predominance of A<sub>2B</sub> receptors. In the presence of adenosine, NECA and CGS 21680, the cAMP accumulation was increased with a rank order of potency of NECA > adenosine > CGS 21680. These findings were also found in MSCs and are compatible with cells expressing recombinant or endogenous adenosine A<sub>2B</sub> receptors (Feoktistov and Biaggioni, 1998; Klotz et al., 1997; Linden et al., 1999).

Besides coupling to the adenylate cyclase dependent signal cascade, adenosine receptors have been demonstrated to be able to couple to at least one subfamily of MAPK in a variety of cell types, including rat smooth muscle, human endothelial cells, embryonic kidney (HEK-293) cells, mast cells and melanoma cells (Feoktistov et al., 1999; Gao et al., 1999; Merighi et al., 2002a; Robinson and Dickenson, 2001; Sexl et al., 1997). The data reported in this chapter shows that adenosine receptors can also be coupled to the MAPK pathway in osteoblasts.

In 7F2 cells, NECA caused time and dose dependent ERK phosphorylation, which was rapid and transient, peaking at 5 minutes, and returning to the basal level after 60 minutes; a similar transient stimulation has been reported in HEK-293 cells (Gao et al., 1999). The duration of the ERK signal is regulated by different upstream events in the ERK signal cascade and is

thought to be linked to different cellular responses (Ebisuya et al., 2005). The concentrations of NECA used to stimulate ERK phosphorylation were comparable to those used in the activation of cAMP suggesting that the ERK is also an important signalling pathway for adenosine receptors. These observations were in agreement with other findings showing that the EC<sub>50</sub> for NECA induced ERK activation is in the lower micro-molar range (Gao et al., 1999; Grant et al., 2001). As with the cAMP responses in 7F2 cells, NECA induced ERK activation also appears to be mediated through the A<sub>2B</sub> receptor as selective agonists for the A<sub>1</sub> and A<sub>2A</sub> receptors failed to have an effect and NECA activation of ERK was inhibited by a selective A<sub>2B</sub> receptor, but not by an A<sub>1</sub> or A<sub>2A</sub> receptor, antagonist. The coupling of A<sub>2B</sub> receptors to ERK signalling have been demonstrated in HEK-293 cells, mast cells and retinal endothelial cells (Feoktistov et al., 1999; Gao et al., 1999; Grant et al., 2001; Schulte and Fredholm, 2003a).

A<sub>2B</sub> receptor linked ERK activation can be mediated through multiple signalling cascades that are either cAMP dependent (Gs/cAMP/PKA → B-Raf → MEK1/2 → ERK1/2) and (Gs/cAMP → PI3K → MEK1/2 → ERK1/2) or cAMP *independent* (Gq11 → PLC/Ca<sup>2+</sup>/DAG → tyrosine kinase → Ras → B-Raf/C-Raf → MEK1/2 → ERK1/2) and (Gq11 → PLC/Ca<sup>2+</sup>/DAG → PKC → MEK1/2 → ERK1/2). The precise pathways involved may depend on the cell type (Feoktistov et al., 1999; Gao et al., 1999; Grant et al., 2001; Schulte and Fredholm, 2000; Schulte and Fredholm, 2003a). Although it is clear that the A<sub>2B</sub> receptor acts through cAMP in 7F2 cells, the adenylate cyclase inhibitor, DDA, only partially reduced the NECA stimulation of ERK activation. The

incomplete inhibition of the ERK response suggests that a pathway independent of cAMP is also involved. This suggestion is supported by experiments that showed the PLC inhibitor, U-73122, inhibited NECA induced ERK1/2 phosphorylation. Such findings are in agreement with that of Gao et al who demonstrated that  $A_{2B}$  induced ERK activation in HEK-293 cells required PLC (Gao et al., 1999). Moreover, as expected, the MEK inhibitor, PD 98059 completely abolished ERK activation. Ras/B-Raf has been described previously to be intermediate signal molecules in the PLC/MEK induction of ERK activation via the  $A_{2B}$  adenosine receptor in HEK-293 cells (Gao et al., 1999). This pathway may also be important in osteoblasts. The PKC inhibitor RO-32-0432 and the PI3K inhibitor, Wortmannin failed to inhibit NECA stimulated ERK phosphorylation. The inhibitory action of RO-32-0432 on PKC was confirmed by demonstrating the loss of PMA induced ERK activation, whereas the PLC inhibitor U-73122 was without effect. Together these data demonstrate that NECA induces phosphorylation of ERK 2 in 7F2 cells by a pathway that is most likely coupled to Gs, and Gq/11 proteins but is independent of PKC and PI3K. Similar findings has been previously described by Gao (1999) and colleagues in HEK-293 cells.

An important target that is activated by both ERK and cAMP/PKA pathways is the transcription factor CREB (Johannessen et al., 2004). Since  $A_{2B}$  receptor is shown here to activate both cAMP and ERK pathways in 7F2 cells, it is likely that one or both of these pathways subsequently activate the phosphorylation of CREB in these cells. NECA caused a rapid and transient increase in CREB phosphorylation which was inhibited by PD 98059 and U-

73122, consistent with its inhibitory actions on NECA induced ERK signalling. A similar effect was seen with the PKC inhibitor, RO-32-0432, suggesting that PKC may be an intermediate step downstream of ERK and upstream of CREB signalling. The weak or lack of effect of DDA and Wortmannin on CREB signalling was in agreement with that seen on ERK phosphorylation. Given the complexity of interactions between the cAMP, ERK and CREB, no firm conclusions can be drawn about the signal pathways associated with NECA activation of 7F2 cells. Nevertheless, my experiments do indicate a possible involvement of the  $A_{2B}$  receptor that is linked to PLC, MEK, ERK, PKC and CREB but less likely with PI3K (figure 5.5.1).

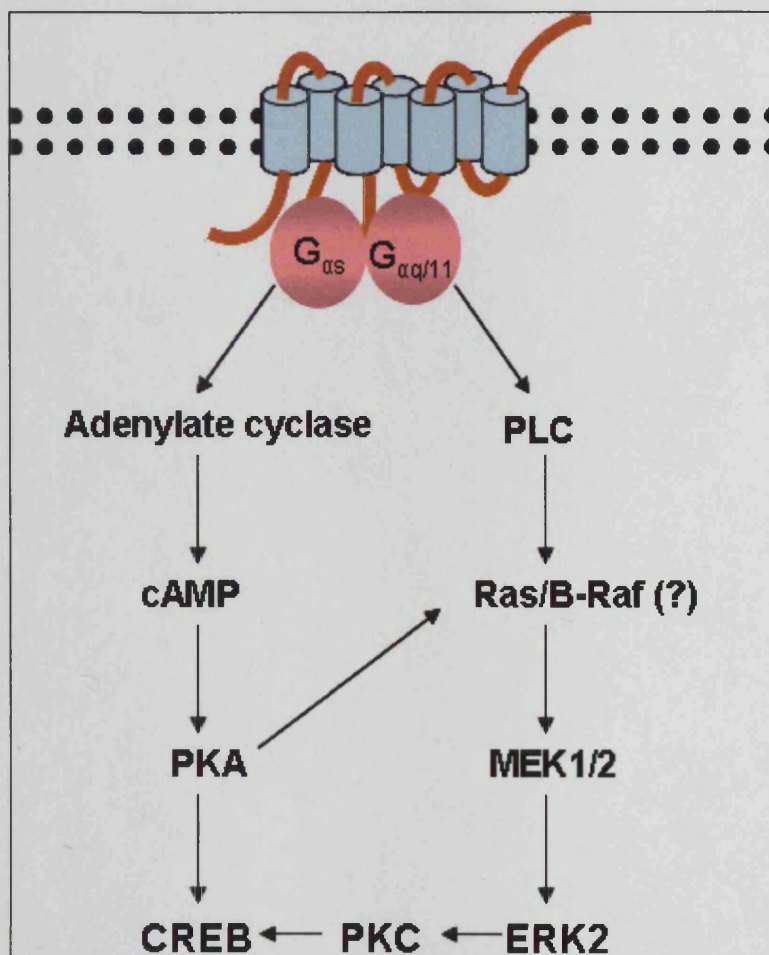


Figure 5.5.1 Possible pathways of ERK and CREB activation by  $A_{2B}$  receptor in 7F2 cells.

### **5.5.2 Adenosine receptor expression and function in the transdifferentiation of 7F2 and HOB cells to adipocytes**

Both HOB and 7F2 cells correspond to mature osteoblasts as evidenced here by expression of ALP and osteocalcin and positive staining for extracellular matrix mineralisation. Induction of adipogenesis in 7F2 cells resulted in the formation of lipid droplets in at least 50% of the cells and strong expression of adipogenic markers PPAR $\gamma$ , C/EBP, LPL and GPDH and loss of the late osteogenic marker, osteocalcin. These findings are in agreement with those reported by Thompson et al (1998). An interesting observation was the progressive reduction in the expression of adipocyte markers (and indeed of the A<sub>1</sub> receptor and the enzyme, CD73) after reaching a peak after 7 days of differentiation. This could be explained by the loss of mature adipocytes from the cell monolayer surface, as they are filled with lipid and can become easily detached. Similar observations have been reported by others and are attributed to the reduction in production of matrix proteins as well as morphologic changes due to lipid accumulation (van Harmelen et al., 2005).

In contrast to 7F2 cells, transdifferentiation of HOB cells was slow and only resulted in conversion of about 1 - 2 % of the cells into lipid containing adipocytes. Furthermore, the loss of osteoblastic genotype *i.e.* osteocalcin expression occurred well before any increase in expression of adipogenic associated genes was observed. This may suggest that cells might have dedifferentiated to a progenitor cell stage prior to redifferentiation into adipocytes. Dedifferentiation prior to transdifferentiation of osteoblasts has been previously reported by Song et al (2004 and 2006) who showed that fully



differentiated MSCs (*i.e.* mature osteoblasts, adipocytes, and chondrocytes) lose their characteristic markers prior to transdifferentiation upon withdrawal of the inducing stimulus. Park et al (1999) also showed that mature human adipocytes were able to dedifferentiate and redifferentiate into osteoblasts.

The observation that HOB cells, in our hands, could only be weakly induced to adipocytes may somehow account for the reduced A<sub>1</sub> adenosine receptor expression. Adipocyte transdifferentiation of 7F2 cells was associated with a marked increase in A<sub>1</sub> receptor mRNA expression and an increase (albeit at a lower level) in protein expression. In chapter 4, I also showed that A<sub>1</sub> mRNA expression was increased as rat MSCs were induced to differentiate to adipocytes and this also occurs during the maturation of preadipocytes (Børghlum et al., 1996). The apparent reduction in A<sub>1</sub> receptor expression during adipogenesis of HOB cells is unlikely to be related to the media used for adipocyte induction as the same media was used for HOBs and MSCs. It is possible that HOB cells undergo dedifferentiation with a loss of A<sub>1</sub> receptors prior to undergoing differentiation to adipocytes. In addition weak staining with Oil Red O could suggest that during induced adipogenic differentiation HOB cells only reach the preadipocyte stage and such cells only have low expression of A<sub>1</sub> receptors (Børghlum et al., 1996; Fathollahi et al., 2006; Johansson et al., 2008; Vassaux et al., 1993). This is consistent with the finding that the late adipogenic marker (LPL) was only observed after 17 days of differentiation and even then at a low level in contrast to that seen in 7F2 adipocyte differentiation. Thus the majority of cells in the HOB differentiation experiments were not mature adipocytes.

Transdifferentiation of 7F2 cells was also accompanied with a loss of the  $A_{2A}$  and  $A_{2B}$  receptors at both the mRNA and protein level and putative inactivation (phosphorylation) of the  $A_{2B}$  receptor. Although immunoprecipitation experiments were unsuccessful, possibly due to low expression of the  $A_{2B}$  receptor after adipogenesis, I was able to show co-elution of the putative phosphorylated band with bands labelled with anti-phosphothreonine and Pro Q. Loss of  $A_2$  receptors were also demonstrated, mechanistically, by losses in cAMP, ERK and CREB activation. Similar to these data, Borglum and colleagues (Børglum et al., 1996) also demonstrated a reduction in  $A_2$  receptors during the adipogenesis of Ob1771 preadipocytes. Such findings could suggest that  $A_2$  receptors are associated with an osteoblast phenotype and this was demonstrated when MSCs were induced to differentiate to adipocytes (chapter 3) and on the transfection of  $A_{2B}$  receptors into 7F2 cells. The loss of  $A_2$  receptors (particularly the  $A_{2B}$  receptor) is also likely to be associated with an inhibitory role for these receptors in the transdifferentiation of osteoblasts into adipocytes. Similar observation have been reported for other inhibitors of adipogenesis (such as TAZ) that are downregulated during the adipogenesis process (Hong and Yaffe, 2006).

The increase in  $A_1$  receptor expression during adipogenesis may suggest that adenosine itself is important in the differentiation process. In support of this was the observation that CD73 is upregulated and ADA is downregulated during differentiation. Interestingly these changes also peaked around days 4 - 7 which mirrored changes in adipocyte marker expression. The increase in

CD73 with a concomitant decrease in ADA expression suggests that adenosine levels accumulate on adipocyte differentiation. The presence of increased endogenous adenosine levels may limit the effects of adding exogenous adenosine and could explain the lack of effects of NECA on adipocyte marker expression seen in our experiments. In addition, it should be noted that 7F2 cells undergo strong adipogenesis in the presence of adipogenic inducing agents and this may overshadow any additional effects of adenosine. Nevertheless, adenosine receptor agonists did have moderate effects on differentiation of 7F2 cells in the presence of differentiating media and NECA similarly stimulated an increase in LPL mRNA expression in HOB cells undergoing differentiation. Additional experiments in HOB cells indicated that NECA could inhibit markers of osteoblastogenesis (ALP and osteocalcin) in the absence of adipocyte differentiation media as well as inhibiting mineralisation. These data could also imply that activating adenosine receptors could stimulate adipogenesis.

Overall my data on 7F2 and HOB differentiation as well as that on MSC differentiation to osteoblasts and adipocytes suggest that the  $A_1$  and  $A_{2B}$  receptors are particularly important in the adipogenesis and osteoblastogenesis processes respectively. The  $A_1$  receptor in adipocytes has been extensively studied (see section 1.4.9) and is shown to increase lipogenesis and reduce lipolysis (Cheng et al., 2000; Dhalla et al., 2007b; Johansson et al., 2008; Rice et al., 2000; Sollevi and Fredholm, 1981; Vannucci et al., 1989). Stable transfection of the  $A_1$  and  $A_{2B}$  receptors into 7F2 cells respectively increased and decreased adipogenesis as determined

by lipid accumulation. Tatsis-Kotsidis and Erlanger (1999) similarly reported that  $A_1$  receptor overexpression in murine preadipocytes (ob17) also stimulated adipocyte differentiation. Enhanced  $A_1$  but not  $A_{2B}$  receptor expression also increased markers of adipogenesis. ALP and osteocalcin were also upregulated with the  $A_{2B}$  receptor and overexpression of the  $A_1$  receptor decreased ALP but surprisingly stimulated osteocalcin expression. This could suggest that the  $A_1$  receptor may be linked to other signal pathways which may indirectly affect osteocalcin expression. One possibility is the activation of  $K^+$  channels that has been previously shown by our lab to be involved in the mineralisation of osteoblasts (Henney et al., 2009). To determine whether the  $A_1$  receptor was a necessary factor for adipogenic transdifferentiation, I have also tried to use an siRNA approach to knockdown the  $A_1$  receptors in 7F2 cells. However the effort was hampered by its low expression level even following adipogenesis.

Together the data from this chapter suggest a possible role for  $A_1$  receptors in the differentiation of adipocytes and for  $A_{2B}$  receptors in maintaining the osteoblastic phenotype.

## **Chapter 6**

### **General discussion**

## 6.1 Summary and discussion

There has been increasing interest in recent years in determining the factors that regulate the differentiation of MSCs into osteoblasts and adipocytes. Such information would enable the development of strategies for treating diseases such as osteoporosis that are associated with an imbalance in the differentiation and function of these lineages. Drugs that are in therapeutic use for bone loss target predominantly inhibition of osteoclast activity. PTH is the only anabolic drug currently in clinical use for osteoporosis. Based on previous data which showed that adenosine receptors are important for the function of human osteoprogenitor cells, the data presented here describe the expression and function of adenosine receptors in MSCs and during their differentiation to osteoblasts and adipocytes, as well as in osteoblasts and during their transdifferentiation to adipocytes.

Adenosine exists in all cells as part of the ATP metabolic pathway and concentrations fluctuate on cellular activity, stress and inflammation indicating that the different adenosine receptors which have different  $K_m$  values can be activated according to the nature of the microenvironment. In addition, activation of specific adenosine receptors to either stimulate ( $A_{2A}$  and  $A_{2B}$  receptors) or inhibit ( $A_1$  and  $A_3$  receptors) cAMP could suggest that reciprocal changes in cAMP levels may be important for lineage specific differentiation. The findings presented here show that  $A_1$  receptors play a dominant role in the differentiation and function of adipocytes and that  $A_2$  receptors seem to be important in MSCs and osteoblasts.

Although all four adenosine receptors were detectable (at least at the level of the gene) in MSCs, the  $A_{2B}$  receptor was dominantly expressed and their activation was sufficient to promote differentiation towards the osteoblast lineage as evidenced by increased ALP expression. Interestingly, the  $A_{2B}$  receptor gene and protein expression appeared to be transiently unregulated as cells differentiated into osteoblasts, suggesting this receptor may have a role in early osteoblastic differentiation. On the other hand the  $A_{2A}$  receptor gene and protein expression appeared to increase during later stages of osteoblastic differentiation. From this data one can speculate that  $A_{2B}$  receptors are more important for commitment and differentiation to osteoblasts, and that the  $A_{2A}$  receptors are important for osteoblast maturation and maintenance of the osteoblast phenotype. There was also a progressive increase in  $A_{2A}$  receptor mediated cAMP expression as MSCs were induced to differentiate to osteoblasts which may also support a role for the receptor in later differentiation. Nevertheless, the  $A_{2B}$  receptor remained the dominant receptor throughout the differentiation process and its activation leads to a stimulation of osteoblast associated genes.

The  $A_{2B}$  receptor was also dominantly expressed in 7F2 osteoblasts and shown to be coupled to cAMP, ERK and CREB signalling molecules. Activation of these pathways has been previously implicated in differentiation and function of osteoblasts (Ge et al., 2007; Lai et al., 2001; Pearman et al., 1996; Pekkinen et al., 2008; Siddappa et al., 2008). Overexpression of human  $A_{2B}$  receptors in 7F2 cells resulted in increased expression of osteoblastic markers *i.e.* osteocalcin and ALP, whilst limiting the ability of 7F2 cells to

differentiate to adipocytes (down regulation of lipid accumulation). On the basis of these findings this work proposes that  $A_2$  receptors are important for bone homeostasis by stimulating differentiation towards an osteoblast lineage and the maintenance of an osteoblast phenotype.

The  $A_1$  receptor, in contrast to  $A_2$  receptors, was found to be involved in adipogenesis and adipocyte function.  $A_1$  receptor expression is reported to be low in preadipocytes, but increases on adipogenesis and remains at high levels in mature adipocytes (Børghlum et al., 1996; Fathollahi et al., 2006; Vassaux et al., 1993). My data concur with these findings. I showed that the expression of the  $A_1$  receptor in rat MSCs and 7F2 cells was low and that it increased markedly on differentiation to adipocytes.  $A_1$  mRNA expression increased respectively > 800 and 1600 fold as MSCs and 7F2 cells were induced to differentiate to adipocytes; this was accompanied by increases in protein expression. On the other hand when MSCs were differentiating to osteoblasts there was < 5 fold increase in  $A_1$  mRNA and no change in protein expression. The importance of the  $A_1$  receptor in adipogenesis was also supported by over expression experiments in 7F2 cells; this led to an upregulation of lipid accumulation and an increase in adipocyte marker expression.

Surprisingly this appeared not to be borne out in HOB cell differentiation to adipocytes, where the  $A_1$  receptor appeared to be reduced. These findings have to be treated with caution as only 1 - 2% of cells differentiated to adipocytes. In contrast, over 50% of the cells were found to be adipocytes



when 7F2 cells underwent adipogenesis. The importance of the A<sub>1</sub> receptor in adipocytes has been previously described by several groups (see section 1.6.3). Its overexpression in murine preadipocytes (ob1771) was shown to enhance adipogenesis and treatment with universal adenosine receptor agonists to increase corticosterone-induced differentiation of preadipocytes suggested that A<sub>1</sub> receptors are important for adipocyte differentiation (Børglum et al., 1996; Tatsis-Kotsidis and Erlanger, 1999).

It appears that adenosine can be important for both osteoblast and adipocyte differentiation. When MSCs were induced to differentiate to osteoblasts adenosine stimulated osteoblast marker expression and similarly it also stimulated adipocyte marker expression during induced adipogenesis. Transdifferentiation experiments in 7F2 and HOB cells also showed that adenosine receptors mediate adipogenesis in an adipogenic environment. Which cells dominate will depend on the nature of the microenvironment, the level of adenosine and the type of adenosine receptors expressed. It has also been previously reported that osteoblasts undergo a dedifferentiation step to a more primitive cell prior to transdifferentiation to a cell of a different lineage (Song and Tuan, 2004; Song et al., 2006); whether this occurs in the 7F2 and HOB cell adipogenic experiments is not known.

## 6.2 Future studies

The prime aim of these studies was to demonstrate the importance of adenosine receptor signalling pathways in the differentiation and function of osteoblasts and adipocytes and to identify putative targets which could be

considered for therapeutic intervention. My data thus far clearly show that A<sub>2B</sub> and A<sub>2A</sub> receptors are important for osteoblasts and A<sub>1</sub> receptors are important for adipogenesis.

Some concerns do however need to be noted, for example rat MSCs may behave differently to human MSCs and experiments *in vitro* may not replicate the *in vivo* situation. This is borne out by the studies of Siddappa et al (2008 and 2009) who showed that cAMP increased human osteoblast differentiation *in vitro* and bone formation *in vivo*, whereas in rodent cells, it inhibited ALP, osteocalcin, and collagen type I expression. In my experiments HOB cells, in comparison to murine 7F2 cells only, showed poor adipogenesis so these experiments need to be corroborated. The use of human primary cells, however, could also be problematic due to cell heterogeneity. There may well be differences in growth and differentiation capacity which is further compounded by the age of donors and the cell passage number. In addition cultures after induced differentiation still possessed multiple cell types which may make data interpretation difficult. This could perhaps be overcome by separating out the different cell types either by FACS analysis or magnetic bead separation.

Co-expression of several adenosine receptors in MSCs and osteoblasts make it difficult to determine precisely which receptor/s are involved in differentiation processes and cell specific function. Studies are also hampered by the lack of specific adenosine receptor agonists particularly those that target the A<sub>2B</sub> receptor. Specificity may also be lost when compounds are used at high concentration or during prolonged exposure which could lead to receptor

desensitisation. Thus as well as pharmacological intervention of receptor action a number of other experimental models should be considered, these include receptor overexpression and inhibition (siRNA) as well as gene knockout animals.

Some of my findings also need further clarification: although the  $A_1$  receptor seemed to be only lowly expressed in MSCs and 7F2 cells, incubation with the  $A_1$  receptor agonist CCPA markedly stimulated adipogenesis. In my experiments, a rather surprising finding was the observation that  $A_1$  receptor overexpression in 7F2 cells increased osteocalcin expression. These findings could relate to up or down regulation of other signal molecules, for example  $A_1$  receptors might mediate osteoblast mineralisation through activation of  $K^+$  channels (Henney et al., 2009).  $A_1$  receptors are also involved in lipolysis and lipogenesis (Dhalla et al., 2007b; Johansson et al., 2008; Vannucci et al., 1989) and this needs to be addressed in the context of adipogenesis of MSCs and osteoblasts.

It would also be interesting to assess the concentrations of adenosine produced in the microenvironment during cellular differentiation to either osteoblasts or adipocytes, and whether such concentration alone can influence differentiation or stimulate adenosine receptor expression. This may be particularly pertinent as CD73 appeared to increase, and ADA appeared to decrease, during adipogenesis of 7F2 cells.

The precise role of adenosine receptors in osteoblast or adipocyte differentiation needs to be clarified and this can be potentially addressed by using pure cell populations and if possible of human origin. In the context of osteoblastogenesis, adenosine receptor expression and function in osteoclasts should provide interesting information particularly since bone homeostasis requires a balance in the activities of osteoblasts and osteoclasts. Adenosine receptors have been shown to mediate the release of hormones, growth factors and cytokines from MSCs, adipocytes and osteoblasts (Hasko et al., 2009; Hasko et al., 2008). Such compounds could have both autocrine and paracrine effects and could affect both adipocytes and osteoblasts.  $A_1$  receptors mediate leptin secretion from adipocytes (Rice et al., 2000) and leptin has complex actions on bone, including stimulating differentiation and inducing apoptosis (see section 1.5.2). Expression of these molecules during osteoblast or adipocyte differentiation in response to adenosine receptor stimulation needs to be investigated.

### 6.3 Clinical importance

To date, targeting adenosine receptors for stimulating bone formation has not been considered. The data obtained in this study suggest that stimulation of  $A_2$  and inhibition of  $A_1$  receptors could potentially be useful for stimulating osteoblastogenesis. In terms of therapy involving adenosine receptors there are several compounds in clinical trials that act mainly at the  $A_{2A}$  and  $A_3$  receptors. Many of these compounds rely on their anti-inflammatory properties and aimed at a variety of diseases that include diabetes, cancer, psoriasis, asthma and RA. RA is particularly interesting since MTX, the most commonly

used anti-rheumatic drug induces increased levels of adenosine and a reduction in inflammation. Combination therapy of MTX and CF101 ( $A_3$  receptor antagonist) is more effective than either drug alone (Ochaion et al., 2006). The action of adenosine or which adenosine receptors are expressed in bone in the setting of RA is unknown.

Antagonism of the  $A_{2B}$  receptor has also been considered for diseases such as asthma, diabetes and inflammatory bowel disease. Targeting  $A_{2B}$  receptors is particularly challenging due to the lack of agonist selectivity; two compounds have been recently developed (LUF5835 and BAY 60-6583) that claim to show good specificity but there is no information available on their effectiveness in a clinical setting (Beukers et al., 2004; Chen et al., 2009). The use of adenosine receptor agonists and antagonists need to be approached with caution as receptors are widely distributed and mediate a diverse range of actions. For example the  $A_1$  receptor agonist, selodenoson, and the mixed  $A_1/A_{2A}$  agonist, AMP579, have been removed from clinical trials due to side effects (Jacobson and Gao, 2006). In spite of this the development of more specific agonists and antagonists for adenosine receptors by several pharmaceutical companies is still ongoing.

## **Chapter 7**

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