Functional Characterisation of Acetylcholinesterase in Bone

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Declaration

All the work submitted in this thesis has been performed by me (Colette Inkson) with acceptation to the in vitro and in vivo loading experiments (Chapter 4, thanks to Dr. Raj Grewal, and Dr. Alex Brabbs), generation of AChE transgenic mice (Chapter 5, thanks to Dr. Ellen Duysen and Professor Soreq), and DXA analysis of neonatal $AChE^{-/-}$ mice (Chapter 5, Thanks to Dr. Mark Perry). All works carried out by other persons has been with the purpose of further analysis to be carried out by myself.

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"Whatever you do will be insignificant, but it is very important that you do it."

Mahatma Gandhi

Chapter 1 :

Introduction

1.1 Introduction

1.1.1. The Skeleton

The skeleton is a complex organ that has many purposes. The framework of the skeleton comprises different bone structures connected together by tendons, ligaments and cartilaginous tissues allowing for movement and locomotion (Seeley et al., 1995). As a supportive role, many bones form protective structures around internal organs such as the brain, heart and lungs. In addition, the skeleton performs an essential role, along with the kidneys, in ion homeostasis (Lian et al., 1999). Marrow spaces within the long bones also provide a controlled environment for haematopoiesis to occur during postnatal life (Beresford, 1989). To be able to perform on many levels the skeleton as an organ is required to adapt and remodel in response to changes in its respective roles.

1.1.2. Bone

Bones are made up of two main types of structure, compact (also known as cortical bone), and cancellous bone (also known as trabecular bone). Compact bone makes up approximately 85% of the skeleton (Mundy, 1999). The structure of compact bone is dense, unporous, highly organised, found along the shafts of long bones (including the femur, tibia, radius and ulna), and forms the principal component of flat bones such as those of the skull. Compact bone contains very few interstitial spaces; those present are organised into osteons or haversian systems occupied by blood vessels or haversian/central canal. Cancellous bone makes up the remainder of the skeleton. Cancellous bone is arranged as a scaffold of trabeculae spanning the circumference of the epiphysis of long bones and vertebrae of the spinal column. The trabecular arrangement of cancellous bone assumes alignment in the direction of the major mechanical forces bones receive during locomotion and physical activity (Robey, 1992, Currey, 2002).

Unlike many other tissues, bone is composed largely of inorganic materials. Calcium and phosphorous or inorganic phosphate (Pi phosphate) combine together to form hydroxyapatite [$Ca_5(PO_4)_3OH$] providing approximately 65% of the bone material (Lian et al., 1999, and Boskey, 1992). Mineralised bone also contains a small amount of other ions such as magnesium, fluoride, carbonate, citrate and potassium (Boskey, 1992). A network of collagenous fibres, non-collagenous matrix

proteins and cells make up the organic component of bone constructing a framework for hydroxyapatite deposition. Collagen type I (col I) constitutes 90% of the collagenous material of bone, forming fibrils that are laid down in a distinctive direction (Boskey, 1992). Non-collagenous matrix proteins attach to these collagen fibres creating a protected environment for the initiation of hydroxyapatite crystal formation or a 'nucleation site' (Lian et al., 1999). Hydroxyapatite crystals then accumulate, aligning parallel to the orientation of the collagen fibres. In haversian systems, collagen fibres alternate in their orientation providing the highly organised lamella structure of compact bone (Lian et al., 1999). The structure, organisation, size, distribution and orientation of organic matrix proteins and hydroxyapatite can significantly affect the mechanical properties of bone.

1.1.3. The Bone Microenvironment

The bone microenvironment encompasses many different cell types that communicate and interact, mutually regulating their activity and function. Mesenchymal progenitor cells located in the bone marrow compartment give rise to preosteoblastic cells (Beresford, 1989). In response to systemic and local extracellular cues preosteoblast cells differentiate into mature bone-forming osteoblasts recognisable by their cuboidal appearance, elongated nucleus, prominent golgi body and rough endoplasmic-reticulum (ER) (Lian et al., 1999, Ducy et al., 2000 and refs therein). Osteoblasts are found at the bone surface and produce bone by expressing and secreting extracellular matrix and components required for matrix mineralisation. Fibroblast-like bone lining cells also occupy the bone surface. Compact bone tissue is populated with osteocytes which are numerous and widely distributed (Lian et al., 1999). Osteoclasts are the bone resorbing cells derived from a different cell lineage than osteoblasts. Mature multinucleated osteoclasts are polarised cells that secrete lysosomal enzymes and collagenases to break down bone making way for new bone formation by osteoblasts (Lian et al., 1999, Teitelgaum, 2000). Other cells such as megakaryocytes are also found in the bone microenvironment and may play a role in bone formation (Lian et al., 1999, Compston, 2002).

1.2 Osteoblasts

1.2.1. Defining osteoblasts

Accumulated evidence suggests osteoblast differentiation is a complex process involving numerous interacting factors and mechanisms, many of which are seemingly similar to those of other cell types (Ducy, 2000). The understanding and research of differentiation of other cell types has been facilitated by distinct changes in cell morphology and phenotype at specific stages of differentiation. However, osteoblasts are phenotypically very similar to fibroblasts both in vitro and in vivo. There are very few genes exclusively expressed by osteoblasts, so far making the characterisation of osteoblast differentiation very difficult. The only defining attribute of an osteoblast is the ability of mature terminally differentiated osteoblasts to produce a mineralised bone matrix (Ducy, 2000, Karsenty, 2001).

Despite an extensive search for osteoblast specific factors, only osteocalcin and Cbfa1 have been identified with expression or function that is limited to the osteoblast lineage (Ducy, 2000, Karsenty, 2000, Karsenty, 2001). Osteocalcin is secreted by osteoblasts, accounting for a large percentage (approximately 20%) of the non-collagenous component of the bone matrix (Lian et al., 1999). Within the 6 kDa of the osteocalcin protein, 3 gamma-carboxyglutamic acid (Gla) sites with high affinity to calcium are contained, enabling 2-3 moles of calcium per mole of protein to be bound (Young et al., 1992, Boskey, 1992). There is also a site for apatite binding that is dependent upon calcium binding (Young et al., 1992, Boskey, 1992). The expression of osteocalcin appears late in osteoblast differentiation, expression limited to the mature terminally differentiated osteoblasts (Lian et al., 1999, Karsenty, 2000, Ducy et al., 2000 and refs therein). The expression profile of osteocalcin indicates a bone specific role, restricted to osteoblasts at sites of bone formation. The exact function of osteocalcin in bone is unknown, however evidence suggests that it acts to inhibit osteoblast differentiation, and reduce the mineralisation process by binding calcium and phosphate, sequestering them from interactions required for hydroxyapatite production and matrix mineralisation (Young, et al., 1992, Boskey, 1992, Lian et al., 1999).

Examination of the promoter region of the osteocalcin gene identified two osteoblast specific elements (OSE 1 and 2) that were subsequently linked with the transcription factor Core binding factor alpha 1 (Cbfa1) (Ducy and Karsenty, 1995,

Ducy et al., 1996, for review see Karsenty, 2000). Cbfa1, also known as acute myeloid leukaemia factor 3 (AML3), polymavirus enhancer binding protein 2αA (PEBP2αA), and runt related gene 2 (Runx2), is a transcription factor of the runt family, related to a drosophilia transcription factor that binds to DNA and activates transcription via a conserved domain of 128 amino acids known as the runt domain (Komori and Kishimoto, 1998, Westendorf and Hiebet, 1999, Franceschi, 1999, Komori, 2002). The runt family of transcription factors function as a heterodimeric complex of alpha (Cbf**a**) and beta (Cbf**b**) subunits, both of which are required for activation of transcription (Yoshida et al., 2002, Kundu et al., 2002). Cbfb is a small protein with no DNA binding or transcriptional ability of its own; rather it induces an increased affinity of the runt domain of Cbfa for DNA. Apart from the runt domain, other sites at the N and C-terminus of the Cbfa1 gene product are important in function. The N-terminus contains a polyglutamine and polyalanine repeat region that acts as an activation domain. The C-terminus also contains important activation, repression, and nuclear localisation signals within a stretch of proline, serine, and threonine (PST) residues. Members of the runt family are established regulators of lineage differentiation, e.g. Cbfa2 (also AML1, PEPBα1A and Runx1) is well characterised as a critical element in haematopoiesis, the most common cause of acute myeloid leukaemia occurring via translocation of the Cbfa2 gene locus (Robin et al., 2003). Although not exclusively expressed by osteoblasts, the expression of Cbfa1 displays a very specific pattern relating to that of embryonic bone formation and mature osteoblast function.

Characterisation of Cbfa1 expression during embryonic development revealed that regardless of embryonic origin, Cbfa1 precedes the onset of osteogenesis, and is expressed in all cells destined to become osteoblasts and chondrocytes (Karsenty, 2000, Ducy, 2000). At the onset of osteogenesis, Cbfa1 expression becomes limited to osteoblasts, with expression in cartilaginous tissues rapidly decreasing (Karsenty, 2000, Ducy, 2000). Although homozygous mutants of Cbfa1 produce a lethal phenotype, analysis of the skeletons revealed a complete lack of mature osteoblasts and bone ossification, leading to a normally patterned skeleton made of only cartilaginous elements, and incomplete intramembranous bones (Otto et al., 1997, Lee et al., 1997). The phenotype of Cbfa1 haploinsufficiency as observed in Cbfa1 heterozygous mice is also comparable to the autosomal dominant

human bone disorder Cleidodocranial dysplasia (CCD) that is characterised by hypoplastic clavicles, patent fontanelles and sutures of the skull (Lee et al., 1997, Mundlos et al., 1997, Mundlos, 1999). Numerous clinical presentations of CCD have now been linked to mutations in the Runx2 allele (Yokozeki et al., 2000, Golan et al., 2000, Giannotti et al., 2000, Quack et al., 1999). In adult life, all pre-osteoblasts and osteoblasts express Cbfa1 (Ducy, 2000). In vitro studies demonstrated activation of the osteocalcin gene by Cbfa1 via the OSE 1 and 2 sites, and in doing so controlling osteoblast differentiation (Karsenty et al., 2000). It is clear that Cbfa1 plays an essential role in osteogenesis; factors that influence the regulation of Cbfa1 expression and function are therefore important regulators of osteoblast differentiation.

1.2.2. Osteoblastogenesis

Although only osteocalcin and Cbfa1 have proved to be predominantly osteoblast or bone specific in expression and function, multiple other factors control osteoblast differentiation and therefore osteogenesis. Cells of the osteoblast lineage are derived from a primitive mesenchymal progenitor cell that has multi-lineage potential, generating adipocytes, myoblasts, bone marrow stromal cells, and chondroblasts as well as osteoblasts (Caplan and Brunder, 2001). Osteoblastogenesis occurs under sequential expression of multiple transcription factors, matrix proteins, and induction by a variety of growth factors and signalling systems. The most recent description of osteoblast differentiation defines mesenchymal stem cells that differentiate into osteoprogenitor and pre-osteoblastic cells (Lian et al., 1999, Aubin, et al., 2001, and refs therein). Proliferation of these populations and the production of a type I collagenous matrix is followed by cessation of the cell cycle which induces these cells to differentiate further into mature matrix synthesising osteoblasts (See figure 1.1). At each stage in differentiation, transcriptional control, growth factors and matrix composition can influence the further progression of osteoblast differentiation and function.

1.2.3. Transcriptional Control

As already outlined, mesenchymal cells destined to become osteoblasts express high levels of Cbfa1. Transcriptional control of cell proliferation by transcription factors

Origional in colour

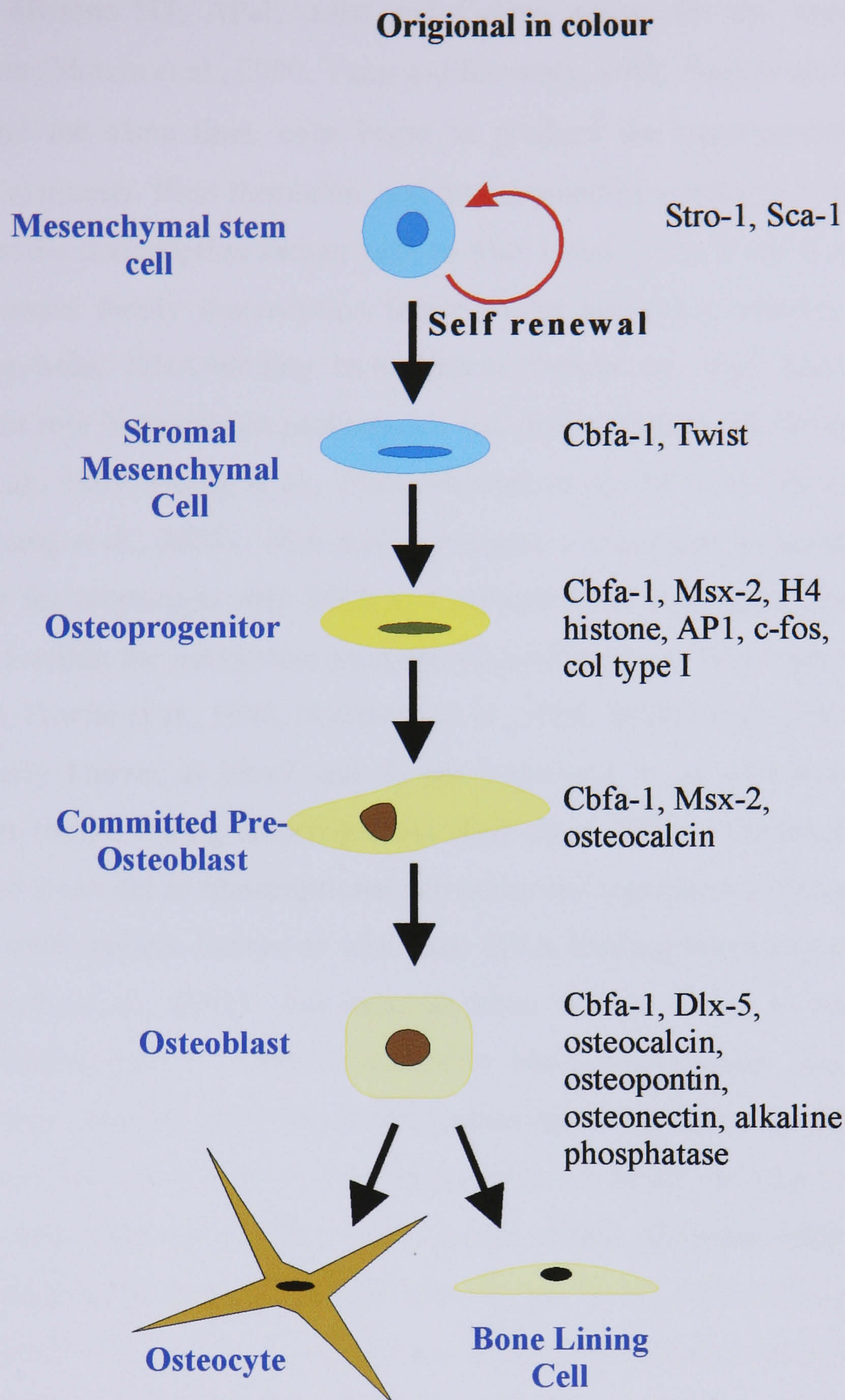

Figure 1.1
Osteoblastogenesis
Osteoprogenitor cells derived from mesenchymal stem cells express the osteoblast defining transcription factor Cbfa1. Proliferation induced by factors such as Msx-2, AP1 and c-fos creates a population of committed pre-osteoblast cells. Synthesis of a collagenous matrix and other transcription factors induce pre-osteoblasts to mature and produce bone matrix proteins such as osteocalcin, osteonectin and osteopontin. Mature osteoblasts produce a mineralised matrix. Osteoblasts that become encapsulated within the bone become osteocytes, and others lie dormant on the cell surface (bone lining cells).

such as Histone H4, AP-1, c-fos and C-myc allows for the expansion of this population (Marzia et al., 2000, Yang and Karsenty, 2002, Harada and Rodan, 2003). At around the same time, cells begin to produce the αpro-peptide required for collagen synthesis, fibril formation, and arrangement of a collagenous matrix. Other more specific transcription factors such as Msx 1 and 2, Dlx 5 and 6 members of the homeodomain family transcription factors, twist and other members of the basic helix-loop-helix DNA-binding transcription factors are also known to play a significant role in osteoblast proliferation and differentiation (Hoffmann et al., 1994, Ryoo et al., 1997, Dodig et al., 1999, Miyama et al., 1999, Orestes-Cardoso et al., 2002, Cheng et al., 2003). Msx proteins require a consensus homeodomain binding sequence for interaction with DNA and activation of target genes, which has been identified within the osteocalcin promoter (as well as in the Wnt-1 gene) (Hoffman et al., 1994, Towler et al., 1994, Hoffmann et al., 1996, Willert et al., 2002). Msx 1 and 2 (formerly known as Hox7 and 8) are expressed in an overlapping pattern in numerous tissues during embryogenesis (Davidson, 1995). Evidence suggests that Msx1 and 2 can act as transcriptional activators and repressors of target genes acting through other protein factors as well their DNA binding homeodomain (Davidson, 1995, Sasaki et al., 2002). For example Msx1 has the ability to interact with the TATA-binding protein (TBP) to initiate or block transcription (Davidson, 1995). Msx proteins play an early role in the patterning of the skeleton and expansion of early osteoblast populations. Over expression of Msx2 in vitro was shown to suppress osteoblast differentiation. In support of this, antisense inhibition of Msx2 expression induced osteoblast differentiation (Liu et al., 1999, Dodig et al., 1999). Mutations of Msx 1 and 2 in humans are responsible for skeletal disorders such as craniosynostosis, and pariteal foramina, thought to reflect their role in cell proliferation within this skeletal region (Wilkie, 1997, Cohen, 2000). Deletion of the Bpx gene induces a similar defect in osteoblast proliferation during the development of the axial skeleton. This suggests that besides Cbfa1, which is expressed in all skeletal elements, transcriptional control by other factors may be specific to the different regions of the skeleton and the type of ossification that will occur.

Dlx6 and 5 also display overlapping patterns of expression and play a significant role in craniofacial development, tooth formation and limb patterning (Robledo et al., 2002). Dlx5 is expressed in all mature osteoblast cells, expression

levels increasing as cells becoming more differentiated. Dlx5 expression in vitro enhances osteoblast differentiation, inducing matrix production and mineralisation, and controlling expression of other transcription factors such as Msx and Cbfa1 (Shirakabe et al., 2001, Tadic, 2002, Lee et al., 2003). Most recently a reciprocal regulation of Cbfa1, Msx2 and Dlx5 has been described. Firstly, Msx and Dlx proteins can form heterodimeric complexes via their homeodomain DNA binding domains, antagonising the action of these transcription factors (Zhang et al., 1997, Newberry et al., 1998). It is thought that the level of expression of each protein can therefore more readily regulate downstream factors. As Dlx5 is expressed after Msx2 during osteoblast differentiation, induction of Dlx5 expression could regulate Msx2 activity and target gene expression. Secondly, it was identified that that Msx2 can bind to Cbfa1 and repress its activity; therefore binding of Dlx5 to Msx2 could elevate the repressive action, regulating activity of both Cbfa1 and Msx2, as well inducing its own transcription (Shirakabe et al., 2001). Most specifically a mutation of Msx2, which prevents Dlx5 binding, can induce Boston-type craniosynostosis (Shirakabe et al., 2001).

1.2.4. Growth factor control

Multiple signalling systems are established in bone that can affect the different stages of osteoblast differentiation by acting upon the transcriptional control of each stage, and therefore can exert multiple effects via down stream consequences on gene expression. Members of the transforming growth factor beta (TGFβ) superfamily including the Bone morphogenic proteins (BMP) BMP2 and 4, and TGFβ-1 and 2 have been implicated in influencing expression and function of Cbfa1, Msx1 and 2, and Dlx5 via direct interaction with Smads (Chen et al., 1998, Hoffmann and Gross, 2001, Valcourt et al., 2002). Smads are transcriptional regulatory proteins that can directly bind to the intracellular components of the TGFβ superfamily receptors, and to transcription factors to mediate their actions (Wrana, 2000, Miyazono et al., 2001). Consequently, effects on osteoblast synthesis and secretion of bone matrix proteins, and expression of cell surface integrins have been demonstrated by TGF-β super-family growth factor treatment (Harris et al., 1994, Horner et al., 1998). Fibroblast growth factor (FGFs) signalling, a known regulator of bone formation and patterning, can also effect and regulate Cbfa1, Msx1 and 2 and Dlx5 activity (Pitaru et al., 1993,

Goldfarb et al., 1996, Galzie et al., 1997, Debi et al., 1998, Scutt and Bertrom, 1999, McIntosh et al., 2000, Mansukhani et al., 2000, Ornitz and Marie, 2002). Multiple other hormonal and growth factor signalling systems are involved in regulating osteoblast function and differentiation (e.g. PTH, oestrogen, Vitamin D, IGF, Wnt), effects of specific growth factor signalling systems will be discussed further in Chapter 4.

1.2.5. Extracellular matrix proteins and osteoblast differentiation

The runt domain of Cbfa1 that was found to selectively bind to the OSE elements of the osteocalcin promoter, has subsequently been identified in the genes of many other proteins already known to play significant roles in osteoblast differentiation including type I collagen, osteonectin, and osteopontin (Sato et al., 1998, Tsuji et al., 1998, Thirunavukkarasu et al., 2000, Tyson et al., 2003). Although it is known that type I collagen provides the framework for bone formation, the precise function that non-collagenous proteins play during bone development is not fully understood. It is believed that in addition to a structural role, the temporal and spatial expression of many non-collagenous proteins is instrumental in regulating osteoblast activity, and therefore critically important in the maintenance of bone mass. In particular, osteoblast differentiation is heavily reliant upon the cell-matrix interactions mediated by these non-collagenous proteins. As already discussed, osteocalcin is the most abundantly expressed non-collagenous bone matrix protein. However, its expression appears late in osteoblast differentiation. Earlier during osteoblast differentiation and matrix production other proteins such as osteopontin, osteonectin, and thrombospondin are expressed (Ducy, 1999). Although bone matrix proteins were originally thought to simply play a structural role in bone formation and mineralisation, the significant effects upon osteoblast adhesion, migration and chemotaxis, and the subsequent effects upon osteoblast function and differentiation demonstrate the complexity between cells and the extracellular environment in which they reside. The roles of specific extracellular matrix proteins in bone will be discussed further in a later chapter (see section 3.1.2.1).

1.3 Bone remodelling

1.3.1 Maintenance of bone

As the main support for the body in movement and locomotion, bone is put under a great amount of stress and strain. It is therefore essential that bone maintain its strength and mass to minimise the risk of fracture. To sustain a healthy skeleton, bone is continuously remodelled. Old or damaged bone is resorbed by osteoclasts followed by new bone formation by osteoblasts. Bone resorption and formation are tightly coupled to ensure the maintenance of bone mass; disruption of this remodelling cycle can lead to skeletal disorders such as osteoporosis.

1.3.2 The ARF sequence

The activities of osteoclasts and osteoblasts are closely regulated to ensure appropriate bone turnover. At sites of remodelling, resorption and formation follow a consecutive arrangement known as the **A**ctivation – **R**esorption – **F**ormation or **ARF** sequence. ***Activation*** of osteoclasts is followed by osteoclast adhering to the bone surface and bone ***Resorption*** occurring (Baron, 1999). The removal or 'Reversal' of osteoclasts from the bone surface is followed by macrophage/monocyte-like cells sealing the resorbed surface with a 'cement-line', marking the end of bone resorption. This cement line acts to bind the old bone with the new bone during bone ***Formation*** by osteoblasts as they fill in the resorbed area with new bone (see figure 1.2). Remodelling of cancellous bone follows the ARF sequence with sites of remodelling occurring on the trabecular bone surfaces. Remodelling of compact bone is however more complex. Remodelling occurs in packets within the bone called haversian systems or osteons, which are recognisable by layers of bone surrounding a blood vessel or interstitial space (Baron, 1999, Burr, 2002). At these sites of remodelling, osteoclasts resorb a long narrow canal that is subsequently occupied by invading blood vessels and filled in with layers of new bone formed by osteoblasts (see figure 1.3).

1.3.3 Osteoclasts and bone resorption

Bone resorption occurs at discrete sites on the endosteal surface of bones or within haversian systems (Baron, 1999, Currey, 2002). The exact stimulus that initiates bone resorption is largely unknown. However a number of different factors are

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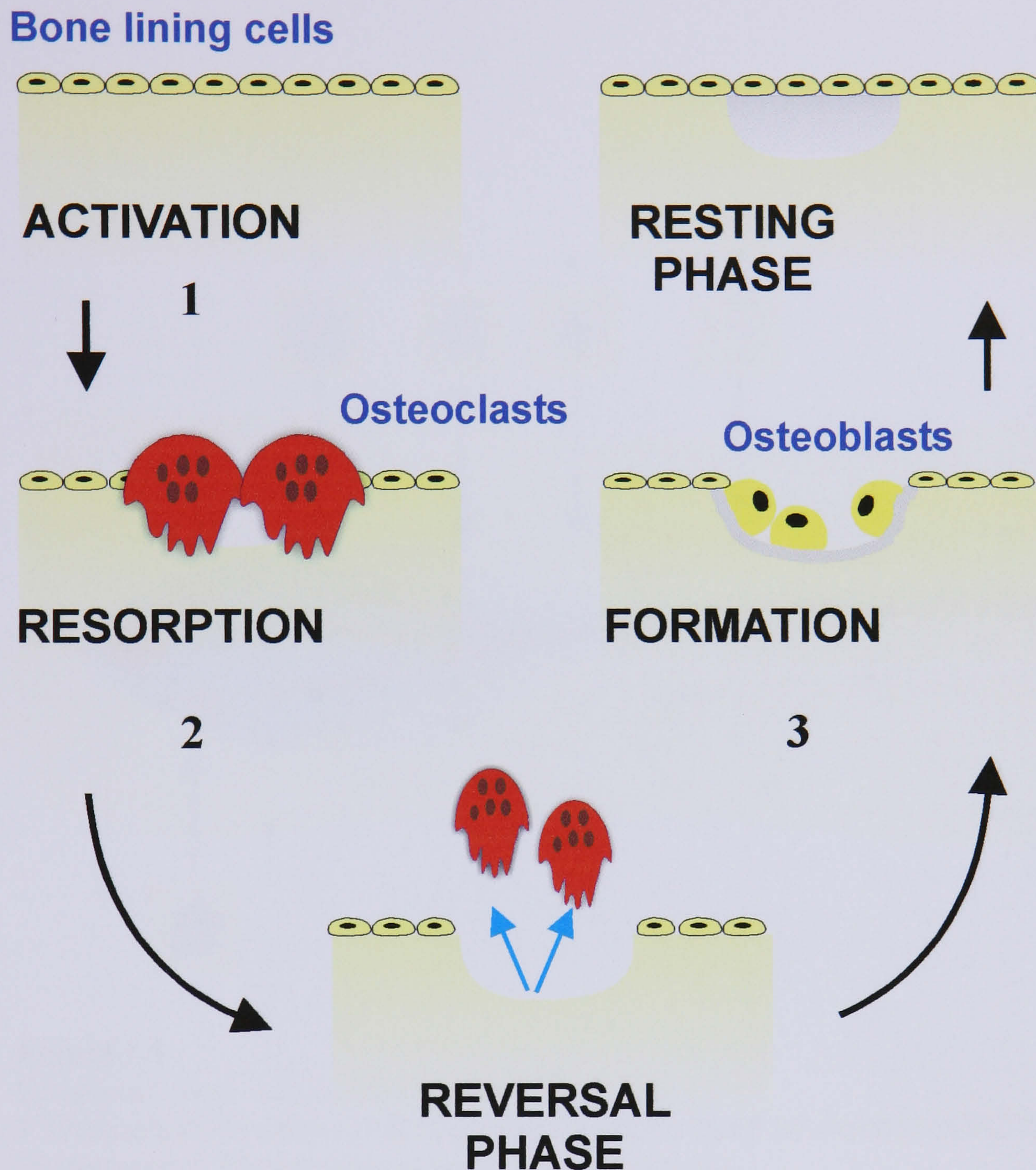

Figure 1.2
The ARF sequence
1. **Activation** - osteoclasts become activated and are recruited to dormant bone surafces

2. **Resorption** - Osteoclasts bind to and resorb the bone surface.

Following removal of osteoclasts from the bone surface the zone of resorption is sealed with cement line.

3. **Formation** - Osteoblasts invade the area and fill in the resorped region with new bone.

(Diagram redrawn and modified from Baron, 1999, Primer on the metabolic bone diseases and Disorders of Mineral Metabolism, 4th edition)

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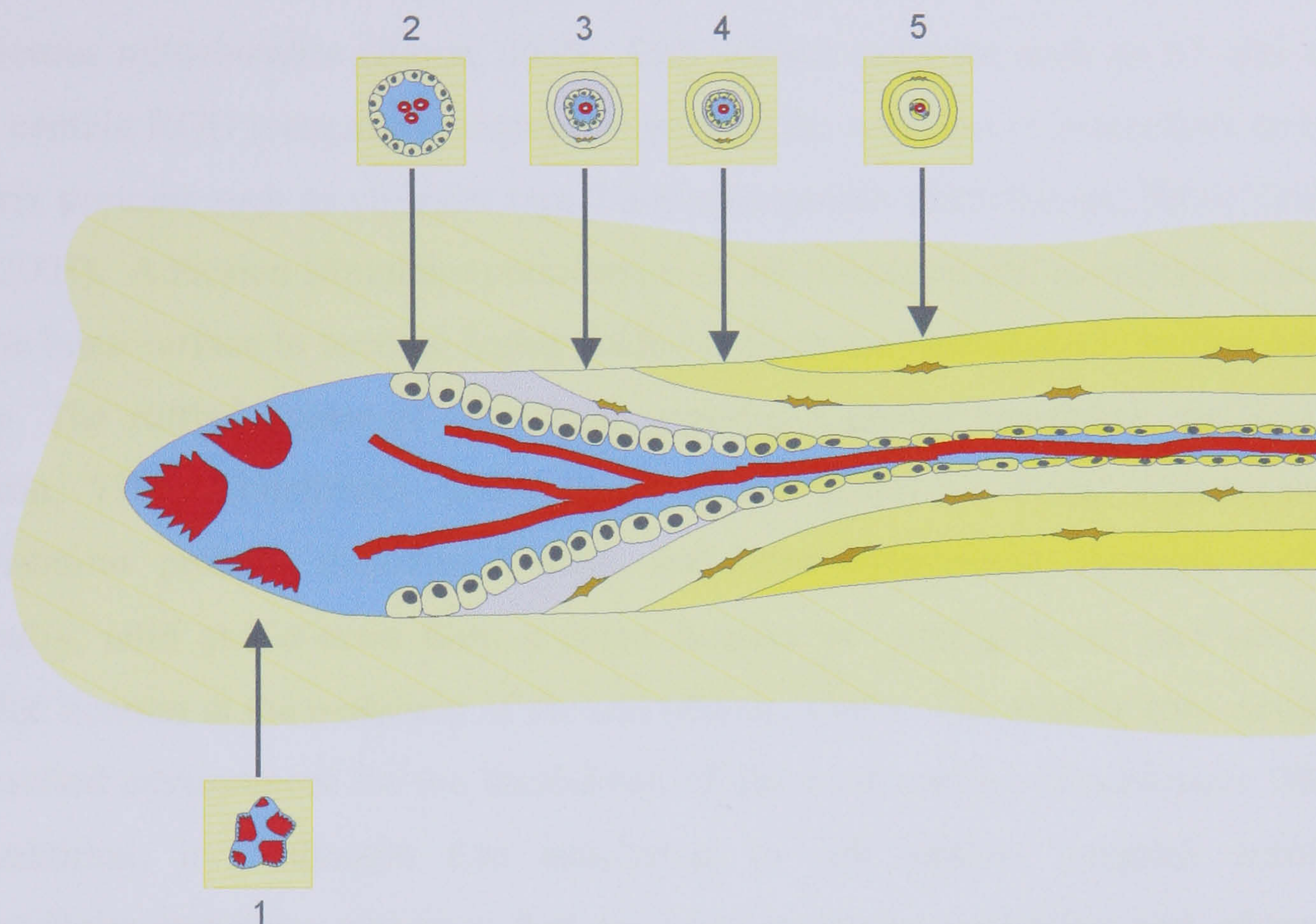

Figure 1.3
Cross section of a haversian system
1. Osteoclasts resorb a canal of cortical bone, the front of which is called the 'cutting cone'. The canal is invaded by blood vessels.
2. Osteoprogenitor cells populate the resorbed area and begin to differentiate into osteoblasts.
3-5. Osteoblasts form layers of bone filling in the resorbed area.

known to stimulate osteoclast progenitors cells to differentiate into mature osteoclasts (Teitelbaum, 2000, Zaidi et al., 2003). Osteoclasts are highly specialised cells that originate from mononuclear cells of the haematopoietic lineage, most specifically the monocyte/macrophage lineage (Teitelbaum, 2000, Zaidi et al., 2003). Mature osteoclasts are giant multinucleated cells, possessing up to 20 nuclei per cell, which are found in contact with the bone surface in resorptive pits called Howship's lacunae (Baron, 1999). The resorptive nature of osteoclasts necessitates these cells to contain abundant active Golgi complexes, vesicular transport systems and numerous mitochondria (Baron, 1999). Cell surface integrins such as $\beta 1$ and $\alpha_v\beta_3$ that contain RGD recognition sequences mediate the adhesion of osteoclasts to bone matrix proteins such as collagen type I and osteopontin (Teitelbaum, 2000, Zaidi et al., 2003). Adhesion stimulates polarisation of the osteoclast cell membrane apposed to the bone surface to become highly folded to form the characteristic ruffled border zone. The ruffled border of osteoclasts provides a greater resorptive surface area (Baron, 1999, Teitelbaum, 2000, Zaidi et al., 2003). Focal expression of filamentous proteins including F-actin, and other cytoskeletal elements such as vinculin, talin and α-actin form a dense boarder or 'sealing zone' that seals the ruffled boarder at the periphery of the cell (Baron, 1999). The sealing zone creates a controlled environment for the breakdown of the bone surface (Teitelbaum, 2000). In addition, it is thought that attachment to cell surface integrins activates intracellular-signalling pathways that stimulate synthesis, packaging and release of secretion enzymes from the ruffled border (Baron, 1999). Targeting of vesicles to the ruffled boarder is regulated by the mannose-6-phosphate receptor (Zaidi et al., 2003). Proton pumps such as the H^+ -adenosine triphosphate (ATP) are charged coupled to Cl^- channels in the apical cellular membrane enabling the development of an acidic microenvironment between the bone surface and the resorptive surface of the cell (Zaidi et al., 2003). Cl^-/HCO^-_3 and Na^+/Ca^{2+} exchangers found on the distal pole of the cell, maintain a neutralised intracellular osteoclast pH. Vesicular release of acidic lysosomal enzymes such as tartrate resistant acid phosphatase, cathespin K, and matrix metalloproteinases (MMPs) is essential in the degradation process (Baron, 1999, Teitelbaum, 2000, Zaidi et al., 2003). These proteins allow the release of hydroxyapatite crystals from their immobilised position in the extracellular matrix and the acidic environment enables their dissolution. Once the mineral component of

bone has been removed, collagenases and other MMPs break up and remove the organic component. The degraded bone matrix is phagocytosed by the osteoclasts and transported to the distal pole of the cell surface for release (Mostov and Werb, 1997, Baron, 1999). The exact mechanism that controls the level of bone resorption is unclear, but it is thought that high calcium levels in the Howship's lacunae produced by demineralisation initiate breakdown of the sealing zone and release of the osteoclast from the bone matrix terminating that phase of bone resorption (Baron, 1999).

1.3.4 Osteoblasts, bone formation and mineralisation

In mature, fully developed bone, the formation of new bone tissue occurs at sites of resorption or repair. The recruitment of preosteoblasts to the area is believed to be affected by local release of growth factors, cytokines and chemoattractants such as transforming growth factors (TGF-βs), fibroblast growth factors (FGFs), and bone morphogenic proteins (BMPs) entrapped in the bone matrix and released during resorption (Baron, 1999, Mundy, 1999, Ducy, Schinke and Karsenty, 2000). These factors also stimulate preosteoblasts to proliferate and populate the area, inducing the expression of cell surface adhesion molecules and promoting the differentiation into osteoblasts (Baron, 1999, Mundy, 1999, Ducy, Schinke and Karsenty, 2000, Harada and Rodan, 2003). Secretion of collagens such as collagen type I forms the basis of the matrix in a material known as osteoid, visibly different from the bone matrix in histological sections of bone (Boskey, 1992, Young et al., 1992, Baron, 1999). Adhesion of osteoblasts to the osteoid, or contact with circulating growth factors initiates cell-signalling cascades that activate genes causing cell differentiation and production of other non-collagenous matrix proteins (see section 1.2). The collagenous-matrix binds and incorporates the non-collagenous matrix proteins, filling the spaces between the collagen fibrils (Young et al., 1992, Boskey, 1992, Robey, 1996, Baron, 1999). As the matrix matures, mineralisation occurs at discrete sites. Extracellular matrix vesicles (ECM vesicles) released by osteoblasts aid mineralisation by concentrating calcium and phosphate ions, inhibitors of mineral catabolising enzymes, and acidic phospholipids providing a protective environment for hydroxyapatite formation (Boskey, 1992, Anderson, 1995, Baron, 1999). The site of initial mineralisation occurs with the formation of a small crystal or 'critical

nucleus'. After 'nucleation', subsequent crystal growth and expansion arises from this nucleus, crystals of each discrete nucleus eventually coming together to form a uniform mineral structure (Boskey, 1992, Baron, 1999).

As osteoblasts form bone some of these cells become trapped in the matrix, forming cells know as osteocytes that occupy fluid filled spaces in the bone called lacunae (Lian et al., 1999, Burger et al., 1999, Noble and Reeve, 2000). Large process extensions or canaliculi allow for the communication between newly embedded osteocytes with others embedded deeper in the bone, or cells at the endosteal bone surface such as osteoblasts (Turner and Forwood, 1995, Lian et al., 1999, Burger et al., 1999, Noble and Reeve, 2000). Osteocytes are thought to play an important role in the regulation of bone in its response to mechanical loads and systemic hormones (Burger et al., 1999). Gap junctions between the canaliculi of osteocytes and others cells provide for a network of communication between deeply embedded cells and bone surface (Turner and Forwood, 1995, Noble and Reeve, 2000). Osteocytes are also thought to be highly responsive to the mechanical strains received during locomotion and exercise (Turner and Forwood, 1995, Noble and Reeve, 2000). Although they cannot undergo replication, osteocytes remain active for the life span of the bone in which they reside (Lian et al., 1999). Other osteoblasts cease to be active and lay dormant on the bone surface to become fibroblast-like cells known as bone lining cells (Lian et al., 1999, Parfitt, 2001). However, the conclusion of active bone formation results in most osteoblasts undergoing apoptosis (Hock et al., 2001).

1.4 Osteogenesis

1.4.1 Embryonic Bone Development

The embryonic development of the skeleton is a complex process still not yet fully understood. Patterning of the embryo designates stem cells to specific regions of the developing body, producing all tissue types from one primitive stem cell type. However in man, by the late gastrula stage (around 14-17 days post coitus (dpc)) specific regions of the embryo have become committed to different lineages of cells and tissue (Raven and Johnson, 1990, Olsen, 1999). The skeleton is derived from three different cell lineages - the neural crest, sclerotome, and lateral plate mesoderm (Olsen, 1999). Cells derived from these regions condense and differentiate into

mesenchymal cells that form the pattern of the future skeleton. Neural crest cells, derivatives of the brachial arch, form the craniofacial skeleton (Olsen, 1999, Opperman, 2000). The sclerotome division of somites condense to form part of the axial skeleton (Olsen, 1999). The remaining skeletal elements of the limbs arise from the lateral plate mesoderm (Olsen, 1999). The developed mesenchymal pattern of the skeleton then undergoes further patterning and differentiation forming the shape of future bones. At this point bone development can occur via two processes - intramembraneous ossification, forming the flat bones such as those of the skull, or endochondral ossification to develop the long bones of the limbs and the ribs. The distinct difference between these two processes is in the differentiation of the mesenchymal skeletal pattern. During intramembraneous ossification, mesenchymal cells differentiate directly into osteoblasts (Seeley et al., 1995, Baron, 1999). However during endochondral ossification, mesenchymal condensations first differentiate to form cartilaginous elements that are then mineralised and replaced by osteoblasts and bone (Seeley et al., 1995, Baron, 1999, Kronenberg, 2003).

1.4.2 Intramembraneous Ossification

The development of flat bones such as those of the calvarium of the skull and the scapula requires no intermediate cartilaginous phase in their formation (Seeley et al., 1995). Cells derived from the neural crest, migrate, aggregate, condense and differentiate into mesenchymal structures that resemble the future skeletal elements (Olsen, 1999, Opperman, 2000). This process induces further differentiation through cell-cell contact leading to activated signalling systems that can determine future patterning, cell fate and activation of genes related to the structures involved (Raven and Johnson, 1990). Progenitor cells are found in the mesenchymal precursor tissue, and these cells differentiate into mature matrix synthesising osteoblasts (Opperman, 2000). During the first phase of intramembraneous ossification, osteoblasts produce bone in a disordered manner, with a poorly aligned collagenous osteoid leading to the production of delicate network made up of woven bone that spans the area previously occupied by mesenchymal tissue (Seeley et al., 1995, Olsen, 1999). Ossification occurs at discrete central sites called ossification centres. As bone develops, osteoblasts secrete a matrix of collagenous and non-collagenous matrix proteins that are subsequently mineralised. Remodelling of this mineralised matrix occurs by osteoclasts brought to the area by local blood vessels and those that begin

to invade the matrix bringing cells that will eventually form the haematopoietic bone marrow (Seeley et al., 1995). Osteoblasts replace the resorbed area with the more organised lamella bone, joining together the trabeculae structures into an organised network of trabecular bone. Periosteal cells lining the mineralised matrix also aggregate and specialise to form the periosteum, some cells of which differentiate into osteoblasts that produce a more organised mineralised matrix made of compact bone, creating a bone collar surrounding the trabecular network (Seeley et al., 1995, Olsen, 1999). The centres of ossification enlarge and gradually generate the marrow cavity. Areas of mesenchyme that are covered with a thin epithelial membrane known as sutures or fontanelles are interspaced between the bones of the skull (Opperman, 2000). Sutures are essential for the co-ordination of neural and skeletal elements both during embryonic and postnatal development, and although most sutures are almost closed at birth some remain patent during the first years of life to allow for further growth of the skull (Opperman, 2000). Suture patency is under strict regulation and is a complex process of its own relevance that will be discussed in depth in a later chapter (see section 5.1.1 chapter 5).

1.4.3 Endochondral Ossification

Endochondral ossification is the process by which the majority of the skeleton is developed. Mesenchymal tissue derived from the sclerotome condenses and aggregates to form the pattern of the future elements of the axial skeleton (bones of the thoracic cage, vertebral column, and pelvis) (Seeley et al., 1995, Olsen, 1999, Gilbert, 2000, Wolpert, 2002, Kronenberg, 2003). Limb buds shaped from cells derived from the lateral plate mesoderm, extend posterior to an epithelial layer of cells the apical ectodermal ridge (AER). As the AER expands, the interior limb tissue undergoes further patterning events to form the outline of the future limb bones (humerus, ulna, and radius of the upper limbs, femur, tibia and fibula of lower limbs, metacarpals and phalanges of the hand, and metatarsals of the feet) (Seeley et al., 1995, Olsen, 1999, Kronenberg, 2003).

Mesenchymal condensations differentiate into chondroblasts creating cartilaginous elements or models in the shape of the future bones. Chondroblasts of this primitive bone model then mature, secreting a primary matrix of type I and III collagen, continuing to proliferate expanding the width, length and shape of the bone by interstitial and appositional growth (Kronenberg, 2003). A mesenchymal layer or

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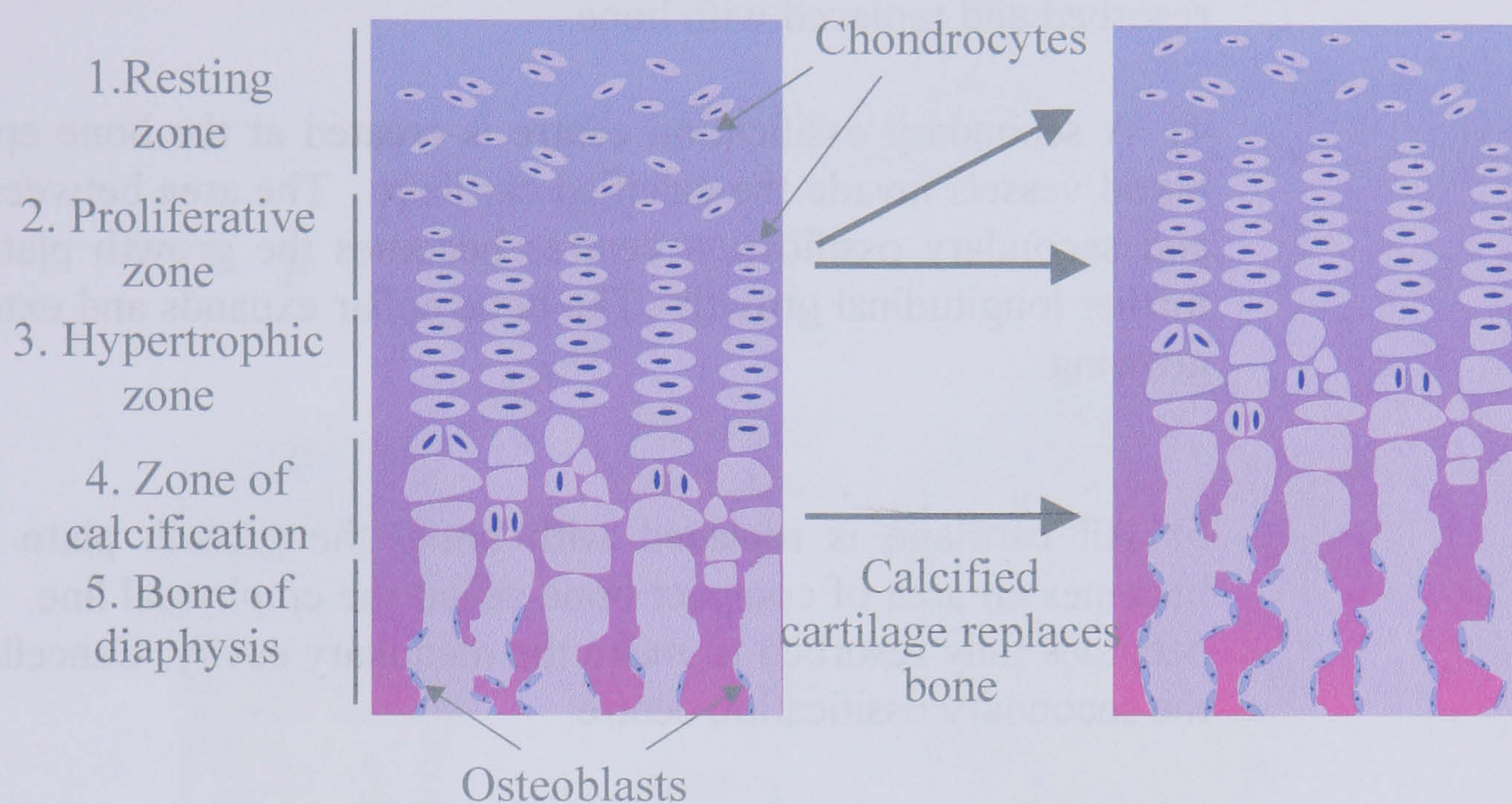
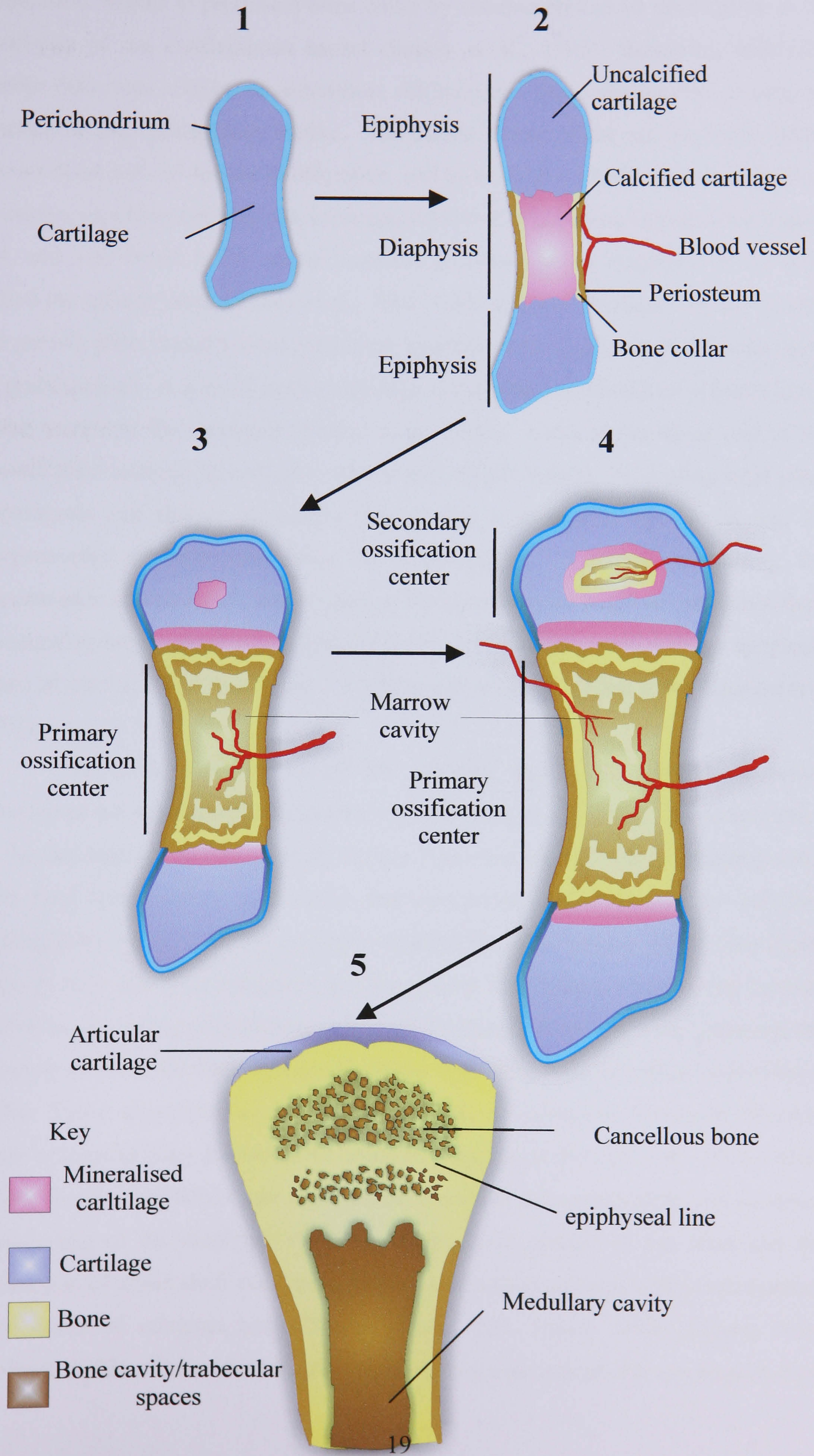


Figure 1.4
The growth plate
The cartilage anlagen of future bones form distinct zones of cells known as the growth plate that allows for bone growth .
1. Zone of resting cells
2. Chondrocytes become active and proliferate.
3. Chondrocytes enlarge, secrete a collagenous matrix and form columns of cells called isogenous groups.
4. Chondrocytes mineralise their matrix and undergo apoptosis.
5. Cartilagenous tissue is replaced by bone.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1
2
Perichondrium
Cartilage
Epiphysis
Uncalcified cartilage
Calcified cartilage
Diaphysis
Blood vessel
Periosteum
Bone collar
Epiphysis
3
4
Secondary ossification center
Marrow cavity
Primary ossification center
Primary ossification center
5
Articular cartilage
Key
Mineralised carltilage
Cartilage
Bone
Bone cavity/trabecular spaces
Cancellous bone
epiphyseal line
Medullary cavity

perichondrium contouring the primitive bone, and containing an osteoprogenitor population, begins to produce a bone collar by intramembraneous ossification at the diaphysis of the cartilaginous model (Seeley et al., 1995). Becoming imbedded within their own matrix, chondroblasts differentiate into chondrocytes, occupying lacunae of the cartilaginous matrix. The elastic nature of the cartilaginous matrix allows these cells to remain proliferative, and to do so in a highly organised fashion. Chondrocytes form columns known as growth cones or isogenous groups (see figures 1.4. and 1.5) found in the region from the epiphysis to the diaphysis of the bone called the growth plate (Seeley et al., 1995, Gilbert, 2000, Wolpert, 2002). Within the growth plate, chondrocytes proliferate upwards to the epiphysis of the bone (zone of proliferation). A zone of resting cartilage, containing chondroblasts is found at the point nearest to the epiphysis (Seeley et al., 1995). Cells at the distal end of the growth plate undergo hypertrophy, where cells rapidly enlarge, presenting large golgi apparatuses and change the matrix they secrete to collagens type II, IX and XI (hypertrophic chondrocytes, zone of hypertrophy). Lacunae containing the hypertrophic chondrocytes also enlarge as the chondrocytes begin to mineralise their surrounding matrix at the metaphysis of the bone before these cell undergo apoptosis (zone of calcification) (see figure 1.4) (Seeley et al., 1995, Olsen 1999, Kronenberg, 2003).

Osteoclasts begin to resorb the calcified matrix allowing blood vessel invasion of the perichondrium and periosteum (Seeley et al., 1995). Vascularisation of the cartilaginous model provides further osteoclast precursor and osteoprogenitor cells (and bone marrow precursors) that commence remodelling of the calcified cartilaginous matrix creating a primary ossification centre (Olsen, 1999) (See figure 1.5). A bone matrix of trabeculae is formed from the enlarged chondrocyte lacunae known as the primary spongiosa, made of disorganised woven bone spanning the circumference of the bone metaphysis at the front of the hypertrophic zone (Olsen, 1999). These trabeculae are remodelled to form an organised trabecular structure made of lamella bone, known as the secondary spongiosa (Seeley et al., 1995, Olsen, 1999). Further interstitial and appositional growth at the growth plate and recurrent remodelling of the diaphysis results in longitudinal growth of the bone and the production of a mid shaft made predominantly of trabecular bone with a surrounding bone collar of compact bone (Seeley et al., 1995, Olsen, 1999, Gilbert, 2000, Wolpert, 2002). The mid shaft trabeculae are then near completely resorbed to form

the medullary shaft, which is invaded by blood vessels and occupied by marrow cells.

A secondary ossification centre is formed at the upper epiphysis where the cartilage mineralises and undergoes remodelling in a similar way as that of the primary ossification centre (see fig. 1.5) (Seeley, 1995, Olsen, 1999). The bone collar of the diaphysis thickens and extends toward the epiphysis as the bone undergoes further longitudinal growth lengthening the mid-shaft. Remodelling continues at both centres of ossification and longitudinal growth by the growth plate continues into postnatal life (Gilbert, 2000, Wolpert, 2002).

1.5 Acetylcholinesterase (AChE)

1.5.1. Role of AChE in cholinergic signalling.

Acetylcholine acetyl hydrolase or Acetylcholinesterase (AChE, 3.1.1.7) is most commonly known for its important role in the termination of cholinergic neurotransmission. Acetylcholine (ACh) is the neurotransmitter used to transmit nerve impulses at cholinergic synapses and at the motor end plates of neuromuscular junctions (Hammond, 2001). Synthesis of acetylcholine by choline acetyltransferase (choline acetylase or ChAT) occurs whereby the acetyl group of acetyl-CoA is transferred to choline (Purves, 2001). At cholinergic signalling sites the presynaptic cell is separated from the postsynaptic cell or the muscle cells by the synaptic cleft (Simmons, 1999). Presynaptic cells concentrate acetylcholine in synaptic vesicles (approximately 10^4 molecules per vesicle) close to the nerve terminal apposed to the synaptic cleft (Simmons, 1999). Arrival of a nerve impulse causes a depolarisation of the presynaptic membrane creating an influx in Ca^{2+} into the cell via voltage-sensitive calcium channels (Hammond, 2002). Increased intracellular Ca^{2+} results in a subsequent release of acetylcholine into the synaptic cleft (Hammon, 2002). Acetylcholine molecules diffuse across the synaptic cleft and bind to abundant receptors found on the postsynaptic membrane (see figure 1.6). There are two known receptor types, nicotinic and muscarinic acetylcholine receptors, distinguishable by the effects of the alkaloids nicotine and muscarine. The two types of acetylcholine receptor produce actions by different mechanisms. Binding of acetylcholine to nicotinic receptors found on the postsynaptic membrane opens the receptor acting as

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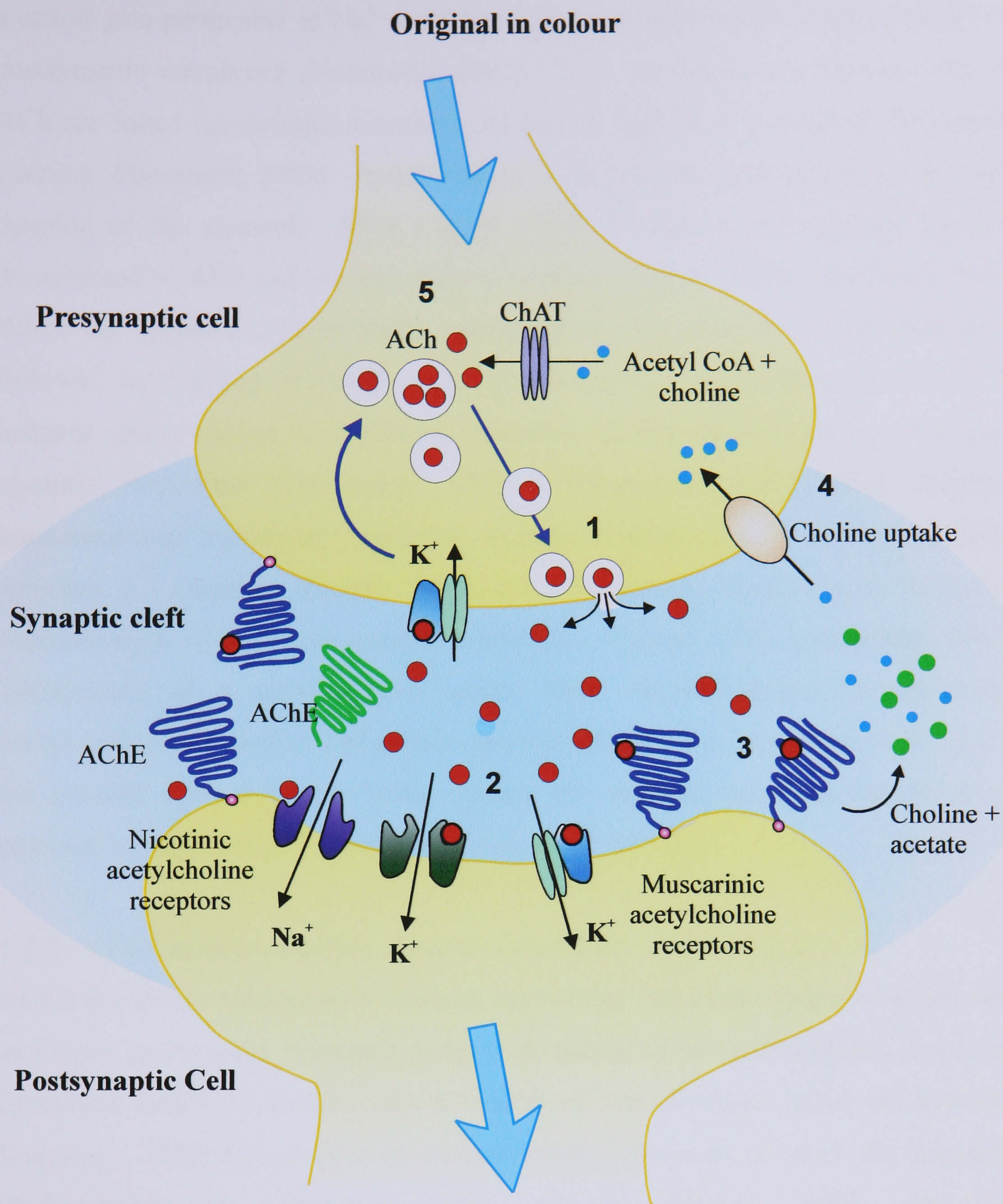

Figure 1.6
Cholinergic neurotransmission
1. Arrival of a nerve impulse at the presynaptic terminal causes vesicular release of ACh into the synaptic cleft.
2. ACh diffuses across the cleft and attaches to nicotinic or muscarinic receptors found on the surface of the postsynaptic cell, inducing intracellular signalling.
3. Acetylcholinesterase (AChE) found soluble within the cleft and bound to the surface of the post and pre-synaptic cell terminates neurotransmission by hydrolysing ACh into choline and acetate.
4. Choline is readily uptaken by the presynaptic cell and combined with Acetyl Co-enzyme A to produce more ACh molecules by ChAT.
5. ACh is packaged and concentrated into synaptic vesicles.

a cation gate permeable to Na^+ and K^+ provoking a rapid influx of ions through the postsynaptic membrane (Hammond, 2002). Two non-interacting binding sites for ACh are found on nicotinic receptors; binding at both sites is required for channel opening (Hammond, 2002). Initial binding of ACh to the receptor causes transitory opening of the channel. After a short period of activity the receptor becomes desensitised to ACh and the molecule is released (Purves, 1999, Hammond, 2002). When the synaptic cleft is highly concentrated with ACh this initial binding is followed by binding of further molecules ultimately culminating in further ion influxes and a change in membrane potential causing depolarisation of the post-synaptic membrane (Hammond, 2002). Muscarinic acetylcholine receptors transmembrane regions are G-protein coupled; binding of ACh to these receptors activates K^+ channels through the coupled G-protein complex also instigating depolarisation of the postsynaptic membrane (Purves, 1999, Hammond, 2002). Termination of a nerve signal occurs when acetylcholinesterase hydrolyses acetylcholine into choline and acetate thereby reducing the concentration of ACh at the synaptic cleft and in so doing closing the channels, restoring the membrane potential to basal levels (Purves, 1999, Hammond, 2002).

1.5.2 AChE protein structure, catalytic capabilities and inhibition.

AChE is a serine esterase with catalysis resembling that of the serine proteases such as lipases, and trypsin, belonging to the large family of serine hydrolyses. The serine hydrolyse family is divided into a variety of sub divisions based on structural homology, AChE belonging to the carboxylesterase division, of which the only other cholinesterase is butyrlcholinesterase (BChE) (Horton, 2002, also see http://bioweb.ensam.inra.fr/ESTHER). Cholinesterases are some of the fastest known acting enzymes, the rate of acetylcholine turnover being $k_{cat}/K_m = 10^8$ M^{-1} sec^{-1} (4000 ACh molecules per active site per second) (Horton et al., 2002). This renders AChE able to hydrolyse vast quantities ACh molecules to enable the rapid termination cholinergic signalling. The AChE protein complex consists of a 12-stranded β-sheet surrounded by 14 α-helices (Sussman et al., 1991). The active centre of AChE comprises two AChE binding sites and the narrow gorge. At the top of the narrow gorge is a negatively charged 'peripheral anionic' binding site for ACh, thought to be involved in substrate inhibition of AChE catalysis (Szegletes et

al., 1999). This site has also been identified as a possible secondary allosteric site for the secondary functions of AChE that will be discussed in depth in Chapter 4. The gorge itself is lined with 14 aromatic residues (Taylor and Radic, 1994). The choline moiety of ACh interacts with these aromatic side chains that serve as 'docking sites' to manoeuvre ACh molecules toward the active site in the correct alignment and orientation for interacting with the active site (Tan et al., 1993, Taylor and Radic, 1994). 20Å from the surface of the protein at the bottom of the gorge, in the centre of the protein complex, lies the active site triad common to serine hydrolyases (Taylor and Radic, 1994). The catalytic triad of cholinesterase is comprised of serine (S_{200}), Histidine (H_{440}), and unlike other carboxyesterases, an aspartate residue is replaced by glutamate (E_{327}) (Shafferman et al., 1992, Taylor and Radic, 1994). During hydrolysis the serine hydroxyl element is disprotonated by the histidine residue that functions as a general base, and the negatively charged glutamate residue balances the positively charged histidine residue (Taylor and Radic, 1994).

The catalysis of the ACh hydrolysis reaction is carried out over multiple steps. Firstly, nucleophilic attack of the carbonyl carbon of ACh by the active site serine residue oxygen moiety causes binding of ACh to the serine residue and formation of an intermediate tetrahedral structure (Taylor and Radic, 1994, Horton, 2002). Cleavage of ACh at the Ser bond liberates the choline molecule from ACh creating an acetyl-enzyme structure (Taylor and Radic, 1994, Horton, 2002). Hydrolysis of the acetyl-enzyme intermediate by nucleophilic attack of the acetyl moiety, this time via the active site histidine residue, results in deacetylation of the enzyme (Taylor and Radic, 1994, Horton, 2002). This final step provides acetate and hydrogen products. A chemical description/representation of ACh hydrolysis by AChE is presented in figure 1.7.

Recently a number of catalytically inactive proteins bearing significant structural and sequence homology to cholinesterases have been identified based on 'signature' sequences of the B-carboxylesterase family relating to the catalytic domain of AChE (See Cousin et al., 1998, amended to http://bioweb.ensam.inra.fr/ESTHER/, also see Taylor, Luo and Camp, 2000 for review). This group of 'esterase-like' proteins show 28-45% sequence homology to the signature sequences found within B-carboxylesterases, lacking the active site

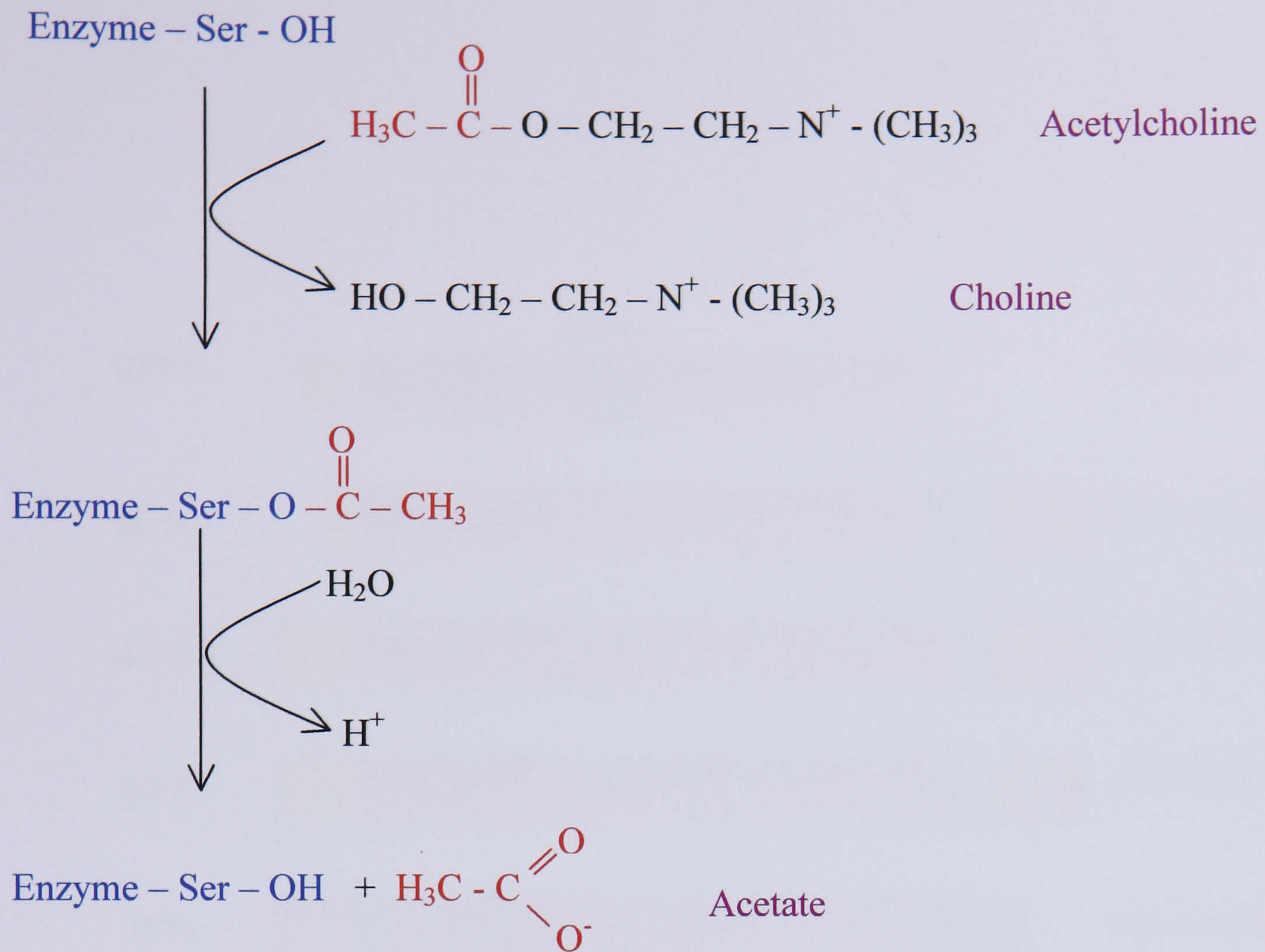

Figure 1.7
Multistep process of acetylcholine hydrolysis by acetylcholinesterase.
Acetylcholine binds to the active site serine, the choline moiety is released leaving an acetylated serine residue. Hydrolysis by an internal H_2O molecule deacetylates the enzyme releasing acetate and H^+ by-products.

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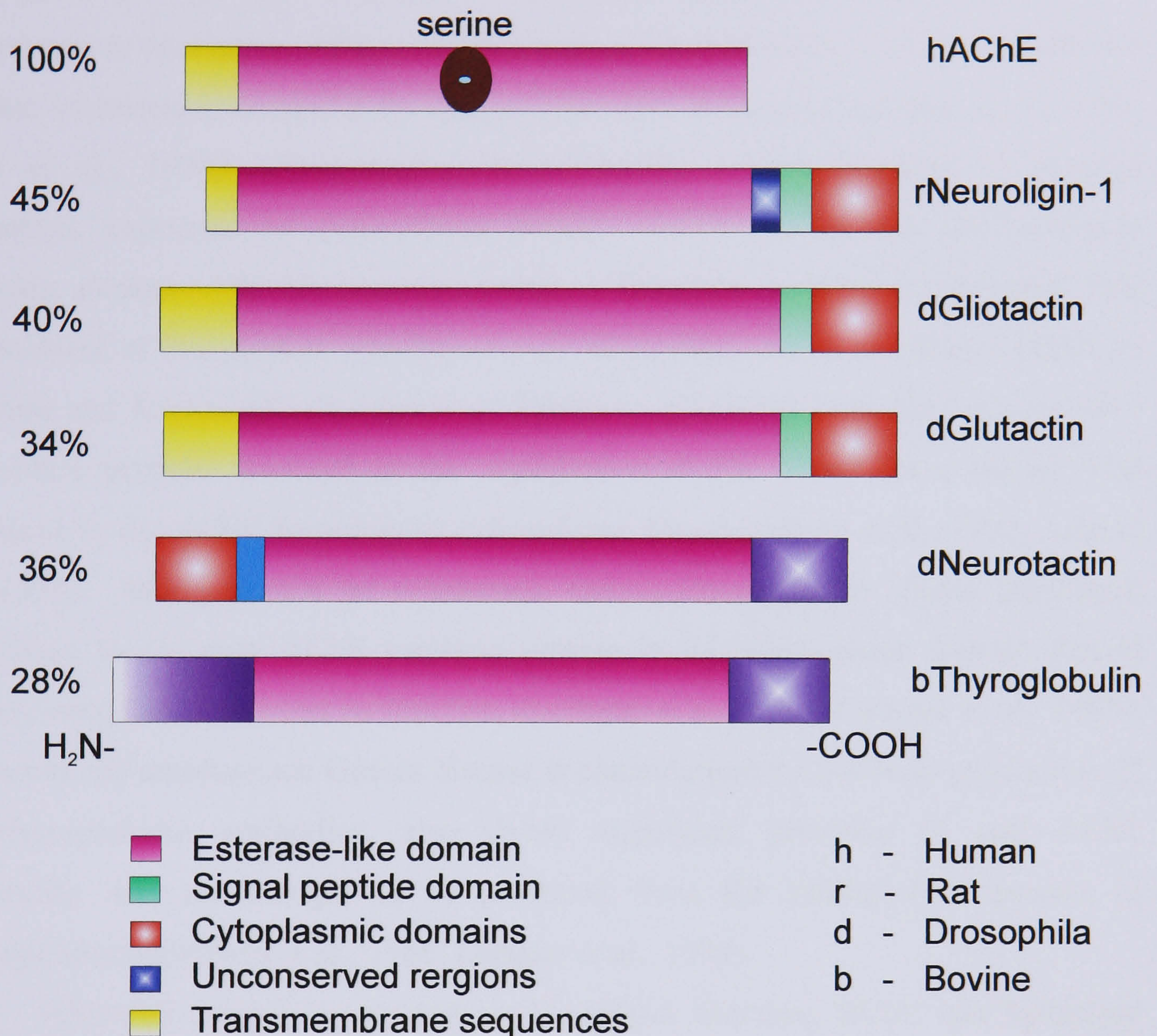

Figure 1.8
Esterase-like family
Proteins displaying sequence homology to AChE in the core catalytic domain, but lacking the active site serine require for catalytic activity.

serine required for catalytic activity, but retaining other residues needed to maintain the structural arrangement (Botti et al., 1998) (see figure 1.8.).

Other than the conserved extracellular esterase-like domain these proteins possess other functional domains such as intracellular segments, and transmembrane regions relating to their specific functions. Interestingly, most family members have a related role in mediating cell-cell and cell-matrix interactions in a variety of different tissues; interactions occurring by their esterase-like domains (Darboux et al., 1996, Botti et al., 1998). Neurotactin, the drosophila protein involved in synapse formation, expresses an extracellular domain homologous to the cholinesterase catalytic domain (De la Escarla, 1998). Mutation analysis established that replacement of this domain with the core AChE protein caused no adverse effect on function and facilitated cell adhesion. Similarly, gliotactin is another esterase-like drosophila protein, involved in the organisation of the blood brain barrier; and mediated by the AChE homologous extracellular domain (Olson et al., 1990, Auld et al., 1995). More specific to vertebrates, human thyroglobulin shares significant homology to the core AChE catalytic protein in its extracellular domain that is already established to exert its function (Swillens et al., 1986, Ludgate et al., 1989). Moreover the autoimmune Graves disease is characterised by antibody production of anti-thyroglobulin antibodies, also shows significant presence of anti AChE antibodies that are thought to be produced from the esterase-like domain of thyroglobulin (Swillens et al., 1986, Ludgate et al., 1989).

Although related in structure and catalytic function, BChE can hydrolyse multiple choline esters (including acetylcholine) as well as butrylcholine, although not all to the same affinity (Chatonnet and Lockridge, 1989). This is unlike AChE that is known as the 'true' cholinesterase for its specific affinity to ACh (Taylor and Radic, 1994). Due to the structure of the AChE protein the ligand specify of this protein is high. The folding of AChE places the active site for enzyme catalysis at the bottom of the narrow gorge, restricting the access of larger less specific esters (Taylor and Radic, 1994). The spatial arrangement of the catalytic triad also makes it impossible for AChE to bind other choline molecules. However, cholinesterases do have the potential to catalyse various other esters such as oxyesters, selenoesters, amides, carbamoylesters and phosphorylesters, creating a great susceptibility for inhibitors (for review see Taylor and Radic, 1994). There are two categories of

cholinesterase inhibition : irreversible and reversible. Covalent bonding of ligands/inhibitors such as Diisopropyl Fluorophosphate (DFP) to the active site serine renders the enzyme irreversibly catalytically inactive (Horton, 2002). Reversible inhibitors prevent the substrate from binding to the active site. This can be either in a competitive manner where the inhibiting ligand binds directly to the active site (tacrine, edrophonium), or a non-competitive manner in which ligands might bind to alternative sites on the AChE enzyme that would prevent the binding of ACh molecules to the active site for hydrolysis (Taylor and Radic, 1994). The peripheral anionic site (PAS) functions as one of these sites acting as a site for allosteric inhibition of the enzyme. Found at the top of the gorge this site can bind ACh as well inhibitors that bind and obscure the entrance to the gorge, (propidium, gallamine). Some inhibitors are capable of binding to both the active site and the PAS and are known as bis-quarternary inhibitors (BW284c51 and decamethonium). Although the crystal structure of many of the known AChE inhibitors has been found it is not yet established if such inhibitors are also capable of causing serious conformational changes to the structure of AChE which could independently inactivate AChE (Taylor and Radic, 1994).

1.5.3. AChE isoforms, tertiary and quaternary associations

Early in AChE research, prior to the cloning of AChE genes, multiple AChE species were identified and characterised according to their physical properties and functional localisation (see figure 1.9). A species of AChE was identified in erythrocytes that contained numerous hydrophobic sequences at its C-terminal cell membranes; hence the term $AChE_H$ was adopted (Massoulie and Bon, 1982). At cholinergic synapses (muscle and brain) a species was identified oligomerised with a collagen-like tail (ColQ) anchor, or attached to a then unidentified transmembrane anchor (PRiMA) (Massoulie 2002). This type of AChE displayed the most resemblance to the well-researched *Torpedo* AChE, and the term $AChE_T$ (for tailed) was adopted. Subunits of AChE are classified as globular (G) AChE (Massoulie, 2002). Globular AChE can be expressed as monomers (G_1), or multiple subunits can form dimers (G_2) and tetramers (G_4) via a conserved cysteine residues found at the C-terminal peptide Massoulie et al., 1998, Massoulie, 2002). Hydrophobic sequences and aromatic residues located at the C-terminus give some globular species amphiphilic properties and are termed amphiphilic monomers (G_1^a) and

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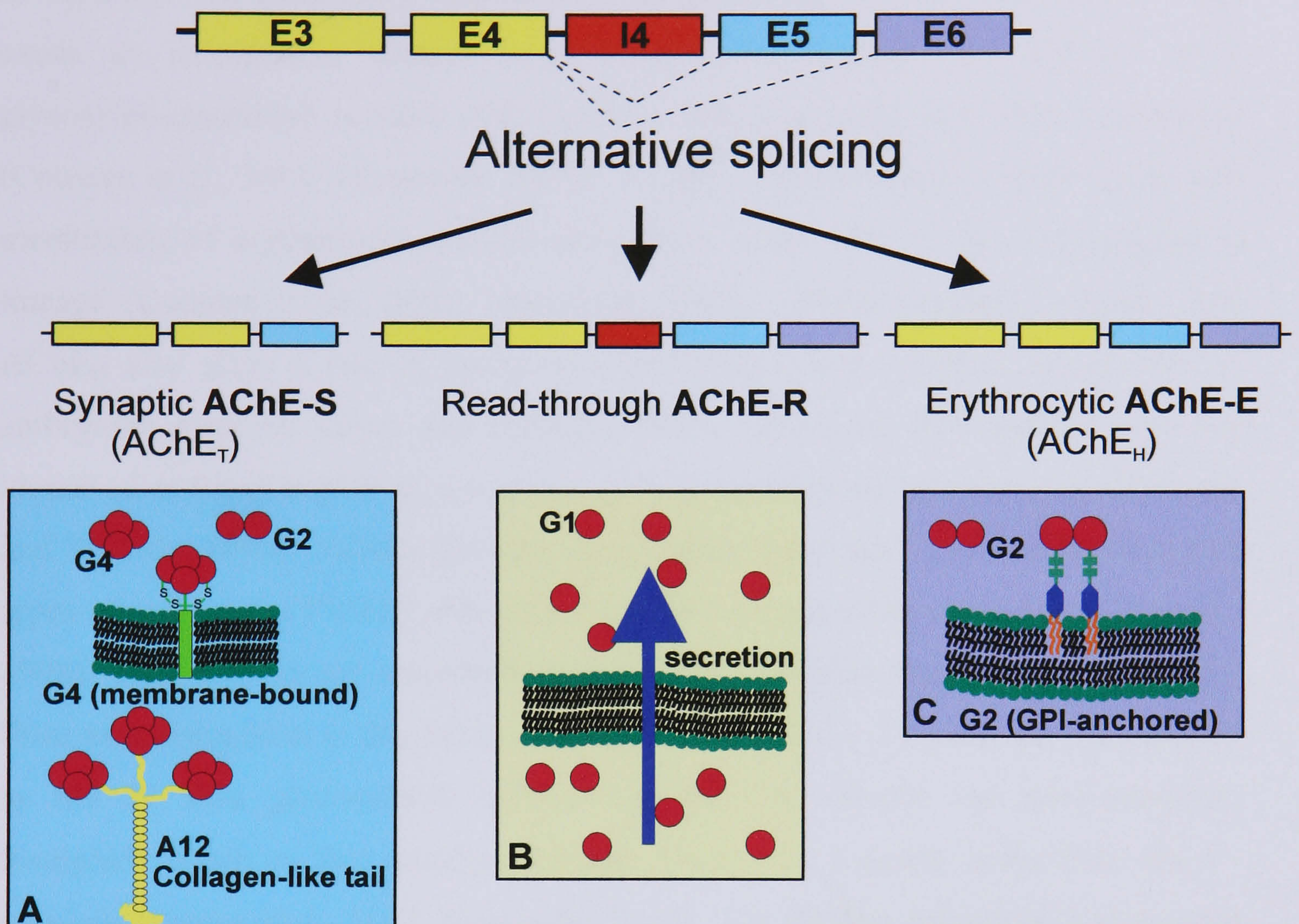

Figure 1.9
The gene for human AChE comprises of 6 exons. Exons1-3 encode the common catalytic domain. Exons 4/5 are subject to alternative splicing to give rise to 3 AChE subunit species (A) synaptic (B) readthrough (C) erythrocytic/GPI-linked. Subunits can be soluble monomers, form dimers and tetramers, or can be membrane bound via GPI-linkage or the hydrophobic PRiMA subunit. Dimers and tetramers can also attach to a collagen like tail.

dimers (G_2^a) that are present for both $AChE_H$ (type I) and $AChE_T$ (Type II) (Massoulie et al., 1998). Secreted subunits of the amphiphilic form are preferentially cell surface associated by electrostatic interactions owing to their hydrophobic nature (Bon et al., 1991).

A number of different AChE forms exist in vivo as a result of oligomerisation of subunits, and quaternary association with anchoring proteins. Globular $AChE_H$ exists as amphiphilic dimers (type I G_2^a) that acquire the addition of a glycosylphosphatidyl inositol (GPI) moiety that integrates with cell membranes (Coussen et al., 2001, Massoulie, 2002). $AChE_H$ is predominantly found in the cell membranes of erythrocytes and possibly some other cells of the haematopoietic lineage (Coussen et al., 2001, Massoulie, 2002). Some evidence suggests that $AChE_H$ also plays a role in myogenesis and muscle development (localisation to embryonic muscles, Layer and Wilbold, 1995). However, this isoform was not identified or found to play an active role at the mature neuromuscular junction (Layer and Wilbold, 1995). $AChE_T$ globular species form quaternary associations with two types of anchor; the PRiMA subunit or the ColQ collagen-like tail (Massoulie et al., 1998). Both anchors are encoded for by genes distinct from that for AChE (ACHE). Prior to translocation to the cell surface, tetrameric $AChE_T$ (Type II G_4^a) is attached to the 22 kDa glycosylated hydrophobic PRiMA subunit via intercanternary disulphide bonds to free cysteine residues located - 4 amino acids from the C-terminus (Perrier et al., 2002, Massoulie, 2002). The PRiMA subunit is then thought to integrate itself into the cell membrane. Other associated proteins in this process are yet to be identified (Perrier et al., 2002). This form of AChE is the main type of AChE found in the central nervous system (CNS) providing approximately 70-90% of catalytic activity of the brain (Layer and Wilbold, 1995). Globular $AChE_T$ can also be secreted from the cell. Secretion of globular AChE results in cleavage of part of the C-terminal peptide and removal of the aromatic residues; these subunits are therefore non-amphiphilic (G^{na}) (Morel et al., 2002, Belbeoc'h et al., 2003). At the neuromuscular junction (NMJ) the predominant form of AChE is the collagen-tailed form. Multiple amphiphilic dimers or tetramers of $AChE_T$ bind to the ColQ to form the asymmetric (A) forms of AChE (A_4, A_8, A_{12}) (Massoulie et al., 198). ColQ (Q standing for the French word for tail 'queue') may also exist as multiple isoforms due to extensive splicing of the ColQ gene (Massoulie et al., 2002). The C-terminal peptides of $AChE_T$ contain a trypyophan (**W**) amphiphilic tetramerization domain or

WAT domain that binds to ColQ via a string of conserved proline residues found at its N-terminus named the **p**roline **r**ich **a**ttachment **d**omain (PRAD) (Bon et al., 1997, Simon et al., 1998, Massoulie, 2002). ColQ is then organised in a triple helical arrangement (Massoulie et al., 1999, Bon et al., 2003). Motifs that would permit binding of AChE to extracellular matrix proteins are located distal to the N-terminus of ColQ (Krejci et al., 1997). Binding to a variety of heparan sulphate proteoglycans such as perlecan and chondroitin sulphate have been identified that allow for localisation of AChE at the basement membrane (Vigny et al., 1983, Rossi and Rotundo, 1996, Arikawa-Hirasawa et al., 2002). The advent of cloning of the AChE gene from a variety of species however brought about new names and terms for the respective AChE isoforms (see figure 1.9).

1.5.4. Gene structure, alternative splicing, and post-translational modification.

Although AChE has retained significant structural homogeneity across species, there is considerable diversity in gene size, number, and intron/exon organisation. In mammals the AChE gene spans 6-7kb of genomic DNA located to chromosome 7q22 in humans, and to the distal part of chromosome 5 in mouse (Ehrlich et al., 1992, Getman et al., 1992, Rachinsky et al., 1992, Wilson et al., 2001). However in other species the AChE gene was found to span a much larger genomic sequence, e.g. 25kb in *Torpedo*. BChE is also encoded for by a single gene that bears resemblance to but is distinct from that of AChE, located to chromosome 3 in mammals (Getman et al., 1992). Despite this diversity these genes do share some similarities (see figure 1.10). In general genes that encode AChE can be divided into two segments; exons encoding the 'core' of AChE protein, and exons that encode the carboxyl terminus or C-terminal. The core AChE protein comprises the catalytic component of AChE. The C-terminal protein governs isoform expression and associated attachment to the GPI, ColQ, or PRiMA anchoring proteins. The catalytic domain of *Torpedo* and human BChE is encoded by a single exon. *C.elegans* have a family of four *ACE* (1-4) genes that encode for AChE, all of which have different exon organisation in the region that encodes the core catalytic domain (For review see Massoulie, 1999). In mammals however the corresponding coding region of AChE is divided in multiple exons. The mammalian AChE gene contains 6 exons. The first exon is an untranslated region connected to promoter (that is spliced out in mature AChE mRNA) (Li et al., 1991, Ben-Aziz Aloya, 1993, Camp and Taylor,

1998, Luo et al., 1998). The open reading frame of the AChE gene therefore begins in exon 2, with exons 2-4 encoding the core catalytic domain of mammalian AChE (Li et al., 1992, Ben-Aziz Aloya, 1993). As a consequence of diversity in gene structure and organisation, differences in the respective catalytic properties have been noted which are thought to reflect the relative variations in the cholinergic systems of these species. The catalytic domain makes up the bulk of the AChE gene and protein.

Differences in gene structure between species can also be observed in the remainder of the AChE gene (see figure 1.10). The 3' end of the AChE gene provides only a small part of the overall AChE protein, C-terminal peptides contributing just 40-67 residues (Grisaru et al., 1999). However these C-terminal regions bear greater significance to the organisation and localisation of the AChE species and their functionality. Putative splice sites were identified at the distal region of exons 4-6 (Li et al., 1992, Ben Aziz Aloya et al., 1993). Alternative splicing of these sites generates multiple AChE mRNA species that produce the specific AChE isoforms differing in their C-terminal peptides and ability to form quaternary associations (Li et al., 1992, Ben Aziz Aloya et al., 1993). In mammals three distinct AChE splice variants exist AChE-S ("synaptic" $AChE_T$), AChE-E ("erythrocytic" $AChE_H$), or the recently identified "readthrough" isoform, AChE-R, which contains the pseudo-intron 4 (see figures 1.9 and 1.10). Splicing of mRNA exon 4 (E4) at the 3'and exon 5 (E5) at the 5' results in the removal of exon 5 and a mature mRNA product containing only the core catalytic domain and exon 6 (E6) (E2, E3, E4, E6). This AChE-S product gives rise to AChE with C-terminal peptides that correspond to the $AChE_T$ isoform and is found in cells of the CNS, NJM and cells of haematopoietic origin (megakaryocytes and lymphocytes) (Soreq and Seidman, 2001, Massoulie, 2002). Splicing of mRNA at a single point at the end of exon 5 (E5) results in a mature mRNA product containing the core catalytic domain and exon 5 resulting in the expression of the $AChE_H$ isoform (E2, E3, E4, E5) (Li et al., 1992, Ben Aziz Aloya et al., 1993). Readthrough of the AChE gene that incorporates the pseudo-intron 4 (I4), originally thought to be part of the non-coding sequence can give rise to an additional splice variant AChE-R (Li et al., 1992, Ben Aziz Aloya et al., 1993). Mature mRNA of AChE-R contains the coding region of the core catalytic domain, intron 4 and all of the remaining exons (E2, E3, E4, I4, E5, E6).

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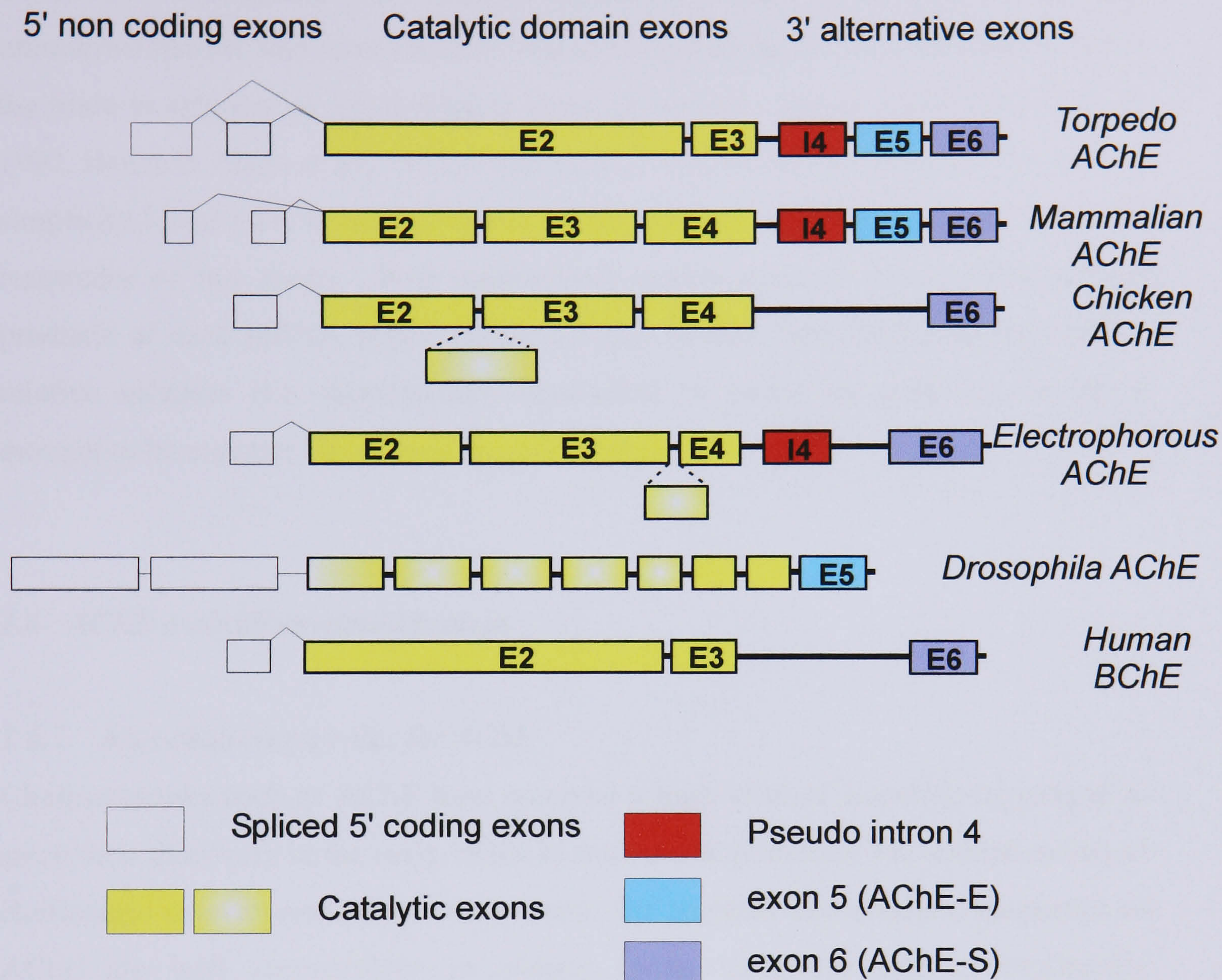

Figure 1.10
Cholinesterase gene alignment.
All cholinesterase genes contain untranslated 5' exons containing transcription initiation sites. These exons are spliced out and do not contribute to the mRNA that gives rise to AChE.

Although the catalytic domain of the AChE protein retains considerable cross species homology it is produce by a varying number of exons, from 3 exons in mammals up to 7 exons in drosphilia. Some species also have coding exons inserted into the sequence.

C-ternimal exons vary greatly across species meaning some species do not have the capcity to produce all the known AChE isoforms (See figure 1.11).

The resulting protein product contains a 26 amino acid extension at the C-terminus, and is subsequently devoid of the free cysteine residues required for subunit oligomerisation and attachment to functional anchoring proteins and is therefore expressed a soluble monomer that is secreted from the cell (Li et al., 1991, Kerem et al., 1993). This isoform of AChE is expressed at high levels in embryonic and tumourous tissues, and more recently was identified as the isoform over-expressed in the brain in response to psychological stress or chronic catalytic inhibition (Li et al., 1992, Ben aziz Aloya et al., 1993, Small et al., 1996, Soreq and Seidman, 2001). For simplicity I will refer to the respective AChE isoforms as AChE-S, E and R for the remainder of this thesis. Post translational modification of the resulting protein products of each mRNA splice variant confers further functional properties of the relative subunits (i.e. dimerisation, attachment to anchoring proteins), which is thought to be regulated in a tissue specific manner.

1.6 AChE a multifunctional protein

1.6.1 Non-cholinergic roles for AChE

Cholinesterases such as AChE have received a high level of scientific investigation since their discovery in the early 1930s in studies that pioneered the understanding of cholinergic neurotransmission. As a result, the physical and catalytic properties of AChE are well characterised in relation to its traditional role in cholinergic neurotransmission. However over 25 years ago a number of observations lead to the idea of 'secondary' functions of AChE unrelated to its cholinergic role. The fact that AChE exists in multiple isoforms and species suggests that AChE could have divergent roles, especially as one identified isoform is thought to be expressed exclusively in erythrocytes, cells known to have no cholinergic innervation or role in neurotransmission (Lawson and Barr, 1987, Roberts et al., 1987). In addition, AChE is expressed in regions of the brain that do not express the acetylcholine synthesising enzyme, choline-acetyltransferase (ChAT) indicating an absence of cholinergic signalling in those regions (Silver, 1974, Henderson and Greenfield, 1987). Non-cholinergic neurons have also been identified to express high levels of AChE (Cheramy et al., 1981, Greenfield et al., 1981, Henderson and Greenfield, 1984, Falugi and Raineri, 1985, Greenfield, 1991, Mesulam, 1995, Small et al., 1996). Also

AChE release into the cerebrospinal fluid (CSF) was noted at rates that do not coincide with the release of ACh (Greenfield et al., 1986). The number of secondary functions for AChE has gradually accumulated with AChE seemingly playing a role in many cellular functions, during development, and in disease states. In recent years, much AChE research has focused on these secondary functions of AChE, proposing that in contrast to immediate assumptions, AChE is in fact a complex multifunctional protein.

1.6.2. Expression of AChE in non-cholinergic tissues

Cholinesterases have been identified in a number of non-cholinergic tissues. Firstly, expression of AChE and BChE was noted during the development and patterning of the brain prior to the emergence of cholinergic signalling and in many cases correlated with the phase of neutrite extension. Layer et al. (1991) identified a role for cholinesterase in the development of the neural tube; additionally many neural tube defects that are a result of incomplete closure of the neural tube are associated with a significant increase in the amount of AChE found in the amniotic fluid (Bonham and Atack, 1983 and Rakonczay et al., 1985, Layer and Kaulich, 1991, Layer and Willbold, 1995). For a number of years the detection of elevated levels of AChE in amniotic fluid has been used as a reliable diagnostic measure of neural tube defects (Muller et al., 1986). More specifically a role for AChE and BChE has been identified in the cell proliferation and migration of neural crest cells and the development of cranial nerve fibres (Layer and Kaulich, 1991). The expression of AChE was noted in approximately 90% of migrating neural crest cells as identified by co-localisation to the HNK-1 epitope a commonly used marker of neural crest cells (Drews, 1975, Cochard and Cotley, 1983, Rickman et al., 1985, Bronner and Fraiser, 1986, Layer and Auber, 1990, Layer and Kaulich, 1991). AChE expression has also been identified in a number of mature brain regions not innervated by cholinergic signalling (Cheramy et al., 1981, Greenfield et al., 1981, Henderson and Greenfield, 1984, Falugi and Raineri, 1985, Greenfield, 1991, Mesulam, 1995, Small et al., 1996). Intriguingly, AChE expression is also noted in various tissues not typically associated with neuronal or muscular tissues or neural signalling in general. Expression was found in numerous cells of haematopoietic origin such as megakaryocytes, erythrocytes, activated B and T- lymphocytes, and thymocytes (Paulus et al., 1981, Lawson and Barr, 1987, Roberts et al., 1987, Richier et al.,

1992). Significant levels of AChE expression and activity have also been identified in liver, testes, ovaries, skin, the adrenal medulla, thyroid, cartilage, bone, tumourigenic and developing tissues of a variety of origins (Li et al., 1991, Karpel et al., 1994, Grisaru et al., 1999, Genever et al., 1999). The significance of AChE expression in such a diverse range of tissues is still poorly understood but has received much attention in recent years. In many cases evidence now exists for complex non-cholinergic roles for AChE.

1.6.3. Non-cholinergic role for AChE in the brain.

Certain brain regions that express very little ChAT and therefore have minimal cholinergic innervation, still express high levels of AChE, such as the cerebellum, lateral hypothalamus, dorsal raphe nucleus, and substrata nigra (For reviews see Appleyard, 1992, Appleyard, 1994, Small et al., 1996, Greenfield, 1998). A number of non-cholinergic neurons have been shown to express and secrete AChE as well as other neuronal related cells such as glia, glioma, dendrites, and astrocytes. Moreover, release of AChE was found to be modulated by the existing neuronal signalling systems of these tissue/brain regions, suggesting that the release of AChE has significance and is not a consequence of cell death or matrix degradation (See Greenfield, 1998). AChE release from dendrites of neurones of the developing substantia nigra was found to be controlled by dopamine in a concentration-dependant manner, and could modulate motor control in a non-enzymatic fashion (Negergaard et al., 1988, Jones et al., 1995). This effect remained unaltered by catalytic inhibition, yet was susceptible to inhibitors of the peripheral anionic site (see Greenfield, 1998). AChE release in the cerebellum also induced effects on the resident signalling systems. AChE was found to sensitise glutamate receptors to their respective ligands and to induce re-uptake of excitatory amino acids in the cerebellum and in vitro (Appleyard, 1988, Greenfield, 1991, Webb et al., 1996, Rodriguez-Ithurralde et al., 1996, and Rodriguez-Ithurralde et al., 1997).

A number of explanations have been proposed for the existence of AChE in no-cholinergic regions of the brain. However, in most cases AChE is only a small part of a complex mechanism involving multiple other factors, and remains significant only to that specific incidence. However, there is compelling evidence suggesting a direct role for AChE in axon guidance and neuronal cell adhesion that could be

applicable to numerous other tissues and may outline a possible secondary function for AChE (Drews et al., 1974, Greenfield, 1991, Umezu et al., 1993, Layer and Willbold, 1994, Bataille et al., 1998, Grifman et al., 1998; Simon et al., 1999, Johnson and Moore, 2000). During embryonic development, cholinesterase expression in the brain (and possibly other tissues) is highly regulated and incomparable to that of the mature brain (Layer and Willbold, 1995). In many developing brain areas, AChE expression precedes synaptogenesis and cholinergic innervation, and more closely corresponds to the major phase of neurite outgrowth (Layer 1983, Layer, 1991, Small et al., 1992). AChE co-localises to areas of expression of a number of different neuronal cell adhesion molecules (NCAMs), and the HNK-1 epitope commonly found on NCAMs is also present on AChE (Layer et al., 1991). Significant evidence exists to suggest that AChE possess neurogenic properties. In primary culture of nervous-system derived cells such as glioma and dorsol root ganglion cells, AChE application has shown to promote neurite extensions (Layer et al., 1993, Dupree and Bigbee, 1994, Jones et al., 1995, Karpel et al., 1996). Transfection of such cells with AChE cDNA also induced these cells to resemble neurons morphogenically and express neuronal markers (Dupree and Bigbee, 1994, Jones et al., 1995, Karpel et al., 1996, Keonigsberger et al., 1997, Bataille et al., 1998). These effects have consistently proved to be insensitive to catalytic inactivation, suggesting that the neurogenic properties of AChE are of a non-cholinergic/non-catalytic nature.

The effects of AChE on cell adhesion have been identified by numerous independent investigations (Jones et al., 1995, Darboux et al., 1996, Johnson and Moore, 1999, Bigbee et al., 1999). It has been suggested that AChE may facilitate heterotrophic interactions in neurons (Song et al., 1999), in a similar manner as that observed between neurexins and their ligands, neuroligins (Grifman et al., 1998, Soreq and Seidman, 2001). Neuroligin 1 and 2 show significant sequence homology to the core AChE enzyme but lack the serine residue required for catalytic activity (members of the esterase-like family discussed in section 1.5). A connection between AChE and neurexins was identified during the characterisation of AChE overexpression. Transgenic mice overexpressing AChE demonstrated significantly suppressed neurexin 1β mRNA specifically during embryonic development (Andres

et al., 1997). In vitro, AChE antisense cDNA expression also induced a significant reduction in neurexin 1α expression; this could however be rescued by neuroligin 1 overexpression indicating a linked role, and possible functional redundancy between AChE and neuroligin 1 in this circumstance (Grifman et al., 1998). It has also been hypothesised that neuroligins may act as signalling molecules via neurexins, which may attach to intracellular components of signalling systems via PDZ domain proteins (Irie et al., 1997, Butz et al., 1998, Missler et al., 2003). It may be possible that AChE competes with/for interaction of neurexin, akin to neuroligins or analogs thereof. The exact site responsible for the adhesive properties of AChE is still to be confirmed, however the peripheral anionic site has been outlined as a possibility. Specific inhibitors or functional blockade of this site induced a significant reduction of the adhesive function of AChE in vitro (Bataille et al., 1998, Jones et al., 1994, Johnson and Moore, 1999 and Simon et al., 1999).

1.6.4. Non-cholinergic role for AChE in Haematopoiesis.

The discovery of non-cholinergic roles for AChE in neuronal tissues was intriguing, but identification of a role for AChE in tissues that have no or limited neuronal innervation, and are derived from completely different cell origins, compounded the idea of non-cholinergic AChE actions. High levels of both AChE and BChE activity are commonly found in human plasma samples, where they are thought to behave as scavenging molecules, to hydrolyse circulating toxins and surplus ACh molecules. Blood BChE has the ability to hydrolyse cocaine, and reduce its toxicity in vivo (Gatley, 1991, Lynch et al., 1997, Duysen et al., 2002, Zhan et al., 2003). AChE expression is found in a number of different cells of the haematopoietic lineage, and some evidence does exist to implicate AChE in ACh mediated mechanisms (Burstein et al., 1980, Burstein et al., 1983, Hu et al., 1990, Costa et al., 1994). However, a growing body of data suggests AChE may have regulatory functions in these tissues independent of cholinergic signalling, or other non-cholinergic actions of ACh. It is well established that erythrocytes express high level of AChE-E, an AChE isoform that is specific to only this cell type (Lawson and Barr, 1987). Presentation of AChE on the cell membrane of erythrocytes has been linked to the immuno-typing of the rare YT blood group (Spring et al., 1992). Study of erythropoiesis demonstrated a correlation between the levels of AChE expression and erythroid cell differentiation

(Samuels et al., 1967, Lawson and Barr, 1987, Barr and Keokebakker, 1990, Paoletti et al., 1992, Soreq et al., 1994, Chan et al., 1998). However, to date there are no data that provides a direct mechanism of AChE action, or reason for such AChE isoform specificity in erythrocytes, and very little evidence suggesting a cholinergic interaction. A correlation between AChE inhibition by exposure to pesticides, and increased risk of leukaemia and other haematopoietic malignancies has been documented on many occasions by a variety of different research groups and governmental studies (Cuneo et al., 1992, Fagioli et al., 1992, Ciccone et al., 1993, Crane et al., 1996, Perry and Soreq, 2001). Moreover, the AChE gene locus is also home to a number of factors essential to haematopoiesis, and is commonly mutated in association with myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML) (Stephenson et al., 1996, Lewis et al., 1996, Perry and Soreq, 2001). In support of this, gene amplification of AChE is also observed not only in cancers of haematopoietic origin, but in a variety of tissues (see below) (Brown et al., 1990, Paouletti et al., 1992, Garry et al., 1994, Greenfield et al., 1996, Small et al., 1996). Amplified or altered AChE expression is also observed in non-malignant blood disorders, in particular platelet disorders and platelet deficiency associated with lupus erythematosus (Lapidot-Lifson et al., 1989, Soreq and Zakut, 1990, Soreq et al., 1992, Zakut et al., 1992, Paoletti et al., 1992). In humans and rats, but not mice, megakaryoblasts the precursor cells of the platelet producing megakaryocytes, express AChE, and expression is correlated with cell differentiation and commitment of the cell lineage (Lev-Lehman et al., 1997). Megakaryocytes secrete large amounts of AChE and AChE activity is associated with platelets (Paulus et al., 1981, Tranhem-Jensen and Behnke, 1981a, Tranhem-Jensen and Behnke, 1981b). Using the colony forming unit-megakaryoblasts (CFU-Meg) derived from bone marrow it was established that AChE expression reached peak levels at the end of mitosis as cells undergo polyploidisation (Paulus et al., 1981). Moreover, antisense inhibition of cholinesterase expression causes a shift in cell population percentages, with cells being driven down the myeloid lineage and a significant reduction in megakaryocytopoiesis (Soreq et al., 1994, Lev-Lehman et al., 1994, Lev-Lehman et al., 1997). As erythrocytes and megakaryocytes are derived from a common precursor cell it is thought that the relative levels of AChE expression could control cell fate and commitment to the specific lineages (Paouletti et al., 1992, McDonald and Sullivan, 1993). More recently, advances in the understanding of non-

cholinergic function of AChE in megakaryocytopoiesis has focused on the role of specific AChE isoforms, and a specific upregulation of AChE-R mRNA during differentiation of megakaryoblastic cells has been identified (Lev- Lehman et al., 1997, Grisaru et al., 2001). Further studies of the AChE peptide sequence revealed a possible cleavage site at the C-terminal peptide of AChE-R (ARP) and AChE-S (ASP) (Velan et al., 1994). Antibodies were developed specifically to recognise these peptides and in vivo production of AChE-R derived C-terminal peptides was demonstrated in haematopoietic cells. The in vitro application of the ARP peptide caused progenitor cell expansion of CFU-Meg cultures, and CD34+ cells derived from umbilical cord blood, which was inhibited by antisense inhibition of AChE-R (Grisaru et al., 2001, Deutch et al., 2002). Transgenic mice overexpressing only the AChE-R isoform also have altered haematopoietic cell numbers, with considerably increased megakaryocytes numbers (Grisaru et al., 2001, Deutch et al., 2002). The authors suggest that in light of the fact that AChE-R expression is upregulated both in response to stress and following catalytic inhibition, it is possible that AChE-R overexpression, or AChE inhibition leading to compensatory overexpression could be the cause of a variety of blood disorders (Grisaru et al., 2001, Deutch et al., 2002).

1.6.5. Transcriptional feedback response.

The neurological effects of stress, acute injury, or exposure to toxic compounds are well documented, leading to cognitive impairment, depression, irritability, defects in motor control and significant fatigue (Kaufer et al., 1998). While it is apparent that numerous signalling systems within the brain could have the capacity to compound the damaging effects of neurological insults, it remains unclear as to the exact mechanisms involved. The effect of inhibiting the cholinergic function of AChE can be fatal and requires immediate clinical intervention, acute exposure causing severe seizure that can lead to permanent brain damage, muscle failure, and death (Karalliedde, 2000, Kwong, 2002). Considering, that there are many naturally occurring cholinesterase inhibitors, it seems plausible that a survival mechanism to circumvent the effects of such inhibitors may have evolved. Similarly, acute stress can result in transiently elevated ACh release causing prolonged or enhanced cholinergic signalling and neuronal activity. It was found that under aberrations of cholinergic signalling (for example cholinesterase inhibitors) there was a rapid induction of genes encoding the cholinergic system as well as immediate early stress

responsive genes i.e. c-fos (Kaufer et. al., 1998, Kaufer et. al., 2000). An increased AChE expression was observed, with a corresponding moderate decrease in ChAT and VAChT expression. Moreover, alterations in AChE alternative splicing lead to an induction of AChE-R over-expression not only in the cholinergic neurons under insult, but also in cells not normally expected to express AChE (Kaufer et. al., 1998). The proposed mechanism is that elevated levels of ACh cause a greater cholinergic capacity leading to constant electrical stimulation, and consequently increased depolarisation and Ca^{2+} influxes. Ca^{2+} responsive elements (CRE) found within the c-fos promoter induce expression, and in turn modulate the transcription of target genes such as AChE, ChAT and VAChT (Kaufer et. al., 2000). However, upregulation of AChE was more profound under catalytic inhibition than with stress conditions indicating additional feedback systems may exist (Kaufer et. al., 1998, Kaufer et. al., 2000). It was suggested that AChE-inhibitor complexes might interact with signalling systems by binding with esterase-like proteins as discussed in the previous section. As well as the immediate effects of aberrations in cholinergic signalling, long-term neurodeterioration is observed and is thought to be the cause of many of the symptoms displayed of depression, cognitive impairment and fatigue (O'Malley, 1997, Ray, 1998). Increased AChE-R expression under these conditions indicated a possible role for this isoform in the regulation of the stress response (Kaufer et al., 1998, Kaufer et al., 2000, Meshorer et al., 2002). In support of this view, transgenic mice overexpressing the AChE-S were found to have a high density of curled neuronal projections, and an accumulation of stress response elements such as heat shock protein 70 within neuronal fragments after neuronal insult, which was not observed in mice overexpressing the AChE-R isoform (Sternfeld et. al., 2000). A similar modulation was later observed within the haematopoietic system (Grisaru et. al., 2001, Deutch et al., 2002, see previous section 1.6.4). Considerable similarity exists between the effects of cholinergic neurological insult and the symptoms of disorders like gulf war syndrome, chronic fatigue syndrome and Myalgic encephalomyelitis (ME) (Clauw, 2001, Kipen and Feilder, 2002). The complexity of these disorders has complicated the identification of the exact cause of these conditions, and as with many other disease states it is likely that multiple factors contribute to their aetiology. Many gulf war veterans were treated with the peripheral acting carbamate cholinesterase inhibitor, pyridostigmine bromide, as a prophylactic in case of chemical warfare (Soreq and Seidman, 2002). Breakdown of

the blood-brain-barrier has been reported as a result of stress (Friedman et. al, 1996, Esposito et. al., 2001), and it has been suggested that the stress of warfare could cause impairment of the blood-brain-barrier allowing the pyridostigmine bromide to act on the central nervous system (Haley et. al., 1997, Relyea et. al., 2001, reviewed in Soreq and Seidman, 2001). A subsequent initiation of the AChE transcriptional feedback response may therefore be causative of the effects of gulf war syndrome and other conditions with similar aetiology.

1.7 AChE and Bone

Support for a role for AChE in bone comes from a number of sources. AChE activity has been observed within developing tissues, in particular limb buds, for many years but was attributed to neuronal innervation, other neuronal sources or vascularisation. Drews and Kussather suggested an embryological role for AChE in chondrogenesis as early as 1971, and Layer and colleagues (1990s) have reported AChE and BChE expression during the embryonic development of chick limbs suggesting a role for BChE in chondrocyte expansion. However, a specific non-cholinergic role for AChE had not been investigated until more recently. Expression of AChE was observed in bone in areas that did not correspond to neuronal innervation, or the neuronal marker MAP2 (Genever et al., 1999). More specifically, expression was identified in osteoblasts and newly formed osteoid at sites of bone formation, in the absence of expression of any other components of the cholinergic signalling machinery, suggesting a non-cholinergic role for AChE in bone (Genever et al., 1999). Subsequently, AChE was found to enhance osteoblast adhesion in vitro, to a similar level of that of established bone matrix proteins, including collagen type I and fibronectin (Genever et al., 1999). Independently, AChE expression was also identified in SaOS-2 osteosarcoma cells, and a role for AChE in osteoblasts was indicated when antisense inhibition of AChE expression induced cell proliferation (Grisaru et al., 1999). In addition, binding motifs for osteogenic factors, including Cbfa-1, 17β-estradiol and vitamin D_3 responsive elements (VDREs) were also located in the upstream extended promotor region of the AChE gene (Grisaru et al., 1999). Application of 17β-estradiol and vitamin D_3; potent regulators of bone

remodelling, caused an increased expression of AChE in osteoblasts (Grisaru et al., 1999).

Organophosphorous pesticides, potent inhibitors of AChE, are components of many commonly used agricultural and household pesticides. Levels of exposure are controlled due to the severe effects of poisoning, however it has been outlined that low-level exposure can also induce toxicity (as discussed in section 1.6.5). Study of farmers using sheep dips containing these pesticides revealed a significant reduction in bone mass and density in individuals following chronic low level exposure (Compston et al., 1999). This alteration in bone mass was noted without any incapacity of individuals due to the effects of inhibitors on cholinergic systems within the CNS or muscles (Compston et al., 1999). More recently, UK Gulf war veterans presenting with symptoms of gulf war syndrome were found to have a similar reduction in bone mass and volume (Compston et al., 2002). However, there are multiple factors involved in bone remodelling and it is difficult to point singularly to exposure to cholinesterase inhibitors during the gulf war, as incapacity was also expected in these individuals. Laws governing pesticide use and testing vary greatly between countries and specific governmental reports are not liable for publication. Regardless, a variety of reports into the effects of these pesticides during embryonic development have identified severe skeletal deformities such as digit fusion, dwarfism, incomplete ossification of pariteal bones and hunched vertebrae in numerous species (Laarson et al., 1974, Beck, 1981, Misawa et al., 1982, Meenley and Wyttenbach, 1989, Cummings et al., 1992). It is also possible that these alterations in the skeleton may be attributable to developmental changes in the surrounding neuronal and muscular tissues. Although these pesticides can inhibit other esterases, and exert deleterious effects upon DNA, these studies supports a role for AChE in bone.

Consistent with a role as a bone matrix protein AChE can modulate cell adhesion, influence cell differentiation by a variety of mechanisms, and has the ability to bind to components of the extracellular matrix (Jones et al., 1994, Small et al., 1995, Bataille et al., 1998, Johnson and Moore, 1999 and Simon et al., 1999, Johnson and Moore, 2003). Intriguingly, osteonectin and AChE are to date the only classically secreted proteins that have been shown to have Ca^{2+}-binding EF-hand motifs (Tsigelny et al., 1998, Tsigelny et al., 2000). Osteonectin is also an established component of the basement membrane, where like AChE it plays an

essential role (Norose et al., 2000, Yan et al., 2003). The collagen tail of asymmetric AChE-S can also associate with heparan sulphate proteoglycans (HSPGs) such as perlecan for localisation at the basement membrane (Peng et al., 1999 Jacobson et al., 2001, Arikawa-Hirasawa et al., 2002). Perlecan and other HSPGs are important components of the bone matrix and alterations in the molecular interactions that regulate HSPG organisation frequently induce osteochondrodysplasias (Forsberg and Kjellen, 2001). Electrostatic interactions of globular AChE with matrix components such as lamanin and collagen have also been observed (Johnson and Moore, 2003). Collectively, therefore the data suggest strongly that AChE is a newly-identified bone matrix protein with fundamental role(s) in bone formation and osteoblast function.

1.8 Aims

The principal aims of this thesis are to :-

- Characterise AChE expression during osteo/chondrogenesis.
- Determine expression and regulation of AChE in osteoblast-like cells and primary osteoblastic cultures.
- Seek a functional role for AChE in bone as a matrix protein.
- Analyse isoform-specific expression of AChE in bone.
- Analyse the effects of aberrations in AChE expression on skeletogenesis in AChE transgenic mice.

Chapter 2 :

General Materials and Methods

2.1 Materials and methods

2.1.1 Materials

Tissue culture plastic ware, media and molecular biology reagents were purchased from Invitrogen. PCR primers were ordered from Sigma genosys, and all other reagents were obtained from Sigma unless otherwise stated.

2.1.2 Cell culture

2.1.2.1 Osteoblast-like cell lines

Osteoblastic cell lines were maintained in a humidified atmosphere at 37°C in 5% CO_2/95% air in culture medium supplemented with 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. Human osteosarcoma cell lines MG63 and TE85 were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal calf serum (FCS). Human osteosarcoma cell line SaOS-2 and murine pre-osteoblast cell line MC3T3-E1 were cultured in α-minimum essential medium (α-MEM) containing 10% FCS. To encourage differentiation of MC3T3-E1 cells toward a more osteoblastic phenotype, medium was supplemented with 100μg/ml L-ascorbic acid phosphate (Wako, Japan) and 5mM β-glycerophosphate as previously described (Franceschi et al, 1994). On reaching confluence, cells were passaged at 1:3 ratio using 0.05% trypsin/0.02% Ethylenediamine tetra-acetic acid disodium salt (EDTA) or media changed every 3-4 days in time course experiments.

2.1.2.2 Isolation of primary rat osteoblasts

Primary rodent osteoblasts were isolated from calvariae taken from newborn (day 2-3) Wistar rats as previously described (Bellows et al, 1986, Malaval et al, 1994). Briefly, calvariae were dissected and cut into small pieces and subjected to sequential collagenase (1mg/ml) and EDTA (4mM, pH 7) digestions at 37°C with agitation at 225 rpm. After an initial collagenase digestion supernatants of subsequent digestions were retained and pooled for centrifugation at 10,000 rpm for 5 minutes. Extracted cells were resuspended and maintained in DMEM containing 10% FCS. Medium was changed after 2-3 hours and cells passaged in a 1:3 ratio once confluent.

2.1.2.3 Isolation of primary human osteoblasts

For isolation of primary human osteoblasts, bone explants of femoral heads from surgical hip replacements (York District Hospital) were maintained as above and cultured in α-MEM, 10% FCS. Media was changed every 5-7 days until confluent when cells were then passaged at a 1:3 ratio. To promote differentiation of primary cells towards a more osteoblastic phenotype, cells were cultured in standard culture medium supplemented with 50μg/ml L-ascorbic acid phosphate, 10nM dexamethasone and 5mM β-glycerophosphate and media changed every 3-4 days in time course experiments.

2.1.2.4 Colony forming unit - fibroblasts (CFU-Fs)

Whole rat marrow extracted from tibiae of 2-month-old male Wistar rats (approximately 200g) was used to isolate CFU-Fs as previously described (Maniatopoulos et al, 1988). Briefly, clean tibias were cut at the growth plate and microfuged at 2000g for 30 seconds in Eppendorphs. Marrow extracts were resuspended in 500μl of DMEM containing 12% heat inactivated FCS and supplemented with 100μg/ml L-ascorbic acid phosphate, 100U/ml penicillin, 100μg/ml streptomycin, 10nM dexamethasone and 5mM β-glycerophosphate. The cell suspension was then diluted in a further 20 ml of medium to provide a suspension of approximately 10^4-10^5 cells/cm^2 which was distributed to 6 well or 24 well plates with cover slips and maintained as described for all other cells. After 5 days in culture non-adherent cells were removed and fresh medium added. Medium was changed every 3-4 days until day 15 in culture when colonies were fixed in 70% ethanol for 5 minutes and used for analysis of osteoblastic markers and colony formation.

2.1.3 Analysis of markers of bone formation

2.1.3.1 Alkaline phosphatase assay

Cell associated alkaline phosphatase activity was determined using a colorimetric microplate activity assay. Cells fixed for 5 minutes in 95% ethanol were assayed for activity using a buffer containing 0.02M sodium hydrogen carbonate ($NaHCO_3$), 3mM magnesium chloride ($MgCl_2$), and 1mg/ml paranitrophenol phosphate (Sigma 104

paranitrophenol phosphate, PNPP) pH 9.5. Colour development was measured at 405nm after 2 minutes when 200µl aliquots were removed to 96 well plates for absorbance determined using a Dynex microplate reader and the revel software package.

2.1.3.2 Stain for alkaline phosphatase

Cells were fixed with 95% ethanol for 5 minutes. Fixed CFU-F colonies were then stained using Fast Red TR salt (1mg/ml) in the presence of alpha napthol phosphate (0.05mg/ml) in Tris-HCl buffer (0.08M) pH 8.0 for 30 minutes. After imaging, colonies were destained in ethanol for 18 hours.

2.1.3.3 Stain for mineralisation and calcium content

Fixed cells as above were incubated with Alizarin red (1mg/ml) pH 6.2 for 30 minutes, stain removed and cells washed in dH_20 and then allowed to dry. After image analysis cultures were demineralised using 5% perchloric acid to remove staining.

2.1.3.4 Stain for total collagen

Fixed cells as above were incubated overnight (18 hrs) in Direct Red (1mg/ml) in saturated picric acid. Cells were washed in dH_20 and allowed to dry. Removal of Direct Red to allow further staining was done by elution of dye with 0.5ml 0.1M NaOH/Methanol (50:50) for several minutes.

2.1.3.5 Stain for total cells

Incubation of fixed cells was carried out on a shaking platform for 5-30 minutes with 1mg/ml Methylene blue in 10mM boric acid pH 8.8 with NaOH (borate buffer). Dye was removed and colonies washed with dH_2O.

2.1.3.6 Analysis

Images of colonies were captured using a black and white digital camera and colonies analysed, the Leica Qwin image processing and image analysis package.

2.1.4 Analysis of protein expression

2.1.4.1 Collection and analysis of protein samples

Whole cell lysates were obtained by treating cell layers with PBS containing 0.1% triton X-100 and 0.05% protease inhibitor cocktail (Calbiochem) on ice for 10 minutes, and harvested using a cell scraper. To remove cell debris, lysates were aspirated through microlance needles (Beckton Dickinson) and then centrifuged at 10,000g for 10 minutes at 4°C, and supernatants removed to a fresh eppendorfs for storage at –20°C. Total protein content was determined by the colorimetric BCA assay (cat No.23227, Pierce). Briefly 25μl samples of whole cell lysates were incubated with reaction mixture for 30 minutes at 37°C in 96 well plates and absorbances were read at 570nm using a dynex microplate reader and the revel documentation system as before. Production of a standard curve by parallel assay of know protein concentrations of bovine serum albumin (BSA) allowed for the determination of lysate total protein concentration.

2.1.4.2 De-glycosylation

For analysis of AChE N-glycosylation state, 50μg whole cell lysates was denatured at 100°C in the presence of 0.1% sodium dodecylsulfate (SDS) and 50mM β-mercaptoethanol for 5 minutes. After addition of nonylphenoxy polyethoxy ethanol (NP-40), lysates were treated with 2U/ml recombinant *flavobacterium meningosepticum* N-glycanase (E-5006, Glyko) and incubated for up to 4 hours at 37°C before diluting and loading onto gels as described below.

2.1.4.3 Western blot analysis

For western blot analyses, samples were diluted with 2% SDS, 50mM β-mercaptoethanol and 1% bromophenol blue loading dye, and between 5-10μg loaded onto 10% SDS/ polyacrylamide gels (5% stacking, 10% resolving) for separation and subsequent transfer to PVDF membrane Hybond P (Amersham Biosciences). Each gel was run with a prestained protein kDa marker (Biorad). Prior to immunodection, blots were blocked with 4% marvel skimmed milk diluted in Tris buffered solution with 0.1% trition X-100 (TBS-T) for 1 hour on a rocking platform. Immunodetection for AChE expression was performed using a monoclonal antibody identifying all AChE isoforms (clone 46, BD Transduction Laboratories, A27320, see table 2.1), followed by

incubation with a horseradish peroxidase (HRP) conjugated polyclonal secondary antibody (Sigma, A-0412). Blots were developed by chemifluoresecent detection using ECL reagent (Amersham Biosciences), followed by exposure to Hyperfilm ECL (Amersham Biosciences). Films were developed using a Xograph machine.

2.1.5 Analysis of mRNA expression

2.1.5.1 Isolation of total ribonucleic acids (RNA)

RNA was isolated from cells and whole tissues using the TRIzol protocol. Cell layers were treated with TRIzol (Invitrogen) at room temperature for 5 minutes and harvested using a cell scrapper. Whole tissues (brain or calvarial bone) were collected and stored in RNAlater (cat. No. 7020, Ambion) and stored at -20°C prior to disruption using a dismembrator. Samples were snap frozen and placed into dismembrator capsules containing ball bearings that had been pre-submerged in liquid nitrogen. Tissues were dismembrated at 2000rpm for 30 seconds. Powdered tissues samples were then treated with trizol until the temperature reached ambient levels. Samples in TRIzol were either stored at -80°C or RNA extracted immediately. To separate protein and RNA fractions 200μl of chloroform was added per 1ml of TRIzol and centrifuged at 12,000rpm at 4°C for 15 minutes in a microfuge (IEC micromax RF). The upper aqueous phase was then transferred to clean eppendorfs and RNA precipitated by the addition of isopropanol (4°C for 15 minutes), followed by centrifugation at 10,000rpm for 10 minutes at 4°C. After removal of supernatant, the pellet was air dried and resuspended in RNA secure (7005, Ambion). RNA concentration and purity was determined by ratio of 260/280/320 absorbances read using a quartz cuvette and a UV spectrophotometer (Ultrospec 2000, Amersham Biosciences). Contaminating DNA was removed using the DNA-free kit (1906, Ambion) where samples where incubated with DNase in a solution of 10mM Tris-HCL pH 7.5, 25mM $MgCl_2$, 1mM $CaCl_2$ at 37°C for 20 minutes.

2.1.5.2 cDNA synthesis

Synthesis of double stranded cRNA was performed using random hexamer primers (oligo dTs) and 5-10μg of total RNA. After preincubation of primers with RNA for 3 minutes at 70°C, cDNA synthesis was carried using superscript II in the presence of PCR buffer, 25mM $MgCl_2$, 10mM 2'-deoxynucleoside 5'-triphosphates (dNTPs), and

0.1mM dithiothreitol (DTT) at 42°C for 1 hour. The reaction was terminated using RNase-H at 70°C for 15 minutes, and cDNA stored at -20°C. Parallel control cDNA samples were prepared using the smae RNA but in the absense of superscript (no RT). cDNA quality was determined by RT-PCR using primers for house keeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, see table 2.2 for sequences) and analysed by agarose gel electophoresis as below.

2.1.5.3 Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine expression of AChE mRNA in a variety of cDNA samples and in comparison to markers of osteoblast differentiation (see table 2.2 for primer sequences). Primers were designed from complete CDs using the primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) primer design package, when possible sequences of various species were aligned using multialign (web address) and primers designed in homologous regions. RT-PCR amplification of cDNA was carried out using platinum Taq polymerase in the presence of PCR buffer, 50mM $MgCl_2$, 10mM dNTPs, 10mM sense primer and 10mM antisense. No RT samples were used in parallel as controls for genomic contamination of cDNA. Primer pairs producing amplification products less than 400bp or high in their GC content were also performed in the presence of dimethyl sulfoxide (DMSO). Appropriate annealing temperatures were determined by performing RT-PCR with annealing temperatures varying between 10°C of the primer pair's lowest melting temperature using a gradient block (Hybaid, PCR express). When possible 1 step RT-PCR was carried out where annealing and amplification temperatures were near to 72°C, and amplification was performed for between 30 and 36 cycles using MJ research Pelyier Thermal Cycler (PTC) 200. RT-PCR products were then run on 1-1.5% agarose gels containing ethidium bromide and product size determined against a 100bp ladder (hyperladder IV, bioline), by UV illumination using an alpha manager 2000 gel documentation system.

2.1.6 Histochemistry

2.1.6.1 Paraffin embedding

Fore and hind limbs were dissected from day 3 postnatal rat pups and skin removed before fixation in 4% paraformaldehyde for 48 hours. Bones were decalcified in a 14% EDTA solution, pH 7.4, for up to 1 week changing the solution daily. For in situ hybridisations, all solutions were prepared using Diethyl Pyrocarbonate (DEPC) treated water (1ml/L DEPC with stirring overnight). Limbs were then processed through serial dilutions of methanol and chloroform before clearing in xylene using a Tissue-tek® VIP tissue processor (Sakura). After immersion in paraffin wax under vacuum, samples were embedded and mounted onto blocks. 10μm sections were cut using a Leica RM2165 microtome and mounted onto polylysine slides (BDH) which were then air-dried.

2.1.6.2 Immunolocalisation

2.1.6.2a Cryosections

Immunolocalisation for AChE on frozen sections was carried out essentially as described previously (Genever *et al*, 1999). Briefly, tissue samples were dipped in 10% polyvinyl acetate (PVA) and snap frozen using isopentene at -80°C before mounting with PVA onto brass chucks and 7-10μm sections cut using an OFT 5000 Bright cryostat, and collected on polylysine slides (BDH). Sections were fixed in 4% paraformaldehyde for 5 minutes and incubated for 30 minutes with hydrogen peroxidase to block endogenous peroxidase activity. To prevent non-specific antibody binding, sections were incubated with 10% goat serum for 30 minutes. AChE expression was localised using anti-AChE antibody (BD Transduction labs) for 30 minutes, biotinylated horse anti-mouse secondary antibody (1:200 dilution) for 15 minutes, and avidin-biotin peroxidase reagent (Vector Labs) for 20 minutes. A mouse IgG was used as an antibody control at the primary antibpdy stage. Incubation with 0.5mg/ml diaminobenzidine and 0.3% peroxidase substrate allowed visualisation of peroxidase substrate, sections were counterstained with haematoxylin and mounted with 9-1 glycerol/ Phosphate Buffered Saline (PBS). Alkaline phosphatase activity was determined on adjacent sections by enzyme histochemistry. Staining was visualised

using standard light microscopy with a Leica DMLA microscope, digital camera and IM50 documentation software.

2.1.6.2b Paraffin sections

Paraffin sections were cleared with xylene and dehydrated through decreasing ethanols. Non-specific peroxidase activity was inhibited by incubation with 3% hydrogen peroxide in methanol for 10 minutes. Prior to immunolocalisation, heat induced antigen retrieval was carried out by emersion of sections in a 10mM citrate buffer brought to a rapid boil by placing in a microwave at maximum power repeatedly for 10 minutes. Slides were then blocked in 10% donkey/horse serum with 0.3% triton/0.05% tween for 1 hour to prevent non-specific binding. Immunolocalisation was performed overnight using either goat polyclonal antibody directed against the AChE-S C-terminal peptide (ASP) (1:500, Santa Cruz, SC-6430), goat polyclonal antibody directed to the common domain of AChE (1:500, Santa Cruz, SC-6432 and SC-6431), or rabbit antibody raised against mouse AChE-R, (3μg/ml, kindly provided by Hermona Soreq, Jerusalem). IgG antibodies raised in the same animal as the primary antibodies used were used as controls. Sections were incubated for 1 hour with biotinylated donkey anti-rabbit (Chemicon), or donkey anti-goat (Jackson) secondary antibodies. Detection and visualisation was carried out as for cryosections (see above), and sections were counterstained with haemotoxylin and mounted with immunomount (Sigma).

Type	**Company cat. No.**	**Type**	**Raised against/ clone**	**concentration**
AChE	BD Transduction laboratories 610267	Mouse IgG1	clone 46 peptide 411-601 amino acids	WB – 1:2000 IH – 1:500 of 1mg/ml
AChE	santa cruz SC-6432 and SC-6431	affinity purified goat polyclonal	E-19 N-19 (N-terminus)	IH -1:100-1:1000 of 200μg/ml
AChE-S c-terminal peptide	santa cruz SC-6430	affinity purified goat polyclonal	C-16 (c-terminal peptide)	IH - 1:100-1:1000 of 200μg/ml
AChE-R	non-commercial	affinity purified rabbit polyclonal	human recombinant AChE-R	IH - 3μg/ml
msx-2	MBL M027-3	Mouse IgG2	2E12	IH - 5μg/ml
GAPDH	Advanced immunochemical corporation	mouse monoclonal IgG2	6C5 rabbit GAPDH	>1mg/ml

Table 2.1 Table of primary antibodies.

Gene	**+ position**	**Accession No. and species cross reactivity**	**Sequences**	**product size base pairs (bp)**
AChE	+1522	as below	5'CGGGTCTACGCCTACG TCTTTGAACACCGTGCTT C'3	
AChE-S	-2003	as below	3'CACAGGTCTGAGCAGC GATCCTGCTTGCTG'5	481bp
AChE-E/R	-1917	as below	3'GGTTACACTGGCGGGC TCC'5	396/474bp
AChE-R	-1939	x56518 human, rat, mouse	3'ATGGGTGAAGCCTGGG CAGGTG'5	418bp
ColQ	+217	AF007583 human,	5'CCGCTTCTCTCCCCAG AC3'	434bp

	-650	rat, mouse	3'TTTGGACCCATTTCAC CTTTC'5	
BChE	+53 -580	Mouse M99492 AF244349 Rat	5'TTGGGAAGTCACACAC TGAAGAAG'3 5'GATCAAATAAACCCAT GTTTCCTG'3	527bp
Neurexin α	+4175 -4667	m96374 rat, human	5'CTGTGACTGTAGCATG ACTTCCT'3 3'CCCTTTGTCGAGTAGC CAT'5	492bp
Neurexin β	+978 -1113	m96375 rat, human	5'GCACCACATCCACCAT TTCC'3 3'ATGTAATTTGTCCACC ACCTTTG'5	135bp
Neuroligin 1	+762 -1497	u22952 rat, human	5'TCTACCACCATTCCAT CTTTCTCCA'3 3'CTTGCCAACACACTCC CATC'5	735bp
Neuroligin 2	+2962 -3633	u41662 rat, human	5'CTTCTCTCCATCCCTTT GGG'3 3'GTTATTTCCTTGCTGCT CC'5	671bp
Osteopontin	136 520	rat (M99252) and mouse (J04806)	5'TGACCCATCTCAGAAG CAGA'3 3'AGGTCCTCATCTGTGG CATC'5	384 bp
Osteonectin	+6 -422	human (BC008011), rat (D28875) and mouse	5'GGCCTGGATCTTCTTTC TCCTTTG'3 3'AGCTTGTGGCCCTTCTT GGTG'5	416 bp
Twist	46 532	human, rat and mouse	5'AGCCTGAGCAACAGCG AGGA'3 3'TGCAGCTTGCCATCTT GGAGT'5	486 bp
GAPDH		BC026907 human, rat and mouse	5'GGTGAAGGTCGGWGT CAACGG'3 3'GGTCATGAGYCCTTCC ACGAT'5	519 bp

Table 2.2 Table of PCR primers.

probe target	Sequence	modifications
mE5 AChE-E	5'- GAGGAGGAAAAGGAAGAAGAGGAGGGACAGG GCUAAGUCCGGCCCGGGC-3'	biotinylated (50mer)
mE6 AChE-S	5'- CCCCUAGUGGGAGGAAGUCGGGGAGGAGUGG ACAGGGCCUGGGGGCUCGG-3'	biotinylated (50mer)
mI4 AChE-R	5' AACCCUUGCCGCCUUGUGCAUUCCCUGCUCCC CCCACUCCAUGCGCCUAC-3'	biotinylated (50mer)

Table 2.3 Table of in situ hybridisation probes

Chapter 3 :

AChE a Secreted Matrix

Protein in Bone

3.1 Introduction

3.1.1 Osteoblasts and bone matrix proteins

The identification of AChE expression in bone in the absence of cholinergic signalling may seem somewhat unusual. However, localisation of AChE expression in bone, specifically to osteoblasts and newly formed bone matrix at sites of bone formation suggested a role for AChE in bone as a matrix protein. In bone, matrix proteins are of significant importance both during remodelling of mature tissues and during osteo-chondrogenesis. Although originally bone matrix proteins were thought to simply play a structural role in bone formation and mineralisation, the significant effects upon osteoblast adhesion, migration and chemotaxis, and the subsequent effects upon osteoblast function and differentiation demonstrate the complexity between osteoblasts and the extracellular environment within which they reside. Although it is known that the collagenous proteins are essential in the make up of the bone matrix, the numerous non-collagenous bone matrix proteins expressed by osteoblasts have also proved to be invaluable components of the bone matrix that impact upon osteoblast differentiation, function and matrix mineralisation.

3.1.1.1. Non-collagenous bone matrix proteins

Analysis of the non-collagenous component of the bone matrix has revealed high levels of expression of matrix proteins such as osteocalcin, osteopontin, osteonectin, tenascin-C and fibronectin but to name a few (Delany et al., 2000, Moursi et al.,1996, Mackie, 1994, Mackie and Tucker, 1992, Young et al., 1992). Although many of these proteins are not expressed exclusively in bone they have proved to be of fundamental importance in normal bone formation and development and often display multiple functions. Osteopontin is a secreted, aspartate rich glycoprotein of approximately 60-75 kDa, containing multiple regions typical of bone matrix proteins (Boskey, 1992). Expression of osteopontin has been identified in the osteoid at sites of preosteoblast expansion and migration, and is expressed abundantly by osteoblasts during the early phases of differentiation and mineralisation (Young et al.,1992). Notably, osteopontin is expressed by osteocytes and is often found within the well-established bone matrix. Osteopontin may immobilise within the extracellular matrix by attachment to structural collagenous proteins and hydroxyapatite (Boskey, 1992 Giachelli and Steitz, 2000, Giachelli and

Steitz, 2000, Sodek, Gnass and McKee, 2000). Heavy phosphorylation of osteopontin yields a highly acidic protein, this along with putative calcium binding sites provide osteopontin with a great affinity for hydroxyapatite (Giachelli and Steitz, 2000, Young et al.,1992). However in solution and in vitro osteopontin was found to be an inhibitor of hydroxyapatite crystal formation (Boskey, 1992). It has therefore been postulated that osteopontin could act to limit hydroxyapatite crystal formation when localised to the mineral matrix (Giachelli and Steitz, 2000). Osteopontin has also been implicated in the chemotaxis and migration of both osteoblasts and osteoclasts, believed to encourage the migration of osteoblast to sites of bone resorption. The common arginine-glycine-aspartate (RGD) sequence is found within cell attachment proteins such as fibronectin, enabling interactions with cell surface expressed integrins, consequently activating intra-cellular signalling system, and mediating osteoblast substrate attachment (Butler et al., 1996). Osteopontin expressed and localised within the matrix at regions of bone formation and resorption can mediate cell attachment of both osteoblasts and osteoclasts via this RGD domain (Giachelli and Steitz, 2000). Osteoblasts produce a number of RGD containing proteins with the ability to mediate cell attachment and signalling. Bone sialoprotein and bone acidic glycoprotein-75 (BAG-75) RGD containing proteins expressed also in bone, show considerable similarity to osteopontin in structure and function, yet display different expression profiles suggesting a specialisation of such proteins to different types of mineralisation or areas of bone formation (Young et al., 1992). Bone gla protein (BGP) for example is predominantly expressed in mineralising tissues, in particular bone, and has been implicated as the principle mediator of hydroxyapatite crystal nucleation in bone (Ganss et al.,1999) (see sections 1.1.3 and 1.3.4).

Expression of osteonectin was also identified during early osteoblast differentiation, where it is limited to osteoblasts and their precursors specifically at sites of matrix deposition, in newly formed osteoid and chondroid. One of the most abundantly expressed non-collagenous bone matrix proteins, osteonectin has a variety of properties that may influence osteoblast activity. Also known as SPARC (secreted protein acidic and rich in cystiene), and BM-40, osteonectin is a 38 kDa protein containing a number of functional domains, has a high affinity to calcium and phosphate, can bind numerous other matrix proteins as well as bind and modulate the activity of certain growth factors (Murphy-Ullrich, 2001,Yon and Sage, 1999).

Osteonectin contains both low affinity ($k_d \sim 10^{-3}$), and high affinity ($k_d \sim 3 \times 10^{-7}$) EF-hand calcium binding motifs which are characteristic of calcium binding proteins such as calmodulin (Murphy Ullrich, 2001, Young et al.,1992, Boskey, 1992). Although osteonectin-null mice develop a normally patterned skeleton, the late onset of osteopenia with significantly reduced bone mass upon reaching adult life suggest a significant role for osteonectin in bone mineralisation (Delaney et al., 1999). In addition, osteonectin can bind to multiple components of the extracellular matrix, most specifically collagens, and is thought possibly to play a role in collagen fibrillogenesis, although this is still to be investigated (Boskey, 1992). Osteonectin is known to play a significant role in matrix organisation in various tissues. Counter adhesive properties have been linked to osteonectin when not immobilised within the matrix, inhibiting cell spreading and causing disassembly of focal adhesions in osteoblasts and other cells (Yan and Sage, 1999, Murphy-Ullrich, 2001 and Bradshaw and Sage, 2001). As well as binding and concentrating growth factors platelet derived growth factor (PDGF) and vascular-endothelial derived growth factor (VEGF) within the ECM, osteonectin also contains a follastatin-like domain that can inhibit TGF-β-like growth factors activin and inhibin (Yan and Sage, 1999). Therefore, osteonectin has the potential to control osteoblast differentiation and activity via a variety of mechanisms, as well as playing a significant role in bone mineralisation.

Matrix proteins in bone hold a number of important functions. Ultimately, they enable the establishment of a mineralised tissue. However, they also perform a number of other important functions. Bone matrix proteins have been suggested to influence osteoblast proliferation, differentiation, migration and adhesion both during development and in mature tissues. More specifically during development cellular interactions are fundamental to normal skeletal patterning during both osteo-chondrogenesis.

3.1.2. Cellular interactions during tissue development

The patterning and differentiation of the developing embryo is a complex tightly regulated process that relies heavily on signalling systems that are activated by interactions between receptors and ligands during cell-cell and cell-matrix

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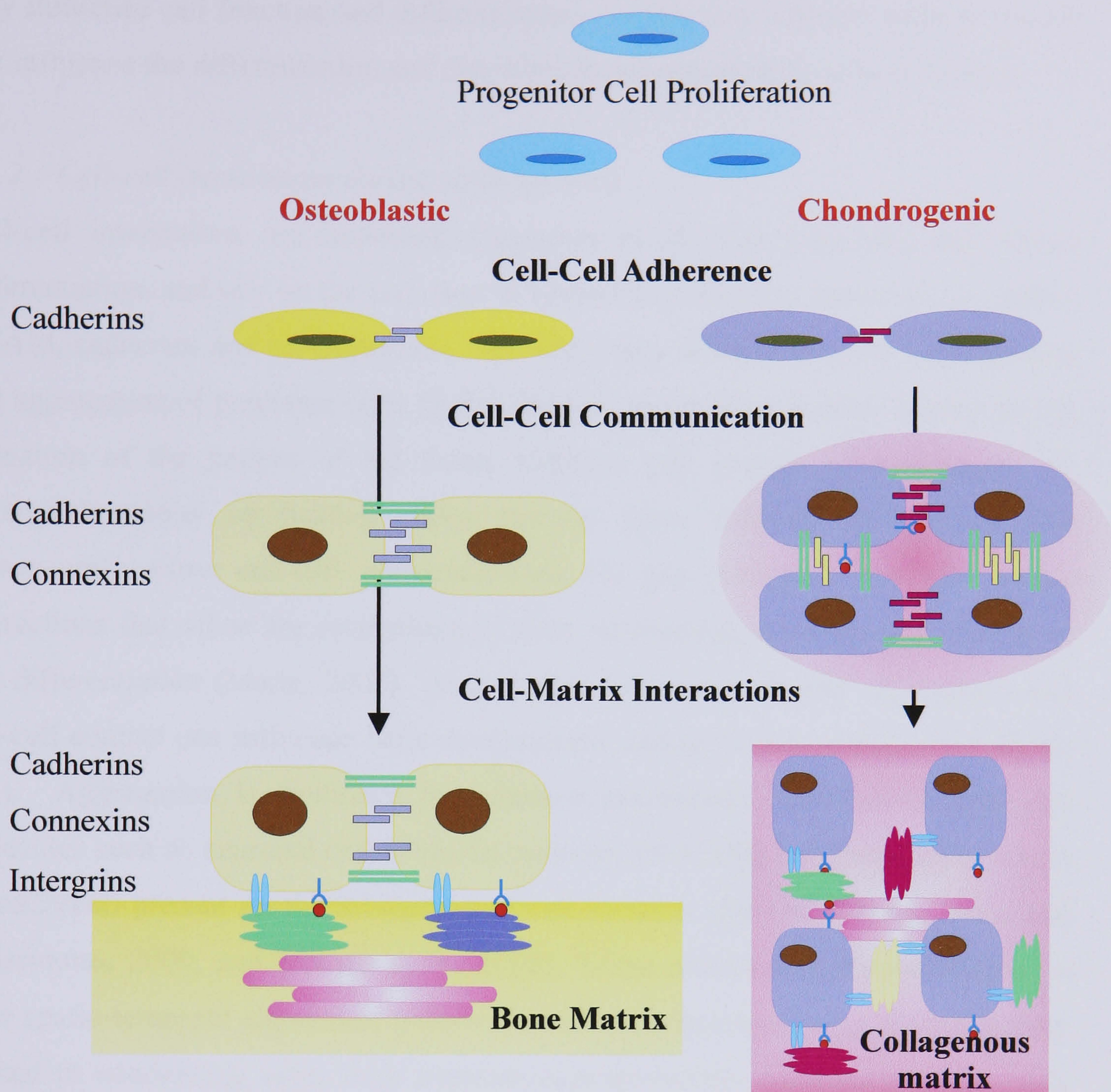


Figure 3.1.1 Cell-cell, cell-matrix interactions during osteoblast and chondrocyte differentiation

During osteoblast differentiation a number of essential cell-cell and cell-matrix interactions occur. Osteoprogenitors cell expansion is followed by osteoblast-osteoblast adherence enabled by interactions between cell surface proteins such as cadherins creating cell aggregation. Adherence can also influence cell phenotype via the activation of cell signalling pathways either directly through cadherins or the bringing together of other cell surface molecules and their receptors. This close proximity of cells also allows for cell-cell communication by gap junctions created by interacting connexins presented on adjacent cells. Cell-matrix interactions permit osteoblast adherence to the matrix as cell surface expressed intergrins bind to matrix components such as fibronectin, Collagen type I and osteopontin. This in turn can activate intracellular signalling cascades with down-stream effects upon cell differentiation, as well as placing cells juxtaposed to growth promoting agents held within the cell matrix.

interactions (see figure 3.1.1). Cell-cell adherence, condensation and aggregation, are prerequisites for organogenesis. Additionally, this solid-state environment places cells in the proximity of growth factors for extracellular signalling. Cell-cell and cell-matrix interactions can also directly trigger intracellular signalling pathways that may influence cell function and differentiation. In this way adjacent cells or tissues can influence the differentiation and development of surrounding cells or tissues.

3.1.2.1 Cell-cell interactions during skeletogenesis

Cell-cell interactions are important regulators of skeletal patterning and tissue differentiation, and rely on the activities of several key adhesion molecules including NCAM, cadherins and proteoglycans. As with many other tissues the condensation and aggregation of precursor cells, in this instance mesenchymal cells, allows for the formation of the pattern of the future skeleton (see section 1.3.3, Chapter 1). Intramembraneous ossification relies heavily upon cell-cell interactions that subsequently allow cell-cell communication via gap junctions, and cell matrix interactions that allow for recognition of their surrounding environment leading to cell differentiation (Marie, 2002). In a similar way mesenchymal aggregation and cell-cell contact can influence bone development during chondrogenesis (see figure 3.1.). Aggregation is facilitated by adhesive interactions mediated by adhesion molecules such as neuronal cell adhesion molecule (N-CAM) and neuronal cadherin (N-cadherin) present on the mesenchymal cell surfaces (for reviews see Perris and Perissinotto, 2000, and DeLise et al., 2000). These adhesion molecules display a clear spatio-temporal expression pattern during skeletogenesis, being predominantly limited to condensing cells, their expression progressively decreasing as the cells become more differentiated, for example in the cartilaginous condensations or mesenchymal condensations forming the calvaria (Marie, 2002, Serge et al., 2000).

Cadherins are members of a super-family of Ca^+ dependant transmembrane molecules that in conjunction with the Ca^+ independent CAMs can modulate cell-cell adhesion in a variety of developing tissues (DeLise et al., 2000). Six sub-classes of cadherins exist, the most common of which are the classical cadherins type I and II. Type I cadherins have the ability to bind to other cadherins expressed on adjacent cells via a conserved extracellular domain containing a His-Ala-Val (HAV) motif (Serge et al., 2000). Cadherins may therefore act in both capacities as ligand, or as a receptor in the absence of other receptor molecules. As transmembrane proteins,

cadherins not only have the ability to merely mediate cell-cell association via their extracellular domains, but can also modulate the formation of an organised cytoskeleton, via binding of intracellular domains to cytoskeletal elements including β and γ catenins (Ferrari et al., 2000, DeLise et al., 2000, and Marie, 2002). β-Catenin also plays a role in complex intracellular signalling systems such as those of the Wnt signalling pathway, and notch signalling which represent important signalling systems involved in early cell differentiation and tissue development in a variety of organs including bone (for review see DeLise et al.,2000). In this way it is thought that cadherin-catenin binding may diverge upon intracellular signalling pathways, subsequently influencing downstream gene expression and cell differentiation. In addition to N-cadherin and N-CAM, developing bone tissues express a moderate repertoire of other cadherin and CAM molecules. Cadherin 11 has been identified in condensing mesenchymal cells during chondrogenesis and osteogenesis (Oberlender and Tuan, 1994, Marie, 2002). Cadherin-4, cadherin-6 and epithelial cadherin (E-cadherin) have also been identified in osteoblastic cells from different sources, with distinct expression patterns (Serge et al., 2000). Expression profiles of N-cadherin and cadherin-11 display the most similarity, suggesting that there may be a functional-overlap between cadherin interactions in development. Most cadherin knock out mice have proved to be lethal, apart from the cadherin-11 null mice that appear to have no overt skeletal phenotype (Kawaguchi et al.,2000). However, upon closer inspection cadherin-null mice do display a marked reduction in bone density at sites of intramembraneous ossification such as the calvaria, and cortical bone at the metaphyses (Kawaguchi et al., 2000, and Marie, 2002). Furthermore, characterisation of cadherin 11 expression in bone revealed that expression decreases with age (Groomer et al., 1998). This suggests that cadherin-11 in particular may play a role in early osteoblast differentiation, and suggests a functional overlap of family members.

Cell adhesion molecules (CAMs) such as N-CAM are members of the large immunoglobulin domain superfamily of Ca^{+} independent cell membrane proteins. N-CAM has proved to be of significant importance in cell-cell association during bone development. N-CAM expression follows a similar pattern to that of N-cadherin, and is lost in mature cartilage and bone matrix. It is believed that like N-cadherin, N-CAM may play a role in early osteoblast differentiation and cell-cell

communication (DeLise et al., 2000). A variety of N-CAM isoforms can be produced by alternative gene splicing and post-translation glycosylation. Inhibition of N-CAM expression in developing cartilaginous tissues leads to a reduction in cell aggregation and a consequential loss of cell differentiation (DeLise et al., 2000). Vice versa over-expression of N-CAM in vitro leads to increased mesenchymal cell aggregation and differentiation (Widelitz et al., 1993).

In addition to the well-characterised interactions mediated by CAMs and cadherins, it has become evident that bone cells express a large number of other cell surface proteins that may interact with cell surface expressed receptors on adjacent cells. In particular, cell surface proteoglycans have proved to be of particular importance in embryonic development (Schachner and Martini, 1995, Selleck, 2000, Turnbull et al., 2001). Glycan moieties of cell surface expressed proteins can present as novel mediators of cell-cell interactions acting as recognition molecules, or modifying proteins to allow for ligand-receptor interactions (for review see Sellek, 2000). The HNK-1 epitope has been identified on glycans found on a number of cell adhesion molecules and in many cases is localised to glycan moieties (Schachner and Martini, 1995). Removal of these glycans leads to a reduction in cell-cell and cell-matrix interactions. For example the removal of the oligosaccharide part of osteonectin reduces its capacity to cause cell matrix interactions (See section 3.1.2.1, Xie and Long, 1995). More specifically, modified or specialised glycan moieties can be characteristic of certain families of proteins that have overlapping roles. Heparan sulphate proteoglycans are proteins with glycan moieties that are highly sulphated (Sellek, 2000, Turnbull, Powell and Guimond, 2001). These proteins represent a large family of surface expressed and matrix proteins that play fundamental roles in matrix organisation and cell-matrix association. In developing bone numerous sulphated proteins such as decorin, perlecan, and chondroitin sulphate are expressed (Sellek, 2000, Turnbull et al., 2001). Proteins involved in matrix mineralisation (bone sialoprotein, matrix gla protein, osteocalcin) possess highly sialyated glycan moieties (Boskey, 1992). Sialyation is thought to contribute to the acidic nature of these proteins in relation to their role in matrix mineralisation (Boskey, 1992, Young et al., 1992). Cell-cell contact is an essential initiator of synthesis and secretion of a specialised extracellular matrix by osteoblastic and chondrogenic cells (Marie et al.,2002).

3.1.2.2. Cell-matrix interactions during skeletogenesis

The extracellular matrix surrounding condensations of mesenchymal cells during chondrogenesis and osteogenesis displays a regulated pattern of expression. During mesenchymal aggregation of developing limb tissues, a matrix composed primarily of collagen type I, hyaluronan, and non-collagenous matrix proteins such as tenascin and fibronectin is produced (De-Lise et al., 2000). The extracellular matrix can influence cell differentiation via a variety of mechanisms. In the developing limb, where cartilaginous matrix is constantly being remodelled, cell phenotype may be directly affected by change in cell surface protein expression (De-Lise et al., 2000, Marie et al., 2002). Collagen type I, and non-collagenous matrix proteins like fibronectin, osteopontin and tenascin bear motifs such as the RGD domain that is capable of binding to integrins expressed on the surface of osteoblasts and mesenchymal progenitor cells (Makie and Turner, 1992, Moursi et al., 1996, Butler et al., 1996, Yan and Sage, 1999). Integrins are transmembrane receptors composed of α and β subunits forming heterodimeric complexes that bind to the extracellular matrix via their extracellular domains (Boudreau and Jones, 1999, Damsky, 1999, Ridley, 1999). The interaction of integrins with the cytoskeleton via their intracellular domain can also provide a physical link between the inside and the outside of the cell (Bourdreau and Jones, 1999). To date 16α, and 8 β subunits have been identified which can potentially produce a plethora of complexes that are capable of interacting with a large variety of extracellular matrix proteins (Bourdreau and Jones, 1999). Binding of integrins to matrix components enables cell adhesion to the extracellular matrix; as well as leading to the activation of intracellular signalling pathways that promote cell differentiation (Gronthos et al., 1997, De-Lise et al., 2000, Cheng, 2001). Activation of intracellular signals by integrins is thought to be mediated by the intracellular portion of the integrin molecules. Attachment of the intracellular component of integrins to cytoskeletal elements can influence cell phenotype through a number of mechanisms. Firstly, it is known that integrin binding to the extracellular matrix can directly activate intracellular signalling via the FAK and MAPK pathways to induce downstream signalling stimulating cell proliferation or cell differentiation (Ridley, 1999, Bourdreau and Jones, 2000). In

addition, interaction of integrins with cytoskeletal elements may stabilise or alter the cytoskeletal organisation to enable cell surface presentation of other receptor complexes, or allow the activation of other receptors by placing them in the proximity of activity altering proteins i.e. FGFRs (Boudreau and Jones, 1999). Furthermore, many ECM components have the ability to bind to a variety of different integrin complexes e.g. fibronectin recognises 6 different integrins, therefore the same specialised ECM may trigger unique signalling pathways in different cell types (Moursi et al., 1996). Integrin binding has proven to be of significant importance to both embryonic and mature bone development, osteoblasts expressing 4 of the fibronectin binding integrins alone ($\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 1$) (Moursi et al., 1996, Gronthos et al., 1997). Fibronectin expression is also abundant in the developing cartilaginous condensations of the limb (DeLise et al., 2000). As well as providing a base for cell attachment, and further matrix assembly, proteins found within the matrix can anchor growth-promoting agents to enable interaction with cell surface receptors. In some circumstances anchoring within the matrix or attachment to matrix components is fundamental to the signalling process. For example it is essential that FGFs are attached to heparin sulphate proteoglycans found within the cell matrix to enable docking and interaction with the cell surface expressed FGFR ligands (McIntosh et al.,2000, Turnbull et al., 2001).

3.1.2.3. Matrix organisation

Remodelling of the extracellular matrix is also an important process during the development of tissues (Blobel, 2000, Sage, 2001, Murphy-Ullrich, 2001). Importantly in tissue patterning and modelling, it is not just the ability of a cell to bind to ECM but also to be able to unbind, and migrate to specific regions (Murphy-Ullrch, 2001). Cells can do this by a number of means. Firstly, cells of developing tissues can express proteins that promote cellular de-attachment (Sage, 2001). For example hyaluronan is associated with cell migration and movement, and is part of the machinery involved in the migration of neural crest cells as well as during chondrogenesis (Perris and Perissinotto, 2000). Expression of ECM components such as hyaluronan is therefore integral to early tissue patterning. Secondly, cleavage of cell surface molecules by MMPs enables cellular de-attachment and promotes cell migration (Blobel, 2000). Later in cell development, aggregation and

formation of 'the solid-state environment' takes precedence in tissue differentiation. Consequently, expression of hyaluronan is progressively lost in the condensing mesenchyme of the developing cranial and long bones (De-Lise et al., 2000). Additionally, condensations of mesenchymal cells maintain high levels of hyaluronidase activity that prevent further cell migration, allowing for the creation of further cellular interactions. Expression of MMPs can also play an important role in creating cell matrix interactions during remodelling in which the matrix is broken down to make way for the production of a more mature organised matrix of proteins that will interact with different cell-surface expressed molecules (Blolbel, 2000). In developing bone this can be important at many stages. For example, as cartilaginous elements become progressively more mature they change the collagenous make up of their extracellular matrix. MMPs breakdown early collagens such as type I to make way for collagens type II, type IV and type V (Blobel, 2000). These diverse collagens have varying affinities for specific integrin complexes, and other cell-surface molecules that may activate signalling pathways distinct to those of the less mature matrix. Non-collagenous matrix protein expression can also be regulated by MMP cleavage or degradation leading to tissue maturation e.g. altered proteins matrices are required to promote cellular hypertrophy at the growth plate, and matrix mineralisation. Again, when the cartilaginous matrix is replaced by bone by the ARF sequence, MMPs play an important role in remodelling the extracellular matrix to make way for a more mature matrix suitable for the interactions required by the osteoblast for cell adhesion (See section 1.3.2 and figure 1.2. of Chapter 1). Although it is clear that signalling pathways are essential in development; equally a specialised extracellular matrix can influence primitive cells to induce gene transcription of downstream target genes, and directly influence tissue maturation (Boudreau and Jones, 1999).

Taken together the processes of cell migration, cell-cell and cell-matrix interactions are essential factors involved in bone remodelling and development. Proteins that have the capacity to mediate these processes are therefore of fundamental importance in bone.

3.1.3 AChE as a matrix protein

In bone, matrix proteins are often required to mediate cellular interactions, and to do this must be localised in the matrix. Significantly, AChE has been implicated in the

mediation of cell-cell interactions, and possesses the ability to bind to components of the extracellular matrix in several tissues. The cholinergic capacity of AChE necessitates that it must be localised at cholinergic synapses adjacent to receptor complexes either in the matrix, on the cell surface or in the extracellular environment. Therefore the mechanisms of AChE secretion and cellular localisation are well established in other tissues, known to be influenced by post-translational modifications such as glycosylation, and tissue specific isoform expression. These same mechanisms of secretion and localisation of AChE characterised for a cholinergic role for AChE may also provide a basis for its function as a bone matrix protein.

3.1.3.1. AChE glycosylation

It is well established that post-translational modification such as glycosylation, can create fundamental changes to protein structure impacting upon biological functions, protein stabilisation, intracellular sorting and transport, and recognition by receptor molecules (Velan et al.,1993, Meynial-Salles and Combes, 1996, Parodi, 2000). Glycosylation of a protein creates a protein carbohydrate hybrid molecule, where groups of oligosaccharide side chains are covalently attached to the surface of the protein at specific sites. This is a highly intricate, sequential process that occurs within the golgi and ER system, but can vary between tissues or cell types (Pfeffer and Rothman, 1987). Proteins can be subject to two distinct types of glycosylation, differing in the type of glycosidic bond in place; N-glycosylation or O-glycosylation (Meynial and Salles, 1996). N-glycosylation creates links between asparagine residues found on the protein to a N-acetyl-glucosamine attached to the oligosaccharide backbone of the carbohydrate side chain. A variety of O-glycosylated bonds have been identified to date, the primary type linking a N-acetylgalactosamine of a carbohydrate to a hydroxylated chain of either a serine or threonine residue of the protein. N-glycosylation can only occur at sites which contain a signal amino acid sequence of Asn-X-Thr/Ser, X being any amino acid except proline, and the third amino acid either threonine or serine (Meynial-Salles and Combs, 1996). AChE is subject to multiple post-translational modifications such as phosphorylation, proteolysis and glycosylation (Treskatis et al., 1992, Velan et al., 1993). Glycosylation of AChE has proved to be of integral importance to the protein thermo-stability, retention and degradation, intracellular transport, and secretion

compatible folding (Liao et al., 1992, Velan et al., 1993). Mammalian AChE has various glycosylation sites and differing glycosylation patterns are thought to contribute to their tissue specific properties (Liao et al., 1992). As with many other glycosylated proteins, glycosylation of AChE varies greatly between species and cell type. However, three conserved N-glycosylation sites found at positions 265, 350 and 464 within exons 1 and 2 of the AChE gene have been identified within all species (Velan et al., 1992). Most AChE proteins were not found to be subject to O-glycosylation even though some mammalian species do have a consensus site for this type of glycosylation (Liao et al., 1992). Human, rat and mouse AChE contain only the conserved N-glycosylation sites, all of which are functional, to provide different catalytic, but mainly structural properties (Velan et al., 1993).

It is thought that AChE protein glycosylation can provide specialised functions. The HNK-1 epitope, commonly associated with cell adhesion molecules involved in development is located on AChE isoforms from a variety of sources (Layer and Wilbold, 1995). Work carried out by Layer and Willbold showed close co-localisation of AChE to the HNK-1 binding lectin peanut aggultin (PNA) in migrating neural crest cells of the developing brain and condensing retinal cells of the eye (Layer and Alber, 1990, Layer and Kaulich, 1991, Alber et al., 1994, Layer and Wilbold, 1995). Furthermore they showed that AChE could bind to PNA and suggested this may be via conserved oligosaccahride side chains (Alber et al., 1994). Modification of oligosaccharide side chains has also been shown to be of significance to AChE function. AChE from plasma and red blood cells is glycoylated and highly sialyated, and sialyation can contribute to the circulatory residence and clearance of AChE and BChE (Saxena et al., 1997, Chitlaru et al., 2002). Glycosylation has also proved to be of significance to AChE's role in Alzheimer's disease. Differential glycosylation of AChE and BChE in the Alzheimer's brain and plasma is thought to impact upon the ratio of AChE species resident in the plaque regions from the G4 to the G1 (for review see Layer and Wilbold, 1995). This could affect the functionality of AChE in the brain, influencing the aetiology of the disease. N-glycosylation of AChE has also been related to assembly and secretion of the enzyme (Kerem et al., 1993). Studies of mutated AChE isoforms indicated that elimination of N-glycosylation sites had a pronounced effect on AChE secretion in vitro (Velan et al., 1993). N-glycosylation of AChE has also been shown to contribute to the secretion-compatible folding and thermo-

stability of the AChE polypeptide (Kerem et al., 1993, Kronman et al., 1995). Indeed N-glycosylation is of significant importance to many other proteins in processing and secretion (Parodi, 2000)

3.1.3.2. Secretion of AChE

In cholinergic tissues the specific localisation of AChE has proved to be essential for functionality; clustered close to ACh receptors either in the cell membrane or in the extra cellular matrix. It is therefore clear that an intracellular transport system for AChE exists to enable efficient regulated externalisation. The secretory system of most eukaryotic cells is very similar. After protein synthesis, the sorting, packaging and transport of proteins is carried out by the ER-Golgi complexes. This system acts upon signal peptides sequences found upon the protein adding peptides that provide resistance to degradation and promote protein stability (Pfieffer and Rothman, 1987). AChE contains a variety of signals that determine the extent of oligomerisation, attachment to anchoring proteins and secretion (Rotundo et al., 1988, Campany et al., 1992). Newly synthesised AChE transverses the ER-golgi system, and subunits attached to anchors are sorted and transported to the surface for incorporisation into the membrane, or externalisation in the case of ColQ (Rotundo, 1984, Rotundo et al., 1988, Campany et al., 1992). The remaining synthesised AChE is subjected to degradation (Rotundo, 1988). It is thought that this degradation prevents over secretion of AChE. However, in the presence of high intracellular calcium all stored and synthesised AChE is secreted from the cell (Haenou et al., 1993). Calcium influxes such as those caused by arrival of action potentials at a motor-nerve terminal can induce such rapid secretion of AChE (Bursztajin et al., 1991). In this way the regulation of cholinergic neurotransmission by AChE can be reactive to the polarisation of the cell. As most of the early work into AChE secretion was carried out on cells that possess a cholinergic signalling machinery the exact mechanism of AChE secretion in other non-cholinergic cells is still unclear. However, AChE secretion has been observed in other cell types i.e. megakaryocytes, lymphocytes, dopaminergic neurons and astrocytes, in some case in response to stimulation by other signalling mechanisms (Henderson and Greenfield, 1984, Murphy and Greenfiled, 1991, Webbond and Greenfield, 1992).

As data indicated the possiblity of a non-cholinergic capacity for AChE in bone as a matrix protein, it was first important to characterise the expression of AChE in bone in relation to the function(s) that matrix proteins might have in skeletogenesis and bone formation. Therefore with the aim of identifying a potential involvement of AChE in osteoblast function and differentiation, the work in this chapter describes the localisation of AChE during embryonic development, as well as expression, and regulated secretion during osteoblast differentiation.

3.2 Materials and methods

3.2.1 Characterisation of AChE isoform expression in bone

3.2.1.1 In situ hybridisation

In situ hybridisation using probes to specific AChE isoforms was performed to characterise expression in developing day 3 rat limbs. We used non radioactive cRNA probes directed specifically to mE5 AChE-E isoform, mE6 AChE-S, or mI4 AChE-R isoform, designed by Shlomo Seidman, Jerusalem Israel (see table 3.1 for sequences). Probes were 5'-biotinylated and 2'-O-methylated to protect from nucleolytic degradation. For controls probes of an inverse sequence to the AChE-S probe was used. Paraffin sections were cleared with xylene and dehydrated through serial dilutions of ethanol. Degradation of DNA and permeablisiation of the tissue was carried out by incubating the slides with proteinase K (10µg/ml) for 10 minutes at 37°C. Slides were prehybridised in a humidified chamber with 50µg/ml yeast tRNA, 50µg/ml heparin in a buffer of 50% formamide in a saline-sodium citrate (SSC), pH 4.5, performed for 10 minutes at 60°C. Hybridisation with 10µg/ml RNA probes was carried out overnight as above and subsequently bound to streptavidin alkaline phosphatase-conjugate (Sigma). Prior to detection of probe, slides were blocked with 1% skimmed milk solution containing the alkaline phosphatase inhibitor levamisol (2mM). Detection was carried out with fast red (Sigma) for 1-3 minutes, and the reaction was stopped by incubating the slides with 25mM EDTA, 0.05% Triton X-100 solution with 1mM levamisol. Slides were mounted with immunomount (Shandon Inc.), and hybridised probes visualised with standard light microscopy.

3.2.1.2 Immunohistochemistry

AChE expression was characterised in developing calvaria using cryosections taken from embryonic day (E)18 and E21 rat calvaria as described in Chapter 2 (section 2.1.6.2a). Analysis of AChE expression in developing human bone was performed on cryosections of week 12 human embryonic bone kindly provided by Dr Chan, Institute of Child Health, London, UK. Expression of specific AChE isoforms by immunohistochemistry was determined on paraffin sections of postnatal day 3 rat fore

and hind limbs as detailed in Chapter 2 (section 2.1.6.1 and 2.1.6.2b). See table 2.1 for antibodies used.

3.2.1.3 Western blot analysis

Western blot analysis of AChE expression by osteoblastic cells was carried out essentially as detailed in chapter 2 (see section 2.1.4.3.) See table 2.1 for details of antibodies used.

3.2.1.4 RNA extraction and cDNA synthesis

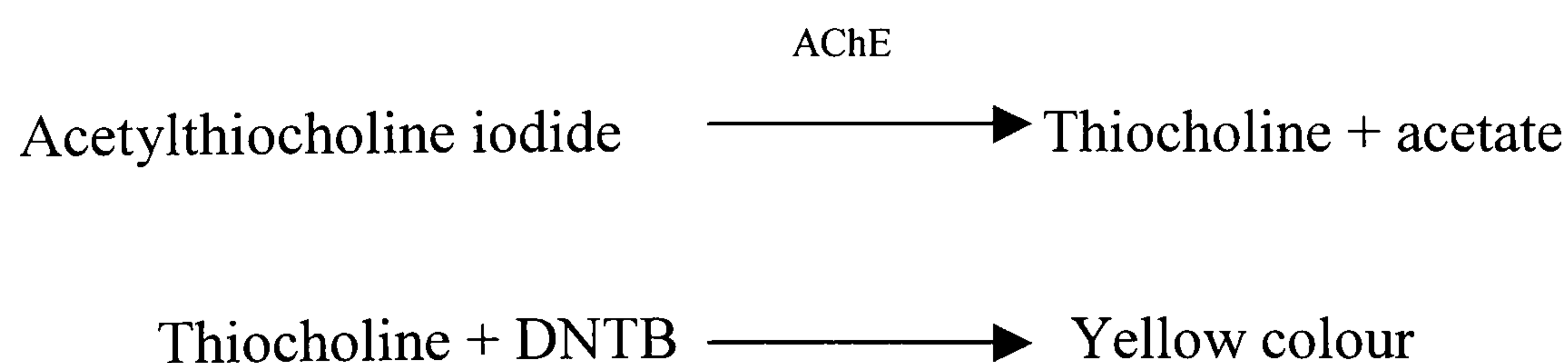
Whole RNA was extracted at three day intervals from primary cultures of rat osteoblasts that were grown under osteogenic conditions as described in Chapter 2 (see sections 2.1.2.2 and 2.1.5.1). cDNA was prepared using 5μg of total RNA essentially as described in Chapter 2 (see section 2.1.5.2).

3.2.1.5 RT-PCR

RT-PCR to identify mRNA expression of AChE was carried out essentially as described in chapter 2. See table 2.2 for details of primers used.

3.2.2 Secretion of AChE by osteoblasts

3.2.2.1 Ellman acetylcholinesterase activity assay



AChE activity was measured using a colorimetric assay of cholinesterase activity (the Ellman method, Ellman *et al*, 1961, see reaction schematic above), modified to allow microplate analysis and high-throughput screening. This assay uses an altered version of acetylcholine (ACh) that incorporates thiol residues, acetylthiocholine iodide (ATCI),

as a substrate. Such thiol residues are capable of binding to 5'5 dithiobisnitrobensoate (DNTB) to produce a yellow coloration. Adaptation of the Ellman method made suitable for a microplate format allowed analysis oif 25 μl samples of either conditioned media or exogenous added AChE. Activity was assayed using 20mM ATCI, 30mM DNTB in PBS pH 7.4 at room temperature. To eliminate the possibility of non-specific cholinesterase activity, samples were assayed in the presence of BChE inhibitor tetraisopropyl pyrophosoramide (iso-OMPA) at 10^{-5}M (unless otherwise stated). Colour development was measured after 10 minutes and concentrations of AChE in samples determined using a standard curve constructed from known amounts of exogenous AChE prepared in culture medium. Phenol red free culture medium was used in all assay experiments.

FCS used in these assays was pre-treated with an irreversible inhibitor of the AChE active site Diisopropyl Fluorophosphate (DFP, 10^{-5}M, 18 hrs), to deplete serum-derived AChE activity. For removal of residual DFP activity, FCS was then refrigerated at 4°C for 48 hours. To ensure sufficient reduction of AChE activity and depletion of DFP activity, samples of treated FCS were assayed for AChE activity alone or in the presence of another AChE inhibitor 1,5-bis(4-Allydimethylammoniumphenyl)pentan-5-1 dibromide (BW284C51) and exogenously added AChE. DFP-treated FCS was used in all assay experiments unless otherwise indicated.

3.2.2.2 Statistical analysis of AChE assays

In all AChE assay experiments 10 samples were taken from each treatment group for analysis. Test of each samples for AChE activity was carried out in triplicate and the average of each used for statstcal analysis. Prior to this all sample values were normalised to total cell protein concentration which was determined by lysis of cells and analysis of lysed solution by BCA assay unless otherwises stated (see section 2.1.4.1 for methods). The statistical significance of AChE assay results were then determined by performing paired t-tests, carried out using SPSS version 10.

3.2.2.3 Immunofluorescent localisation

MG63 and MC3T3-E1 clonal cell lines were grown on coverslips in 24 well plates at a density of 5 x 10^4 cells/cm^2. Following 24 hour incubation in low serum-containing

medium (0.5%), cells were exposed to monensin at 2μM, 5μM and 10μM (Sigma), or Brefeldin A (BFA) at 0.35μM, 0.7μM and 1.4μM (Sigma) for 6 hours. For detection of AChE secretion, samples of conditioned medium were taken and used in the AChE activity assay, described above.

For immunofluorescent localisation of AChE expression, cells were fixed in 4% paraformaldehyde, and non-specific binding blocked with 10% goat serum diluted in PBS with or without 0.1% triton for 30 minutes. AChE expression was determined using a monoclonal mouse anti-AChE antibody (1μg/ml) (BD Transduction laboratories) overnight at 4°C followed by exposure to a goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (1:100) for 45 minutes, and labelling observed by fluorescence microscopy using a leica DMLA microscope with a digital camera and QFluro documentation system. For co-localisation experiments, cells were co-incubated with the secondary antibody and the golgi-specific ceramide dye N-((4-(4,4difuoro-5- (2-thienyl)-4-bora-3a, 4a-diaza-s-indacene-3-yl) phenoxy) acetyl) sphingosine (BODIPY TR-ceramide, Molecular Probes) 5μM.

3.3 Results

3.3.1 Characterisation of AChE expression during bone development

3.3.1.1 AChE expression in developing rodent calvariae

We determined AChE expression during bone development in rats at E18 and E20. Immunolocalisation of AChE on cryosections of E18 calvariae revealed AChE expression in the condensing mesenchyme (Figure 3.3.1 A). This corresponded with areas of alkaline phosphatase activity before calcification was detectable (Figure 3.3.1 C). Intense AChE staining was also observed surrounding calcified calvariae (E20) in periosteal osteoblasts (Figure 3.3.1 B), also following a similar distribution pattern to that of alkaline phosphatase activity (Figure 3.3.1 D). In calvarial sutures, AChE expression was distributed throughout the sutural mesenchyme and in periosteal cells, whereas alkaline phosphatase activity was limited to the cells immediately adjacent to the calcification fronts (Figure 3.3.1 E and F).

3.3.1.2 AChE expression in developing rat long bones

Immunolocalisations on cryosections of developing E16, E18 and E21 rat embryos revealed abundant expression of AChE. In E16 and E18 developing rat ribs, AChE was localised to the condensing chondrogenic tissue and surrounding perichondral cells (Figure 3.3.2 A and B). Expression of AChE in more developed calcified long bones of E21 rat limbs was observed in the periosteal cells adjacent to the diaphysis, and stromal cells of the trabecular compartment (Figure 3.3.2 C).

3.3.1.3 Expression of AChE during human long bone development.

Immunolocalisation of AChE on cryosections of developing human long bones (Week 12) revealed AChE expression in proliferating chondrocytes and surrounding perichondral cells (Figure 3.3.3 C). In more developed calcified sections of bone AChE was expressed in hypertrophic chondrocytes and periosteal osteoblasts (Figure 3.3.3 B). Osteoblasts and condensing cells of the perichondrium also expressed abundant AChE (Figure 3.3.3 A).

Origional in colour

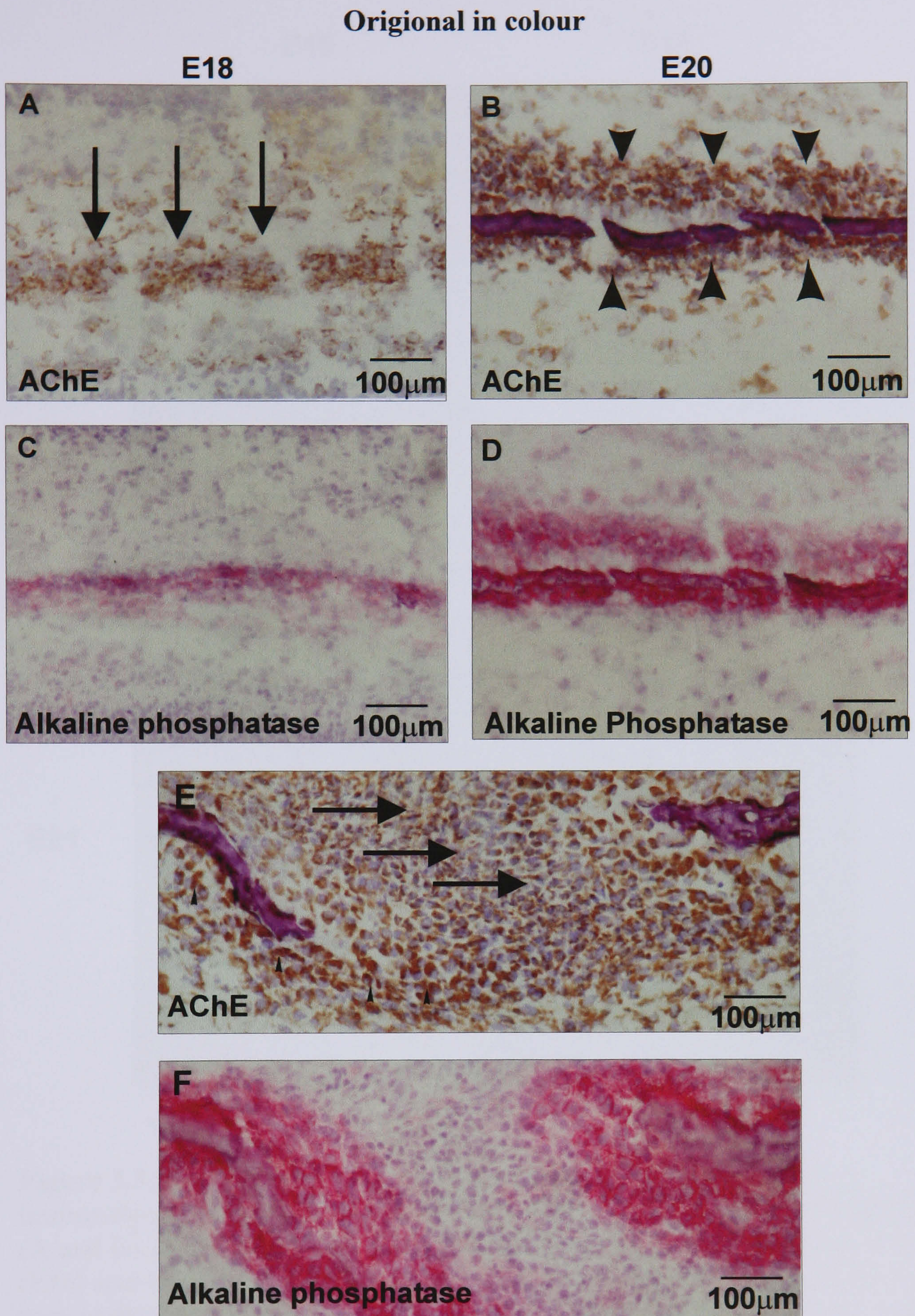


Figure 3.3.1

Immunolocalisation of AChE in rat E18 and E20 calvarial bones

Expression of AChE was observed in condensing mesenchymal tissue of E18 developing calvaria (A arrows), and in periosteal osteoblasts of E20 calvaria (B arrow heads), which corresponded with alkaline phosphatase activity in serial sections (C and D respectively).

At calvarial sutures, AChE expression was observed in cells at periosteal sites and throughout the sutural mesenchyme (E arrows). However activity of alkaline phosphatase was limited to cells immediately adjacent to the calcification fronts in serial sections (F).

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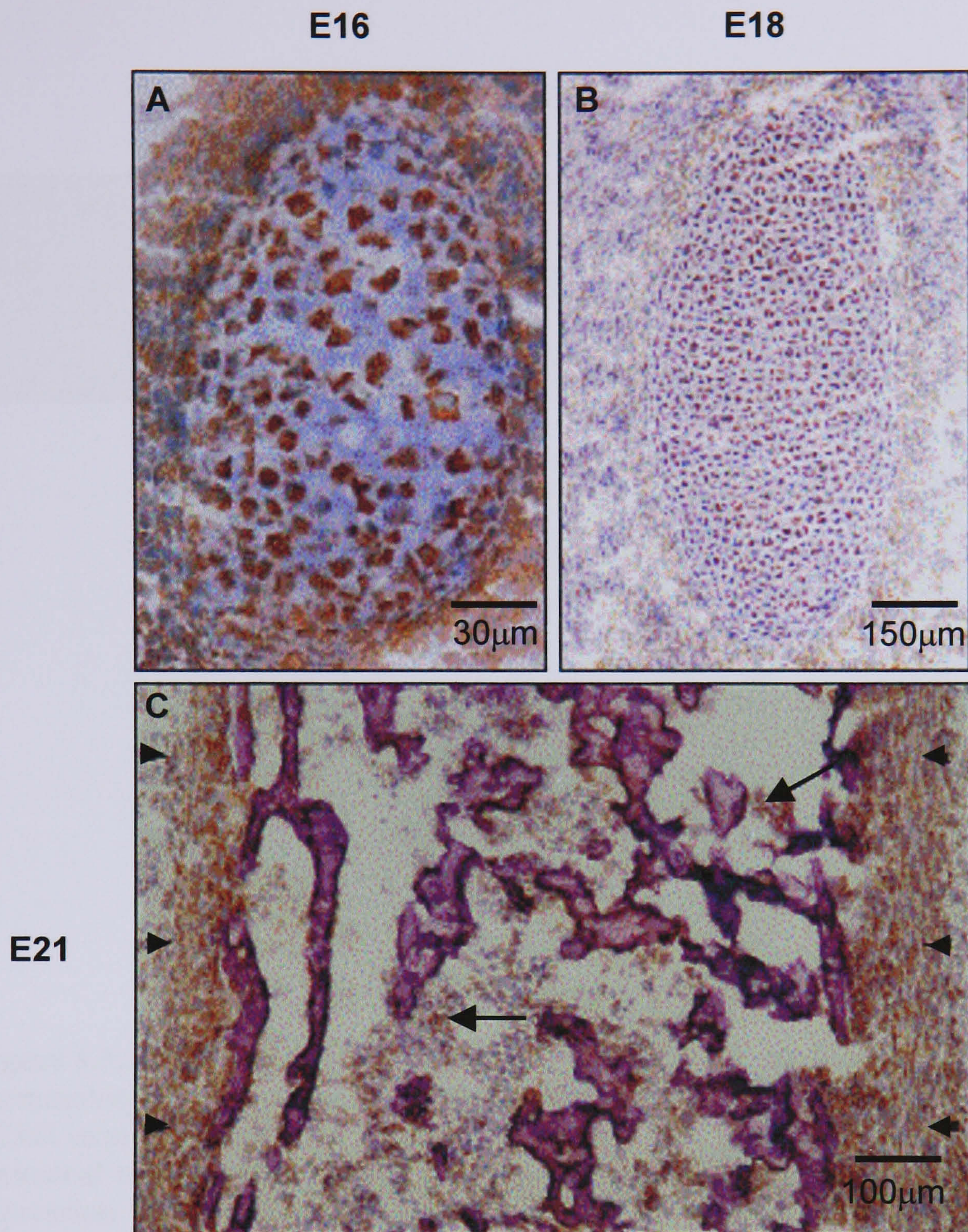

Figure 3.3.2
Immunolocalisation of AChE in developing rat E16, E18 and E21 long bones. (A and B) AChE expression was observed in the chondrogenic tissue of early (E16) and later (E18) developing rat ribs, expression was also observed in the surrounding perichondral tissue. In more developed (E21) rat limbs, AChE expression was localised to the periosteal tissue of the diaphyses (C arrowheads), and within the trabecular compartment (arrows).

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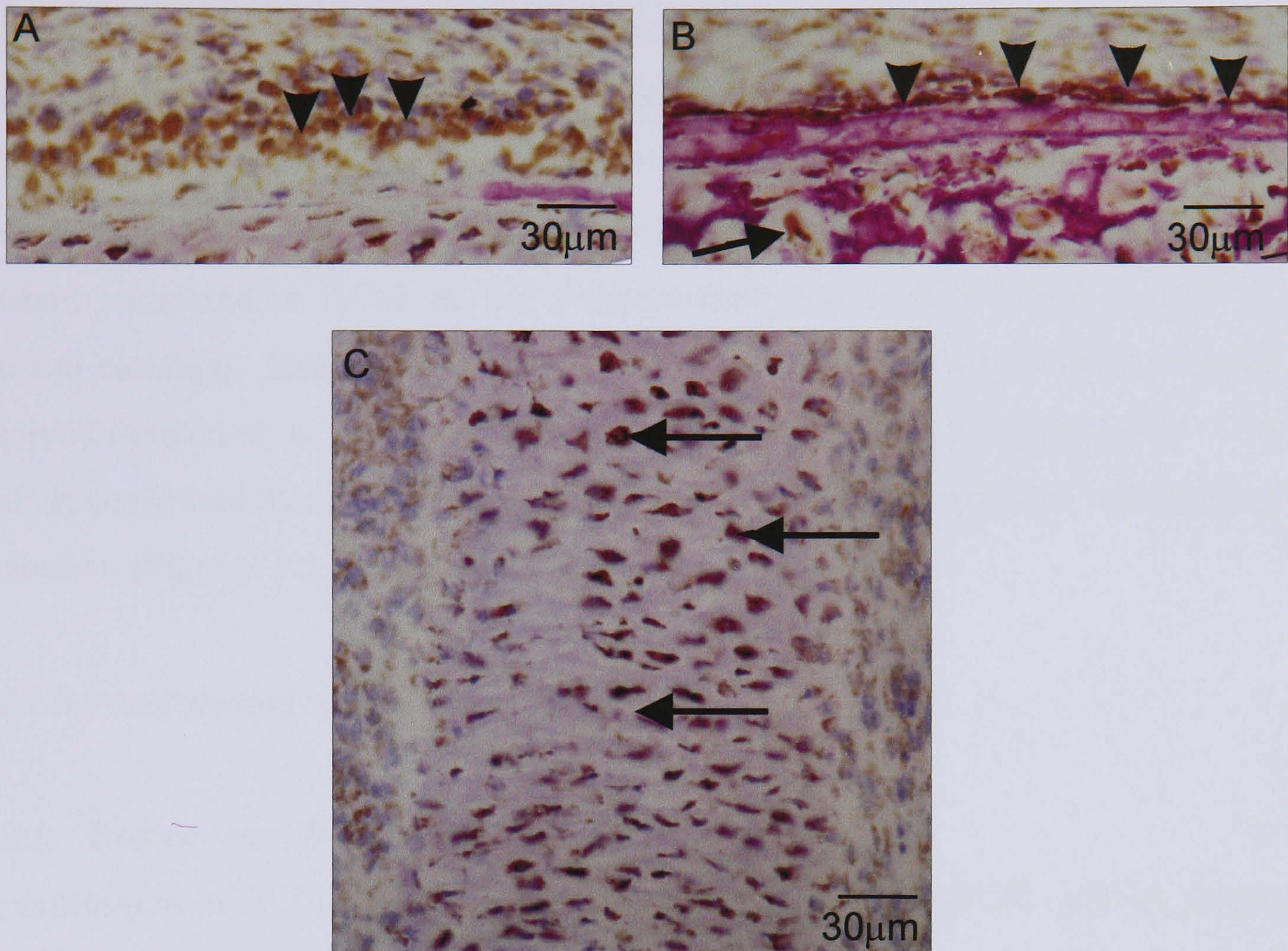

Figure 3.3.3
Immunolocalisation of AChE in developing human long bones (week 12)
AChE expression can be observed in perichondral cells (A arrowheads) and periosteal osteoblasts and matrix (B arrowheads). Intense intracellular AChE expression was also observed in chondrocytes of calcified (B arrows) and uncalcified tissues (C arrows).

3.3.1.4. Isoform-specific expression of AChE in developing rat long bone

In situ hybridisations were performed to determine the expression patterns of specific AChE isoforms in developing bone. Using biotinylated RNA probes on paraffin wax sections of neonatal rat limbs, prominent expression of mRNA encoding the AChE-R isoform was observed, most specifically in the periosteum, perichondrium and endosteal osteoblasts surrounding bone trabeculae (Figures 3.3.4 and 3.3.5). Positive AChE-R staining was also identified in recently embedded osteocytes (figure 3.3.6) and proliferating and pre-hypertrophic chondrocytes (figures 3.3.4 and 3.3.7). Expression of mRNA for AChE-E and AChE-S isoforms was more sporadic and less abundant compared to AChE-R, but demonstrated similar distribution patterns in bone and cartilage. Immunolocalisations for AChE using antibodies directed to the conserved domain of AChE, or to isoform-specific AChE C-termini for AChE -S or AChE-R confirmed that AChE protein expression patterns corresponded with mRNA distribution (Figures 3.3.4, 3.3.5, 3.3.6, and 3.3.7).

3.3.2 Characterisation of osteoblastic AChE

3.3.2.1. Isoform-specific expression of AChE in osteoblasts

The expression of AChE in osteoblasts was determined by RT-PCR. cDNA prepared from total RNA, isolated from day 1-24 primary rat osteoblasts grown under osteogenic conditions, was amplified using primer pairs identifying the AChE. Expression of AChE mRNAs was apparent after 1 day in culture and continued throughout the culture period. Comparison of the amplified product sizes with positive control cDNA prepared from rat brain revealed osteoblastic AChE mRNA was of identical size (Figure 3.3.8). Expression of AChE was also compared to that of specific markers of bone formation osteopontin (early), osteonectin (late) (Figure 3.3.8).

Original in colour

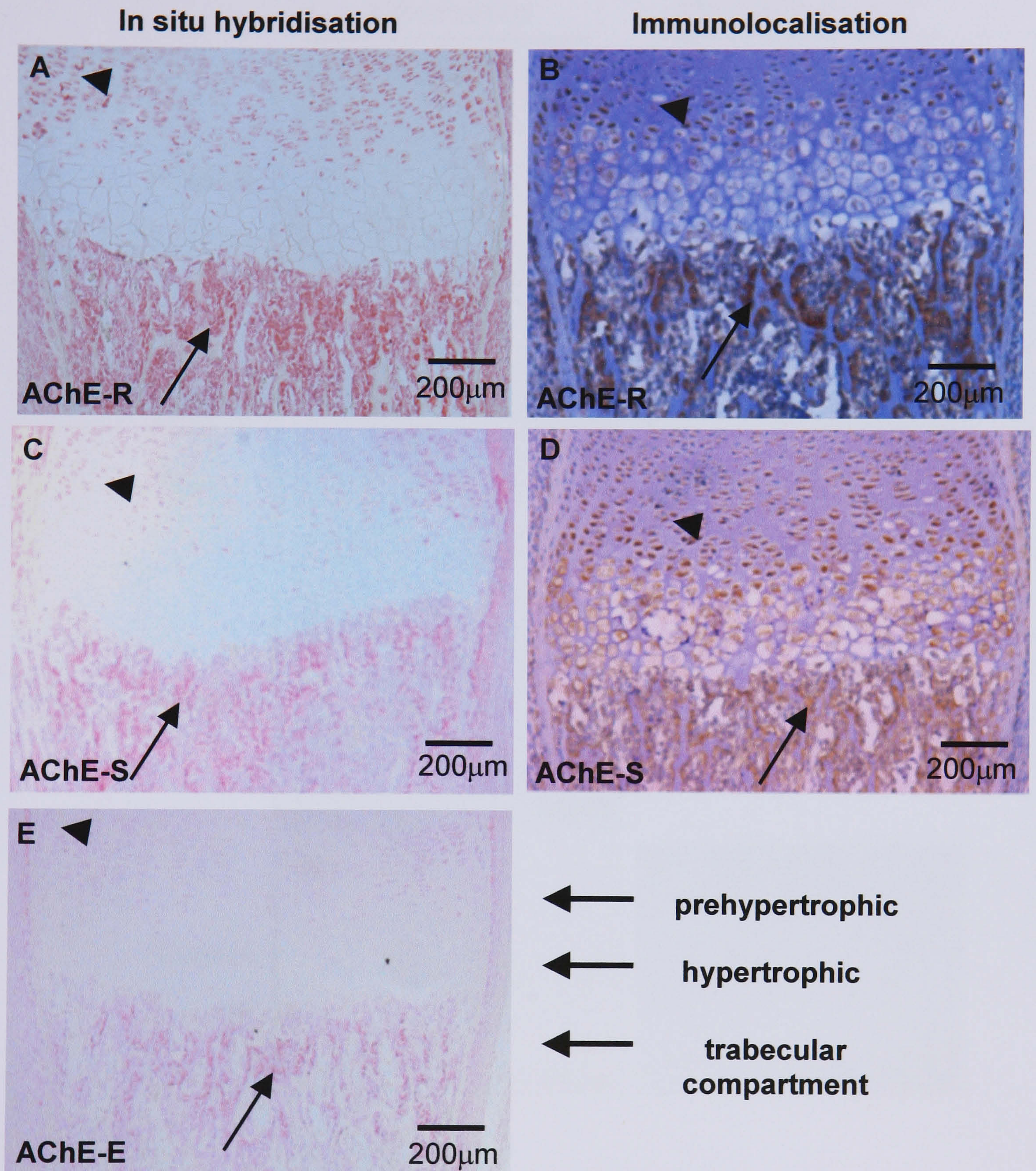

Figure 3.3.4
In situ hybridisation (A, C, and E) and immunolocalisation (B and D) for specific AChE isoforms in the metaphyseal bone.
A and B abundant expression of AChE-R was found in the pre-hypertrophic chondrocytes (arrowhead) and endosteal osteoblast lining trabecular bone (arrows).
C and D and E expression of AChE-S and E appeared to be less abundant but followed a similar pattern of localisation.

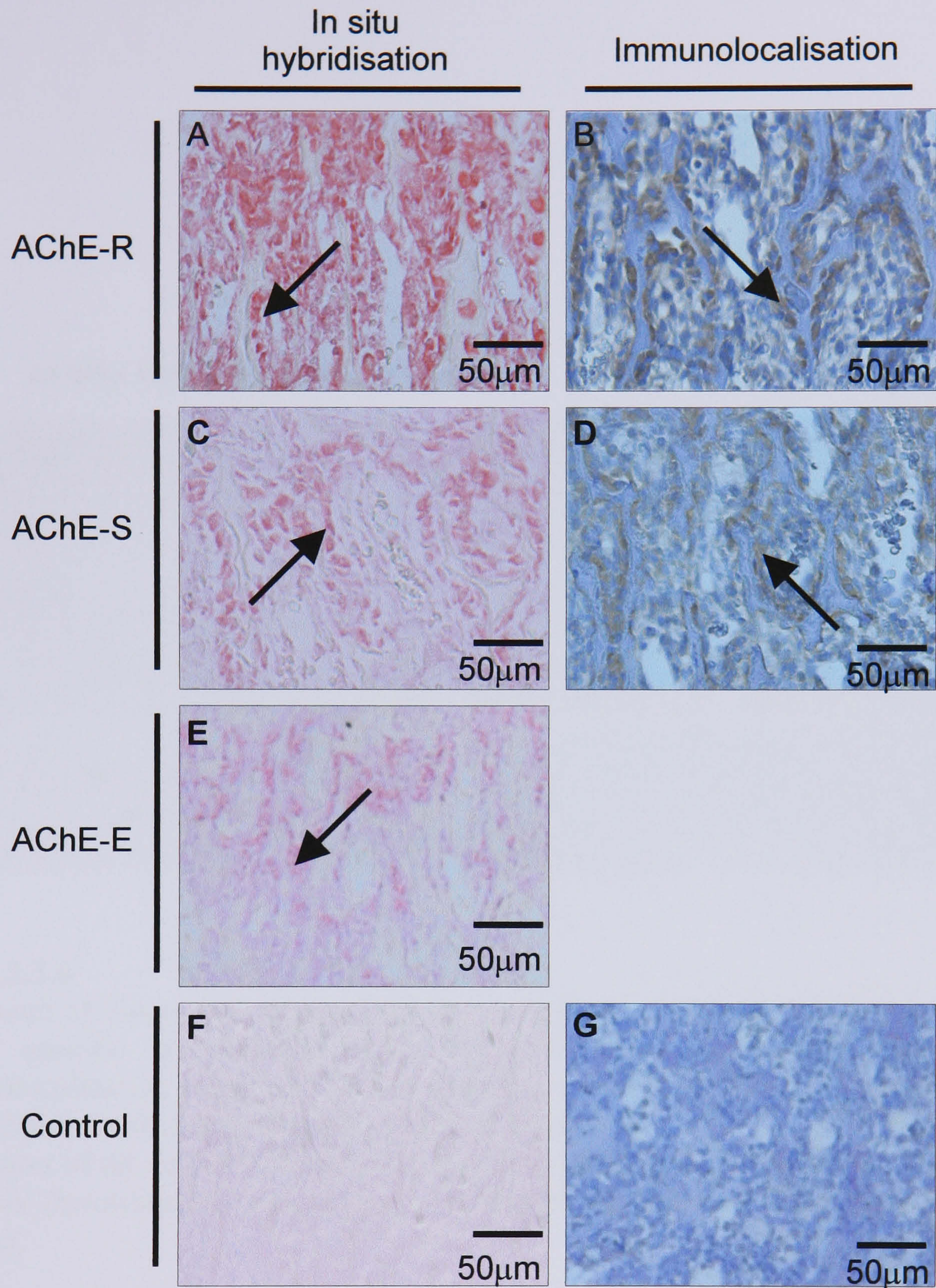

Figure 3.3.5
Trabecular compartment of rat postnatal day three long bones. In situ hybridisation using probes specific to AChE-R (A), AChE-S (C), or AChE -E (E) mRNA, and immunolocalisation using antibodies directed to c-terminal protein fragment of AChE-R (B), or AChE-S (D).
A and B Abundant expression of AChE-R specifically in endosteal osteoblasts lining the trabeculae was observed by both in situ hybridisation and immunolocalisation (arrows).
C, D and E Expression of AChE-S and E appeared to be less abundant but localised to same regions as AChE-R (arrows).
F and G Controls

Original in colour

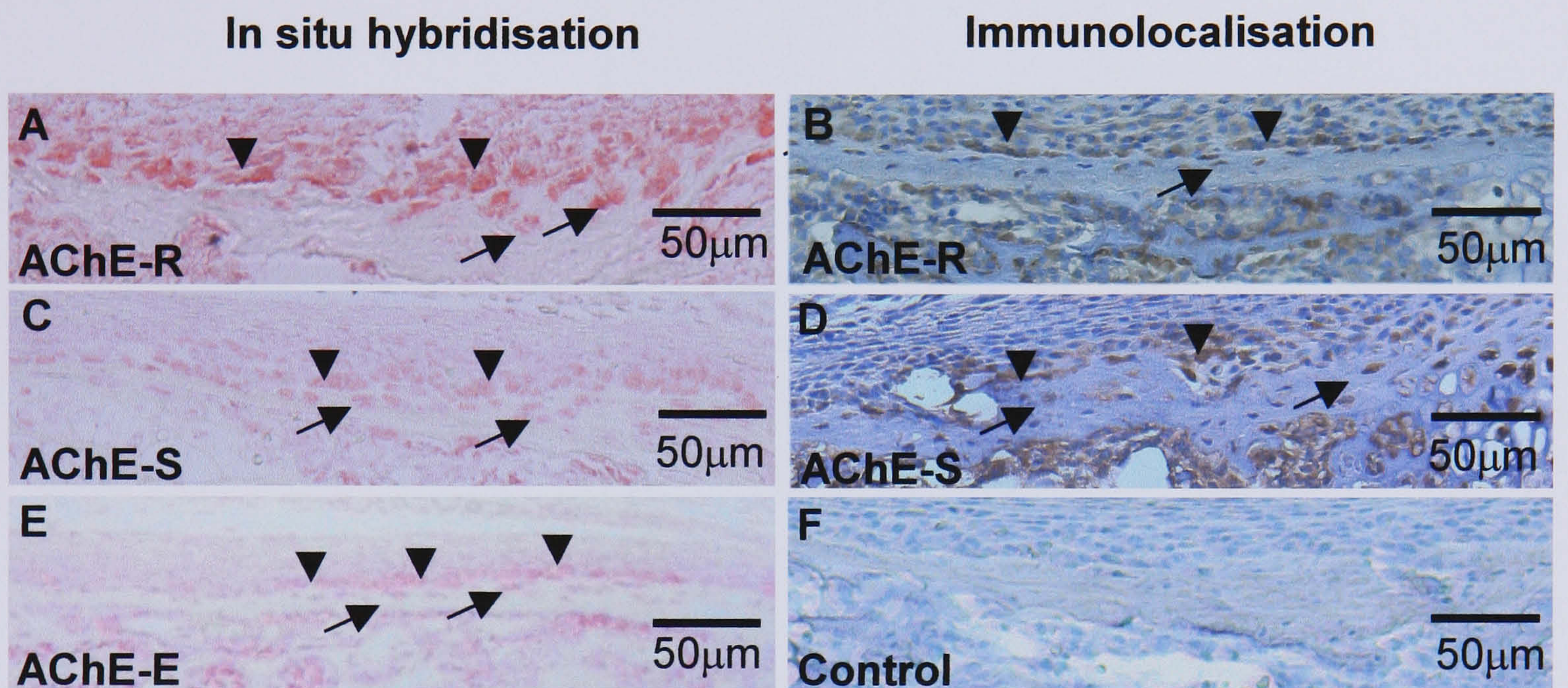

Figure 3.3.6
Periosteum of diaphyseal in postnatal day 3 rat limbs. In situ hybridisation using probes specific to AChE-R (A), AChE-S (C), or AChE-E (E), and immunolocalisation using antibodies specific to AChE-R (B) or AChE-S (D), counterstained with haemotoxylin. Antibody control (F).
Expression of all AChE isoforms was localised to periosteal osteoblast lining the diaphysis (arrowheads), and to newly embedded osteocytes of the same region (arrows).

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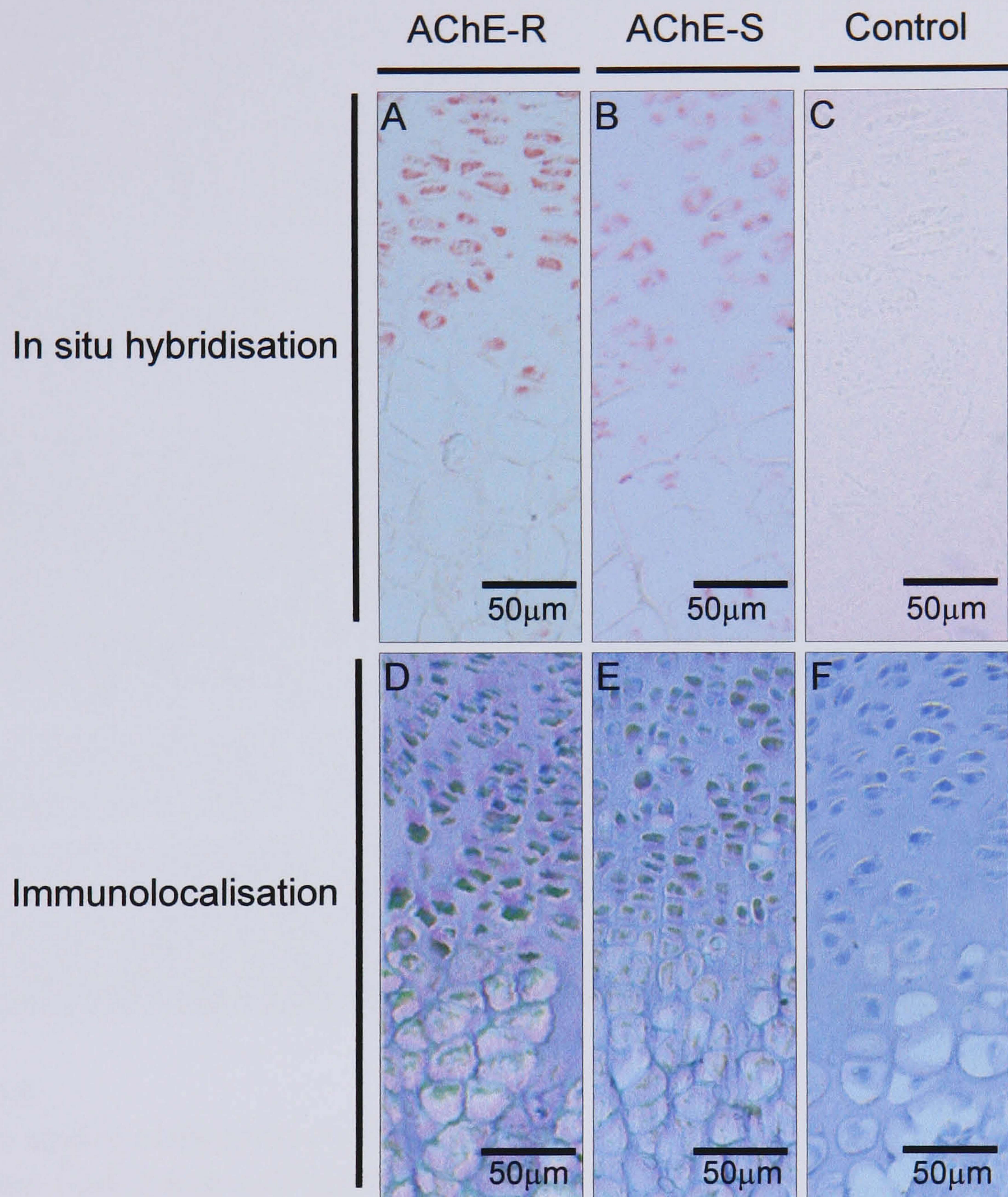

Figure 3.3.7
Chondrocyte columns in the growth plate of postnatal day 3 rat limbs. Immunolocalisation and in situ hybridisation for AChE-R (A and D), and AChE-S (B and E). Expression of AChE-R and AChE-S mRNA and protein expression was observed in cells of the prehypertrophic zone (arrows A, B, C and D). In situ hybridisation control (C). Antibody control (F)

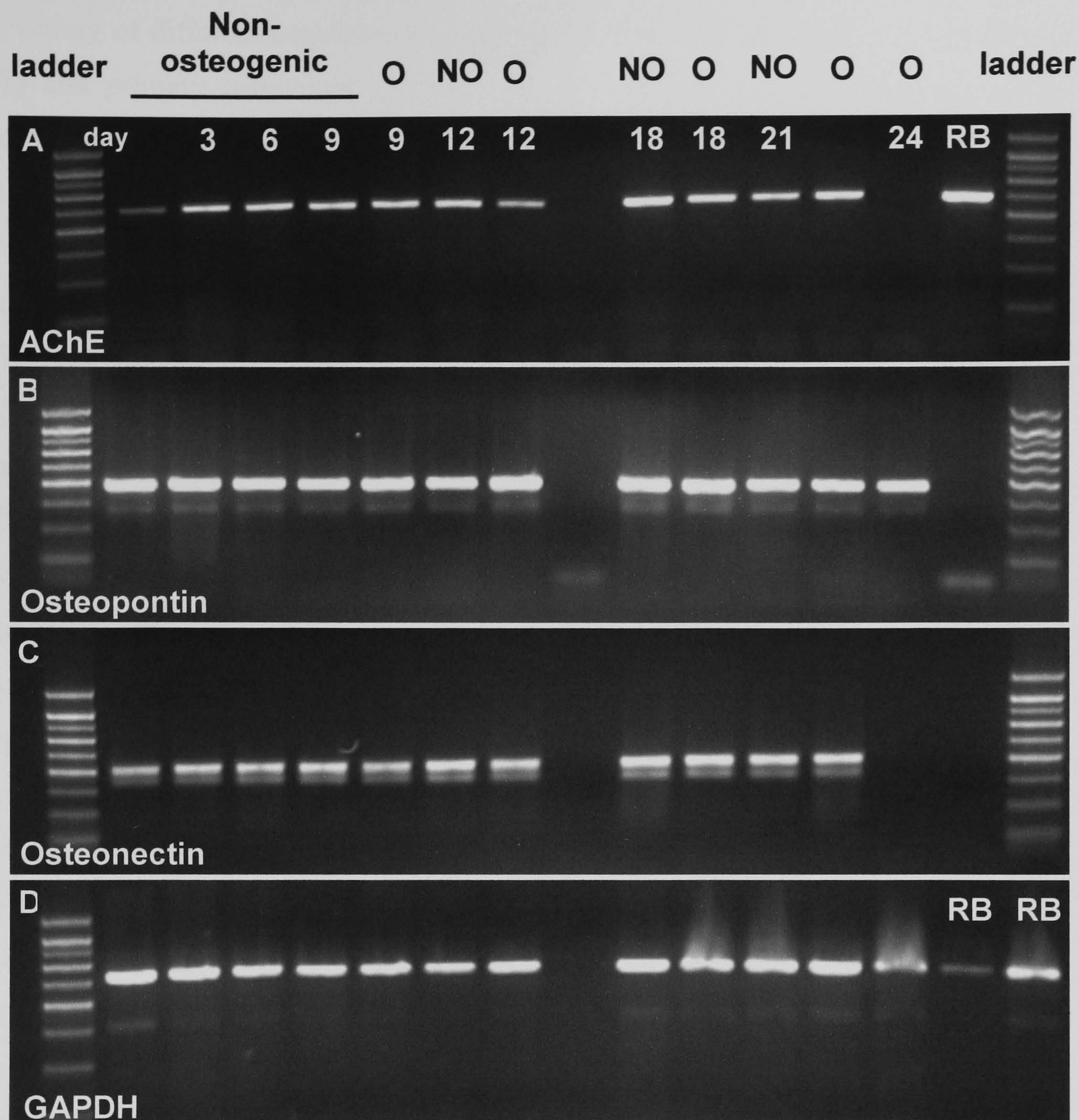

Figure 3.3.8
O – grown under osteogenic conditions
NO – grown under non-osteogenic conditions
Primary rat osteoblast grown under osteogenic conditions until day 24 in culture, total RNA was taken at 3 day intervals and used to prepare cDNA for the use in RT-PCR. Using specific primers for AChE-S a 481bp product was observed at all time points in culture, through till day 24 (A). This corresponded to the 481bp product from rat whole brain cDNA. Expression of mRNA for bone specific proteins osteonectin and osteopontin was also noted, but not expressed in rat whole brain cDNA (B and C).

3.3.2.2. Expression and glycosylation of osteoblast AChE protein

A variety of different osteoblast-like cell lines (TE85, MG63, SaOS-2 and MC3T3-E1) and primary osteoblasts (CFU-F, rat and human) were analysed for AChE expression by western blot analysis (Figure 3.3.9). A major AChE species of 68 kDa, corresponding in size to the synaptic form of AChE, was observed in whole cell lysates of all cell types. Primary osteoblasts and MC3T3-E1 cells cultures that had been treated with osteogenic supplements also expressed an additional AChE species ~55 kDa that was not present in untreated human osteoblast-like cell lines (Figure 3.3.9). In these primary cultures the major AChE species of 68 kDa was expressed uniformly throughout culture, whereas expression levels of the less abundant ~55 kDa AChE species peaked at culture day 15 (Figure 3.3.10 A). MC3T3-E1 cells were grown under osteogenic conditions, whole cell lysates taken at 3-day intervals and treated with N-glycanase to remove N-linked carbohydrate chains. Western blot analysis revealed a shift in AChE kDa in N-glycanase treated cells, which was more apparent as cells became more differentiated (Figure 3.3.10 B).

3.3.2.3. Secretion of AChE by osteoblastic cells.

By western blot analysis, AChE was identified in MG63 conditioned medium (Figure 3.3.11 A). Using the Ellman assay to quantitate AChE activity, it was found that exposure to monensin (10μM) significantly reduced AChE activity of conditioned medium taken from MG63 cultures when compared to untreated cells (Figure 3.3.11 B). In addition, monensin (5μM and 10μM) and BFA (0.7μM and 1.4μM) induced distinct intracellular compartmentalisation of AChE as demonstrated by immunofluorescent localisation in MG63 and MC3T3-E1 cells (Figure 3.3.12i). AChE in MG63 and SaOS-2 osteosarcoma cells also co-localised to the golgi apparatus, which was identified using a specific BODIPY TR ceramide dye (Figure 3.3.12ii).

3.3.2.4. AChE secretion during osteoblast differentiation

Characterisation of AChE secretion during osteoblast differentiation and matrix formation was performed using the modified Ellman assay of AChE activity. Cultures of primary rat osteoblasts grown under osteogenic conditions were assayed for AChE and alkaline phosphatase activity at three-day time points. Increases in

alkaline phosphatase activity, typically associated with differentiation and maturation of the osteoblastic phenotype, paralleled that of AChE secretion in the same cells (Figure 3.3.13). AChE expression peaked at around day 10 in culture, where a 3-fold increase in AChE secretion in cells treated with osteogenic supplements was observed when compared to untreated cultures (Figure 3.3.14).

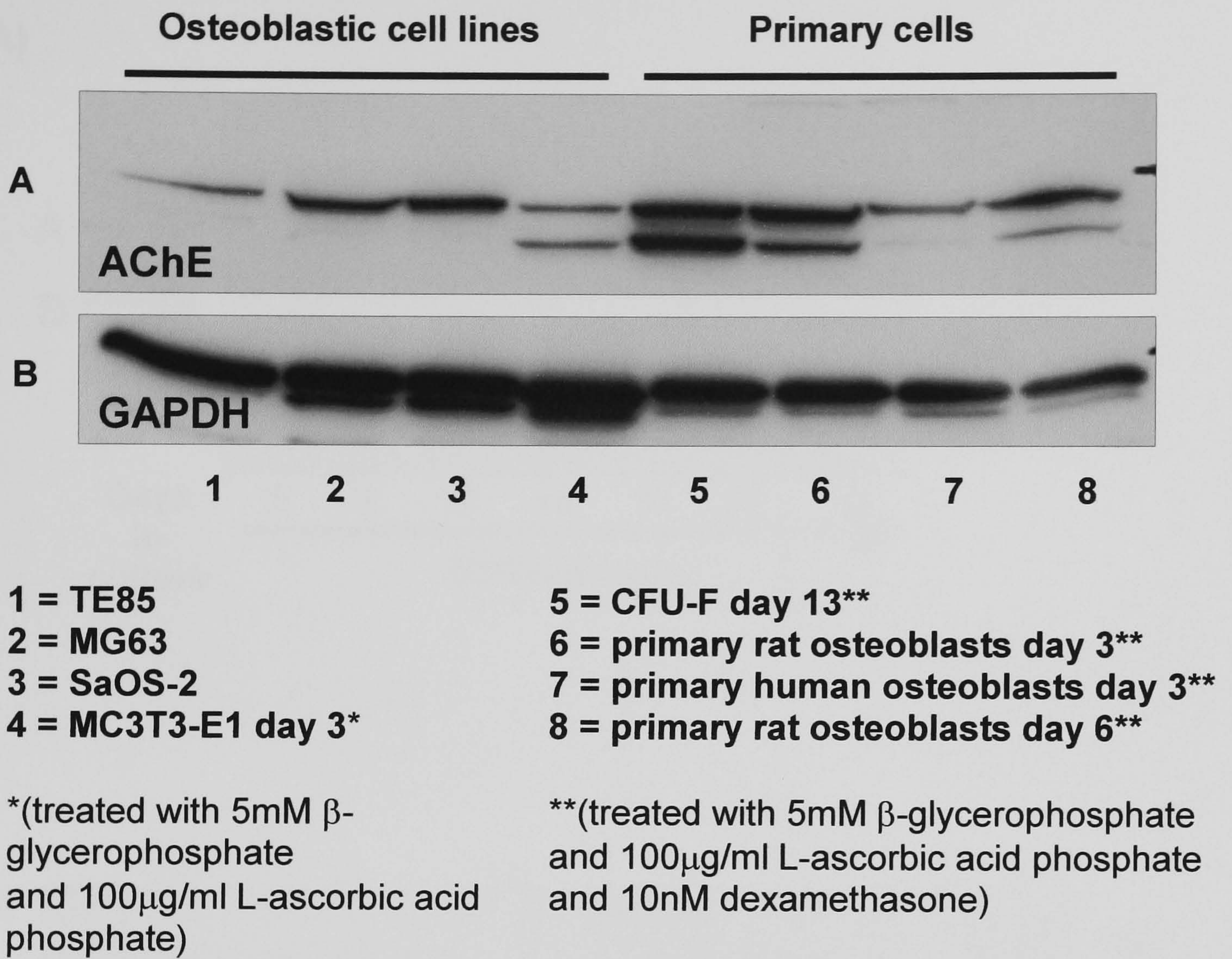

Figure 3.3.9

(A) Western blot analysis using a pan specific AChE antibody. Expression of a 68 kDa AChE species was observed in all osteoblastic cells analysed. A secondary species was also observed in cultures which had been treated with osteogenic supplements (lanes 4-8), not present in untreated osteosarcoma cell lines (lanes 1-3).
(B) GAPDH expression in corresponding lanes.

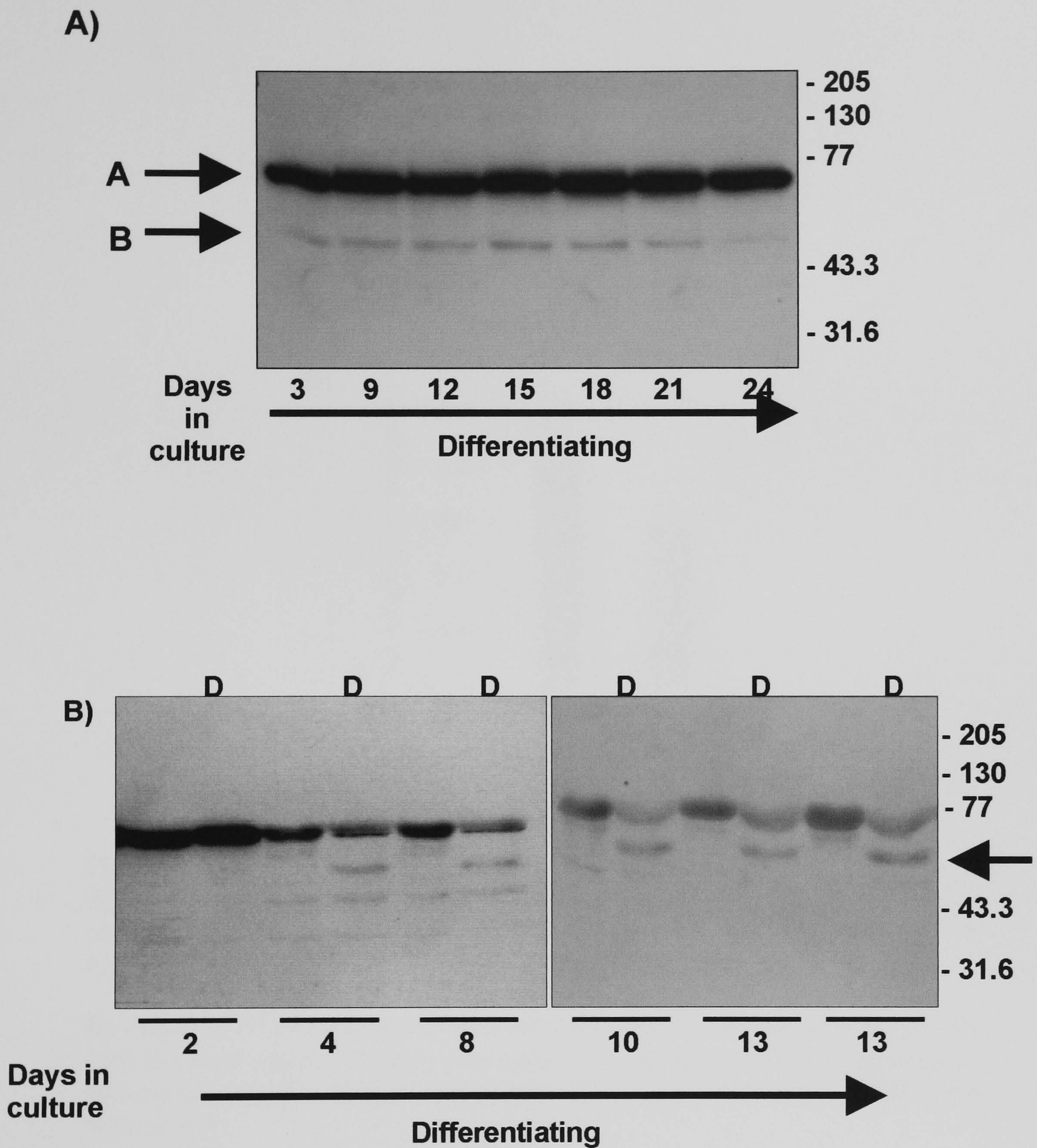

Figure 3.3.10
A) Western blot analysis of whole cell lysates taken at three day intervals from primary human osteoblasts grown under osteogenic conditions untill day 24. AChE expression was analysed revealing two bands, a major band of approximately 68 kDa was expressed uniformly throughout the culture period and corresponded to synaptic AChE (arrow A). Expression of a less abundant minor band of approximately 55 kDa appeared to increase in size between days 15 and 21 in culture (arrow B).

B)Western blot analysis of whole cell lysates taken from MC3T3-E1 cells grown under osteogenic conditions. N-glycanase treatment of samples (D) induced production of a seconday smaller band which become more apparent in cultures of a later time point.

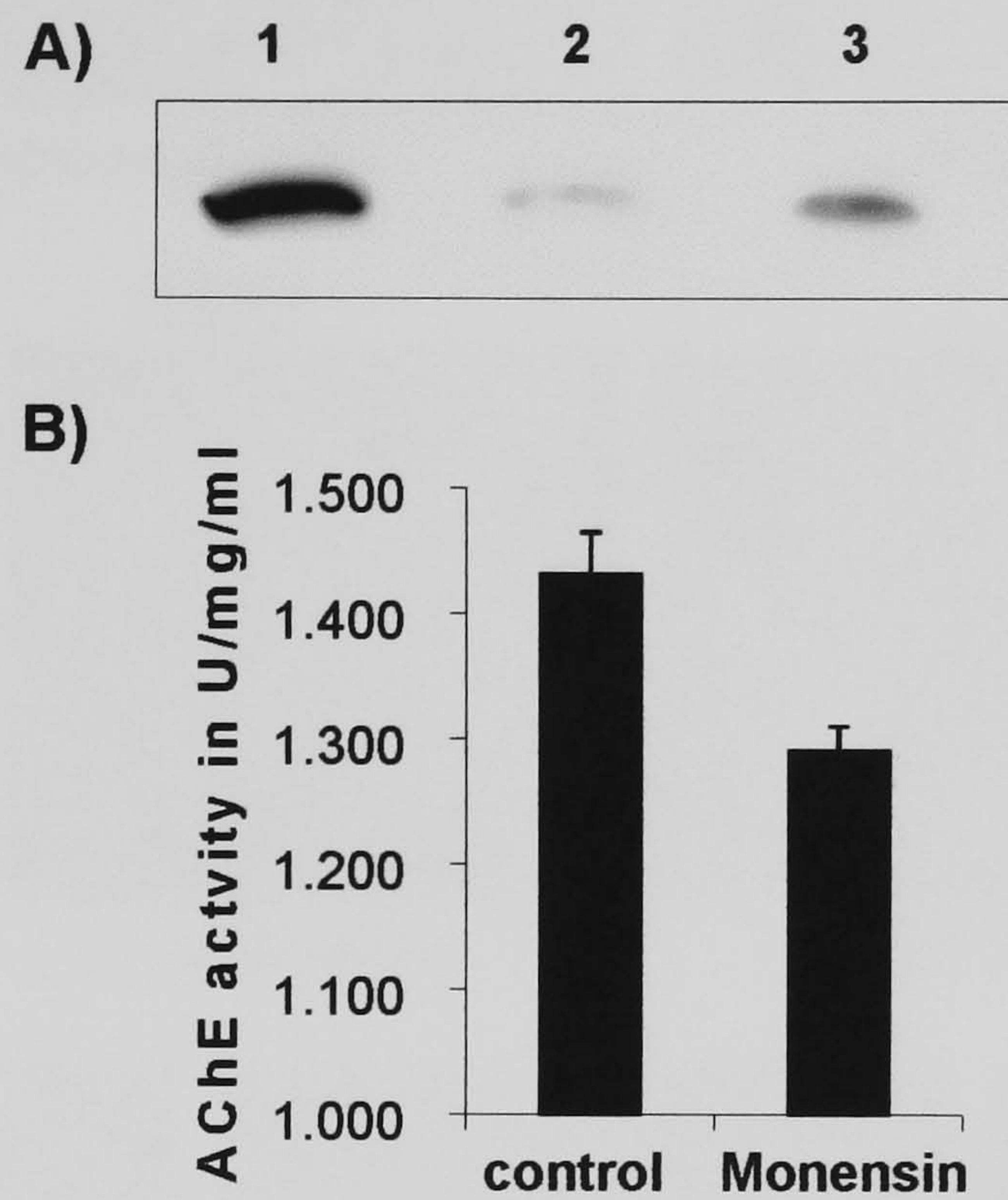

Figure 3.3.11
A) Western blot analysis demonstrating AChE in whole cell lysates (lane 1), unconditioned medium (lane 2) and conditioned medium from MG63 cells (lane 3). Trace levels of AChE in unconditioned medium are derived from FBS.

B) Assay of AChE in conditioned medium taken from MG63 cultures treated with 10μM monensin. A significant reduction in AChE activity was observed in cultures treated after 4 hours, allowing time for residual circulating AChE to be diminished.

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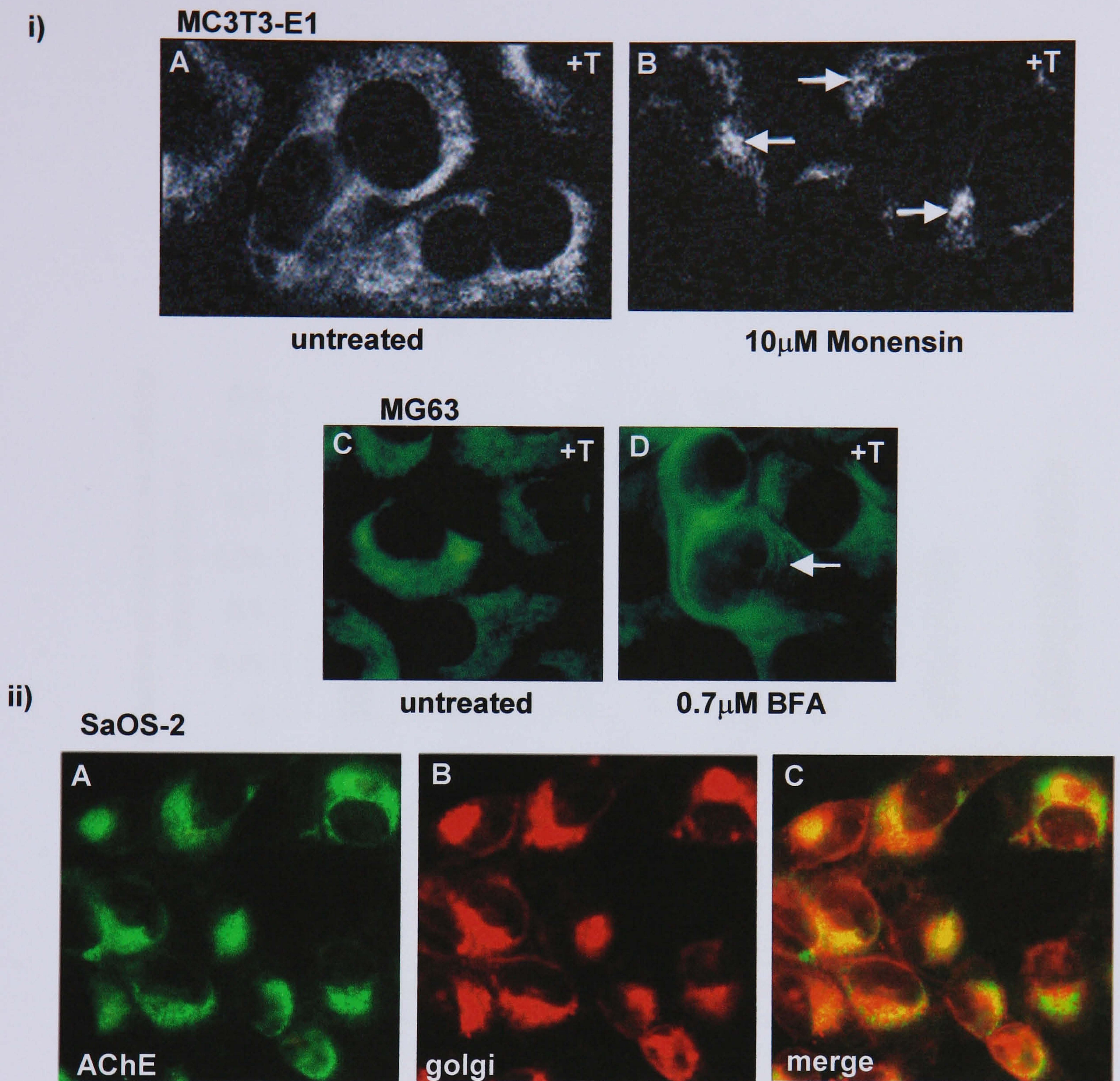

Figure 3.3.12

i) Effect of monensin and BFA on AChE localisation in MC3T3-E1 and MG63 cells. Immunofluorescent localisation for AChE in permeabilised cells revealed that 10μM monensin and 0.7μM BFA induced compartmentalisation of AChE after 6 hours (arrows B and D), in comparison to untreated cells (A and C).

ii) AChE expression (A) also colocalised to the golgi apparatus which was identified using a BODIPY TR ceramide dye (B), and confirmed by yellow staining of the merged image (C).

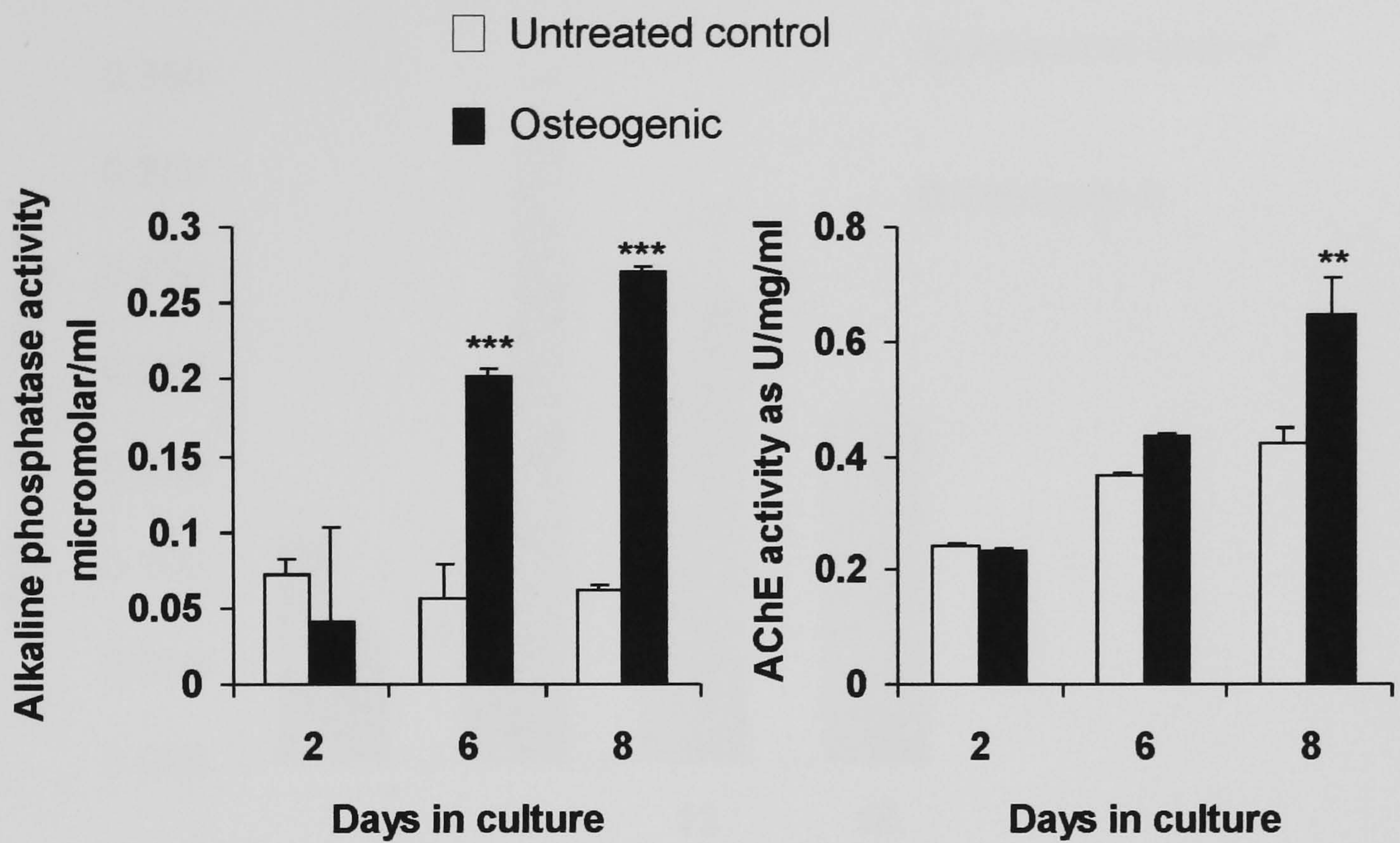

Figure 3.3.13
Alkaline phosphatase and AChE activity of primary rat osteoblasts grown under osteogenic conditions. When assayed at 2 day intervals a significant increase in AChE secretion was observed when compared to control cultures grown in non-osteogenic conditions (n=10, ** $p<0.05$). This paralelled increasing activity of alkaline phosphatase (n=10, *** $p<0.001$).

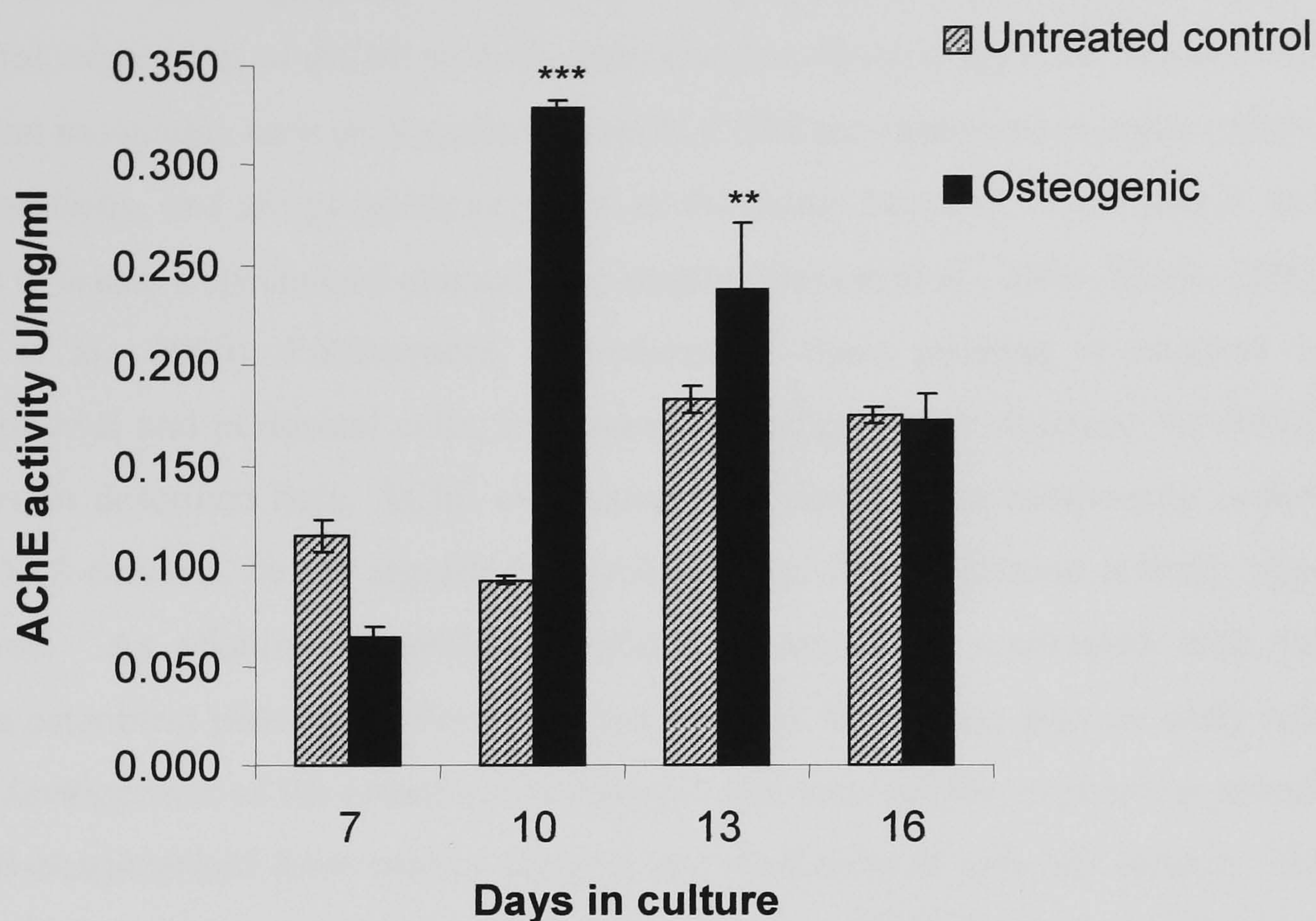

Figure 3.3.14
Primary rodent osteoblasts cultured in the presence of osteogenic supplements 100μg/ml L-ascorbic acid phosphate, 10nm dexamethasone, and 5mM β-glycerophosphate. Conditioned medium was assayed for AChE secretion using the modified Ellman assay at 3 day intervals until day 16 in culture and normalised to total protein. A significant 3 fold increase in AChE secretion was observed in treated cells at day 10 in treated cells (n=10, ***$p<0.001$) when compared to untreated cells. Secretion of AChE in cells treated with osteogenic supplements then declined to levels of untreated cells by day 16 in culture. (**=$p<0.05$).

3.4 Discussion

3.4.1 AChE expression during intramembraneous ossification

It has long been thought that AChE can play significant non-cholinergic roles in embryonic development; indeed Drews (1975) suggested the existence of an embryonic from of AChE (for review see Drews, 1975). Characterisation of AChE expression in developing rat calvaria and long bones revealed a clear spatio-temporal expression of AChE in both early and late developing bone tissues. Cell adhesion molecules such as N-cadherin and N-CAM are expressed in early cellular condensations, and are progressively lost as the tissue becomes more mature and begins to create a specialised extracellular matrix (DeLise et al., 2000, Marie, 2002, Serge et al., 2000). Furthermore, expression of these proteins is retained in perichondrial and periosteal cells, in a manner similar to that observed for AChE expression described here. AChE expression was identified in condensing tissues of rat E18 calvaria, before significant levels of alkaline phosphatase activity were observed. As alkaline phosphatase activity is commonly associated with the mature osteoblast phenotype, these data suggest that AChE may play an early role in the development of the osteoblast phenotype, and parallels the expression pattern of well-characterised bone matrix proteins and mediators of cell-cell contact. The onset of expression of common bone matrix proteins such as bone sialoprotein, osteonectin and osteopontin can vary between the type of bone analysed (Ikeda et al., 1992, Ingram et al., 1993, Sommer et al., 1996, Zhu et al., 2001, Kamiya and Takagi, 2001). However, throughout the skeleton in general, the onset of bone matrix protein expression (around E14-E15) precedes alkaline phosphatase activity (Zhu et al., 2001).

Expression of AChE in mineralised tissues at a later period in development (E20), was more pronounced and could indicate that in addition to influencing early osteoblast phenotype, AChE could be a constituent of the bone matrix. The development of calvarial bones is still not fully understood and is a complex process that involves interaction with surrounding tissues to enable co-ordinated growth. The dura-matter that separates the brain and developing bone tissue is thought to play a role in this co-ordination. Some evidence exists to suggest that cholinergic and catecholaminergic innervation could influence this process, and

may mediate signals between these tissues (Greenwald et al., 2000). It is possible that expression of AChE by condensing calvarial tissues is related to a neuronal innervative role, however such a mechanism is yet to be substantiated, and a specific interaction of cholinergic nerves fibres with osteoblastic cells in this region was not suggested by the authors (the role of AChE in developing calvarial tissues will be discussed further in chapter 5). In addition, an innervative role for AChE in calvarial bones would not account for the expression of AChE found within the osteoid and osteoblasts specifically surrounding these sites, and other bone forming sites of other developing bones.

3.4.2 AChE expression during endochondral ossification.

Previously a number of sources have demonstrated AChE expression in developing limbs (Drews and Kussather, 1971, Drews and Drews, 1972, Drews et al.,1986, Layer and Wilbold, 1992, Alber et al.,1994). A role for cholinesterases in early chick limb development and chondrogenesis has been suggested, in which BChE regulated limb expansion and chondroblast proliferation, with AChE playing an associated role in cell differentiation and patterning of the future limb bone tissues (Alber et al.,1994). In keeping with my observations of AChE expression patterns in calvaria, AChE expression in developing long bones was identified in condensing mesenchymal tissues, and later in osteoblasts and osteoid at periosteal and endosteal surfaces of the bone collar, and trabecular bone. The embryonic periosteum is thought to regulate bone development through interactions with different matrix components such as bone sialoprotein, osteonectin and osteopontin (Shimizu et al.,2001, Fukumoto et al.,2002, O'Driscoll and Fitzsimmons, 2002). It is possible that AChE may play a similar role by interacting with as yet unidentified binding partners, similar to the role AChE plays in synapse plasticity, and axon guidance (Bataille et al., 1998, Grifman et al., 1998; Simon et al., 1999, Johnson and Moore, 2000).

3.4.3 Isoform specific expression of AChE during osteogenesis.

The presence of AChE in such high quantities in bone tissue, with a defined pattern of developmental expression suggests that AChE may be an important regulator of bone formation and osteogenesis. Variety in AChE isoforms, oligomerisation, and attachment to anchoring proteins has proved to be of significance to the functional

localisation of AChE in a number of tissues. As all three isoforms of AChE are expressed in developing and mature bone tissue, it may be that regulation of AChE isoform expression influences any role(s) AChE may play in bone. It was therefore important to determine the expression profile of the different AChE isoforms in developing bone. Immunolocalisation and in situ hybridisations revealed predominant expression of AChE-R in developing rat limb bones. AChE-S and AChE-E were expressed to a much lesser extent but followed a similar pattern of expression suggesting that each isoform may interact in a similar function. This is in contrast to other matrix proteins expressed as multiple splice variants such as tenascin, which expresses different isoforms in mesenchymal, osteogenic and chondrogenic tissues of developing bones (Mackie and Murphy, 1998). Prominent expression of AChE-R may suggest a significant role of a secreted monomeric form of AChE in bone, as this isoform lacks the ability to dimerise or attach to the PRiMA and ColQ subunits. The AChE-S isoform can also be secreted from the cell, however AChE-E is expressed as a GPI-linked membrane protein, this may account for the limited expression of this isoform observed. Early research into expression profiles of the different AChE isoforms indicated that secreted AChE accounted for up to 80% of cellular AChE expressed in muscular and brain derived tissues, with unused membrane bound forms of AChE subject to intracellular degradation (Rotundo et al., 1989). This could indicate that although prominent expression of AChE-R was noted in bone it might be a consequence of the secretable nature of this isoform. Conversely, it could also be possible that monomeric AChE can influence bone formation in a different, or more efficient way that membrane bound AChE. Indeed AChE-R has been suggested as a candidate for the previously described embryonic AChE origionally identified by Drews 1975 (Karpel et al., 1994, Layer and Wilbold 1995, Massoulie et al., 2002). In support of this, AChE-R possesses trophic properties through its cleaved C-terminal peptide in haematopoietic tissues (Grisaru et al., 2001). Several secreted matrix proteins have the ability to influence osteoblast function by acting as a substrate for cell adhesion, interacting with cell surface receptors and activating intracellular signalling mechanisms. This multifunctionality is often regulated by numerous forms of the same protein, including soluble forms competing with membrane bound forms for interaction with surface expressed receptors on other cells, or those found within the matrix. This 'decoy' method could prevent cell

aggregation and allow cell migration. Sequestered AChE within the cell matrix may also in itself mediate cell attachment, as a number of sites are known to influence cell adhesion and spreading in other cells. It is known that processes such as cell migration and aggregation are instrumental to osteogenesis and bone development. Although it is likely that no one protein will prove to be of singular importance in such processes, identification of other mediators can provide a greater understanding of the mechanism involved.

3.4.4 Expression and post-translational modification of AChE in Osteoblasts

Recently binding motifs for Cbfa1, an essential transcriptional regulator of osteogenesis, vitamin D and oestrogen have also been identified in the upstream promoter region of the AChE gene (Grisaru et al, 1999). I characterised AChE expression in osteoblasts and identified expression of two isoforms, one corresponding to the size of synaptic AChE, the other of approximately 55 kDa, which had previously only been identified in recombinant human AChE proteins (Kronman et al., 1993, Scheel et al., 1997). An observed increased expression of this less abundant isoform as cells differentiated toward a more osteoblastic phenotype corresponded with phases of matrix production and secretion in osteoblasts. Post-translational modifications, such as glycosylation, proteolysis and formation of AChE subunits into dimerised and tetrameric forms have previously been identified in other cell types, and are capable of modulating AChE function (Massoulie et al., 1999, Velan et al., 1993, Kerem et al., 1993). Phosphorylation of AChE, at least in vitro, has been shown to weakly induce the expression of a faster migrating AChE species identified in non-denaturing gel electrophoresis due to change in isoelectric charge (Grifman et al., 1997). However, this band was not detectable by immunoblotting under denaturing conditions, and these observations have not been reported in vivo as yet. In addition, the C-terminal peptide of all AChE isoforms contains a signal for proteolytic cleavage, and the resulting peptide from the AChE-R isoform can act as a growth-stimulating factor in at least one tissue (Grisaru et al., 2001). However, as the experiments described here employed a C-terminal directed antibody, it is unlikely that my observations are due to C-terminal cleavage. Protein glycosylation produces proteoglycans (such as glypicans and syndecans), many of which are abundant in the extracellular matrix of connective tissues and the basement membrane (Selleck, 2000). I have

demonstrated that osteoblastic AChE becomes increasingly susceptible to deglycosylation with N-glycanase. As AChE has 3 active N-glycosylation sites, it is likely that the smaller molecular weight identified in primary osteoblast cultures was due to differential or incomplete use of glycosylation sites when processing the protein. This could also indicate that AChE subunits in bone become more heavily glycosylated as osteoblasts mature. Indeed it has been indicated that some glycosylation sequences are more efficient at inducing the addition of glycans than others (Meynial-Salles and Combes, 1996). Moreover, one of the conserved AChE glycosylation sites has been associated with less efficient glycosylation (Kerem et al., 1993, Chitlaru et al., 2002). Differing glycosylation patterns are thought to contribute to the tissue specific properties of AChE (Liao et al., 1992). As the HNK-1 epitope has been associated with AChE it could be possible that the carbohydrate side chains of AChE may play a role in the interaction of AChE with other proteins (Alber et al., 1994). Analysis of AChE carbohydrate composition demonstrated that AChE linked glycans are highly acidic in nature (70-80% acidic), and the majority of N-glycans found on AChE are sialyated (Saxena et al., 1997). Sialytion of AChE glycans has been linked to the rate of clearance from blood plasma (Chitlaru et al., 2002). A number of bone matrix proteins are known to contain large amounts of sialated glycans, including bone sialoprotein, owing its name to its extensive sialytion. The acidic nature of these proteins, along with their ability to bind calcium and phosphate makes them candidates for mediators of matrix mineralisation (Boskey, 1992, Boskey, 1996). AChE contains an EF-hand motif with calcium binding efficiency to the same level as osteonectin; indeed AChE and osteonectin are the only know secreted proteins that contain an EF-hand domain (Tsigelny et al., 2000). As AChE becomes more heavily glycosylated as osteoblasts differentiate, it is possible that glycosylation of AChE in bone provides properties that support matrix mineralisation. The role of AChE glycosylation in other tissues has also been linked to the secretion compatible folding of the protein and signal peptides required for secretion. Mutation analysis of AChE N-glycosylation sites demonstrated that lack of N-glycosylation also prevented secretion of AChE (Liao et al., 1992, Kronman et al., 1992, Velan et al., 1993).

3.4.5 AChE a secreted bone matrix protein?

For AChE to play a role as a bone matrix protein it is essential that osteoblasts not only express but also secrete AChE. Identification of AChE in osteoblast-conditioned medium by western blot analysis indicated that AChE was secreted by osteoblasts. In neuronal and muscular tissues, AChE is secreted via a golgi-ER pathway (Rotundo, 1983, Rotundo et al., 1988). My evidence that monensin, a potent inhibitor of trans-golgi function, induced intracellular compartmentalisation, and significantly reduced AChE activity in osteoblast conditioned medium, indicates that these cells could also secrete AChE via a mechanism similar to muscular and neuronal tissues. These findings are also supported by observations that AChE localised to the Golgi apparatus in osteoblastic cells. While the effects of monensin imply similarities in the storage and secretion of AChE in osteoblasts and other cell types, these inhibitors could impact effects on many intracellular functions. The influence of monensin upon the secretion and production of other bone proteins, and the overall effect of inhibiting protein turnover in osteoblasts must be taken into consideration. However it should be noted that conditioned medium samples tested for AChE activity were done so within the known time for intracellular AChE processing and turnover (6hrs), minimising any indirect down-stream effects that monensin inhibition may have had on AChE processing and secretion (Rotundo et al., 1988). Further characterisations revealed that AChE secretion by osteoblasts increased as the cells became more differentiated, peaking at around day 10 in culture, paralleling increases in alkaline phosphatase activity. This suggests that at least in vitro, AChE secretion is regulated during osteoblast differentiation and bone formation. In addition, this evidence suggests that intracellular levels of AChE do not vary greatly during osteoblast differentiation, however AChE secretion appears to increase markedly in the osteoblast maturation phase and this may be influenced by alterations in the degree of AChE glycosylation that were have observed.

It has been indicated that the secretion of many proteins is calcium dependant, and this may be linked to the high concentration of calcium found within the ER lumen (Pfeffer and Rothman, 1987, Meynial-Salles and Combs, 1996). In cholinergic tissues, AChE secretion is induced by calcium influxes associated with neurotransmitter excitation, in particular the opening of nicotinic acetylcholine receptors (Bursztajn et al., 1988). In a tissue highly enriched in

calcium such as bone, where large quantities are required in the extracellular matrix, it is essential that mechanisms exist to regulate the entry of calcium into the cell. Indeed, intracellular calcium concentration can have an effect upon matrix secretion and deposition (Bursztajn et al., 1988). Although a cholinergic signalling machinery has not been identified in bone, osteoblasts do express a variety of different calcium channels (including voltage gated calcium channels (VGCCs), NMDA type glutamate receptors), that could induce calcium influxes to control the release of AChE by osteoblasts (Duncan et al., 1998). Moreover such receptors have proved to influence osteoblast function significantly (for review see Duncan et al., 1998, Genever and Skerry, 2001, Li et al., 1997). It has also been indicated that AChE secretion in non-cholinergic tissues of the brain is controlled by the resident signalling system for example doperminergic control of AChE release in the substantia nigra (Henderson and Greenfield, 1984, Murphy and Greenfield, 1991, and Webb and Greenfiled, 1992).

Characterisation of AChE expression during bone development has revealed expression patterns suggestive of a matrix protein with role(s) in early osteoblastogenesis. Moreover isoform-specific differences in expression could indicate individuality in isoform function. Further to this, regulated expression and secretion of AChE osteoblast differentiation has been established. Taken together these data suggests a fundamental role for AChE during osteogenesis. However, although AChE displays many of the properties of a matrix protein, functional data suggesting a mechanism in which AChE can influence osteoblast phenotype is required.

Chapter 4 :

A Functional Role for AChE in Bone Development and Osteoblast Function.

4.1 Introduction

4.1.1 Osteogenic stimuli

In the previous chapter it was demonstrated that AChE was secreted by osteoblasts in a differentiation-dependant manner, and appeared to act as a matrix protein. Considering the profound influence osteogenic stimuli have on the expression of other bone matrix proteins, it was hypothesised that AChE expression may also be modulated by these stimuli. Circulating hormones, cytokines, and locally-released growth factors can activate signalling pathways to regulate osteoblast function, proliferation, differentiation and apoptosis, either positively or negatively. A seemingly unending list of signalling mechanisms have been identified that influence osteoblasts and bone formation including PTH, Vitamin D, oestrogen, prostaglandins, TGF-β, FGF, Wnt, and Notch. As the main support for the body, bone must also be responsive to mechanical loads received during everyday life or strenuous activities. Adaptive responses to these loading events enables the body to maintain bone mass that is capable of receiving the increased levels of loads. Modulation of osteoblast function is a main target of mechanical loading and responses to mechanical loading are mediated via the activation of signalling systems that impact on downstream gene expression to regulate bone formation.

4.1.1.1 TGF-β signalling

TGF-β signalling can exert diverse effects on cell function. Modulation of cell proliferation, differentiation, adhesion and apoptosis by TGF-β signalling has been observed in a variety of developing and mature tissues (Massgue et al., 2000, Miyazono, et al., 2001). In addition, abnormal TGF-β signalling is known to contribute to the progression of disease states such as cancer (Massague et al., 2000, Wrana, 2000, Miyazonon et al., 2001). In bone, TGF-β signalling regulates bone remodelling via a variety of effects on osteoclasts, osteoblasts and chondrocytes, and plays an essential role during osteogenesis (Chen et al., 1998, Hoffmann and Gross, 2001, Valcourt et al., 2002). In particular, TGF-β signalling is a major potentiator of osteoblast differentiation, matrix formation and apoptosis (Gehron Robey et al., 1987, Veikiceivic et al., 1989, Harris et al., 1994, Mackie et al., 1998, Geiser et al., 1998, Valcourt et al., 2002). The TGF-β superfamily of cytokines/growth factors includes

TGF-βs 1, 2 and 3, and other TGF-β-like molecules such as members of the bone morphogenic protein family (BMPs), growth differentiation factors (GDFs), activins and inhibins and others (for review see Miyazono et al., 2001). Over 30 different TGF-β–like molecules have been identified so far in mammals alone, making for a diverse signalling system (Miyazono et al., 2001). Members of the TGF-β superfamily are predominantly produced as inactive dimers (Miyazono, 2000). Although all slightly different in structure, members of the TGF-β superfamily predominantly act through serine/theronine kinase receptors to initiate intracellular signalling that can positively or negatively regulate target gene expression primarily through the Smad pathway (Massague, et al., 2000, Miyazono, 2000) (see figure 4.1.1). Activation occurs on proteolytic cleavage of a precursor molecule providing an active ligand composed of the c-terminal fragment that is capable of interacting with TGF-β receptors (Miyazono, 2000). There are two types of receptor capable of binding TGF-β-like molecules, type I and type II receptors, that co-operate to activate signalling. Receptors are composed of an extracellular ligand binding domain, a transmembrane domain, and an intracellular domain that has the capacity to interact with Smad family members and other factors of TGF-β signalling (Miyazono, 2000, Massague et al., 2000, Miyazono et al., 2001, Hoffmann and Gross, 2001). Type II receptors are constitutively active but are unable to initiate signalling alone. However on ligand binding, type II receptors recruit and interact with type I receptors to form a heterodimeric complex. This complex activates type I receptors by phosphorylation of a 30 amino acid glycine/serine rich juxtamembrane region called the GS domain (Heldin et al., 1997, Massague et al., 2000). Activated type I receptors can phosphorylate Smad proteins located close to to the intracellular component of the receptor complex, found in the cytoplasm, or anchored to the membrane via proteins such as Smad anchor for receptor activation (SARA) (Wrana, 2000). There are 5 different types of type I receptor and 7 different type II, making a possible 30 different heterodimeric complexes (Massague et al., 2000, Miyazono et al., 2001, Valcourt et al., 2002). However it has been observed that certain receptors preferentially interact with each other (Miyazono et al., 2001) (see figure 4.1.2). In addition, some ligands have a greater affinity for certain receptor types, or complexes than others and therefore may exert differential effects (Hoffmann and Gross, 2001). It is

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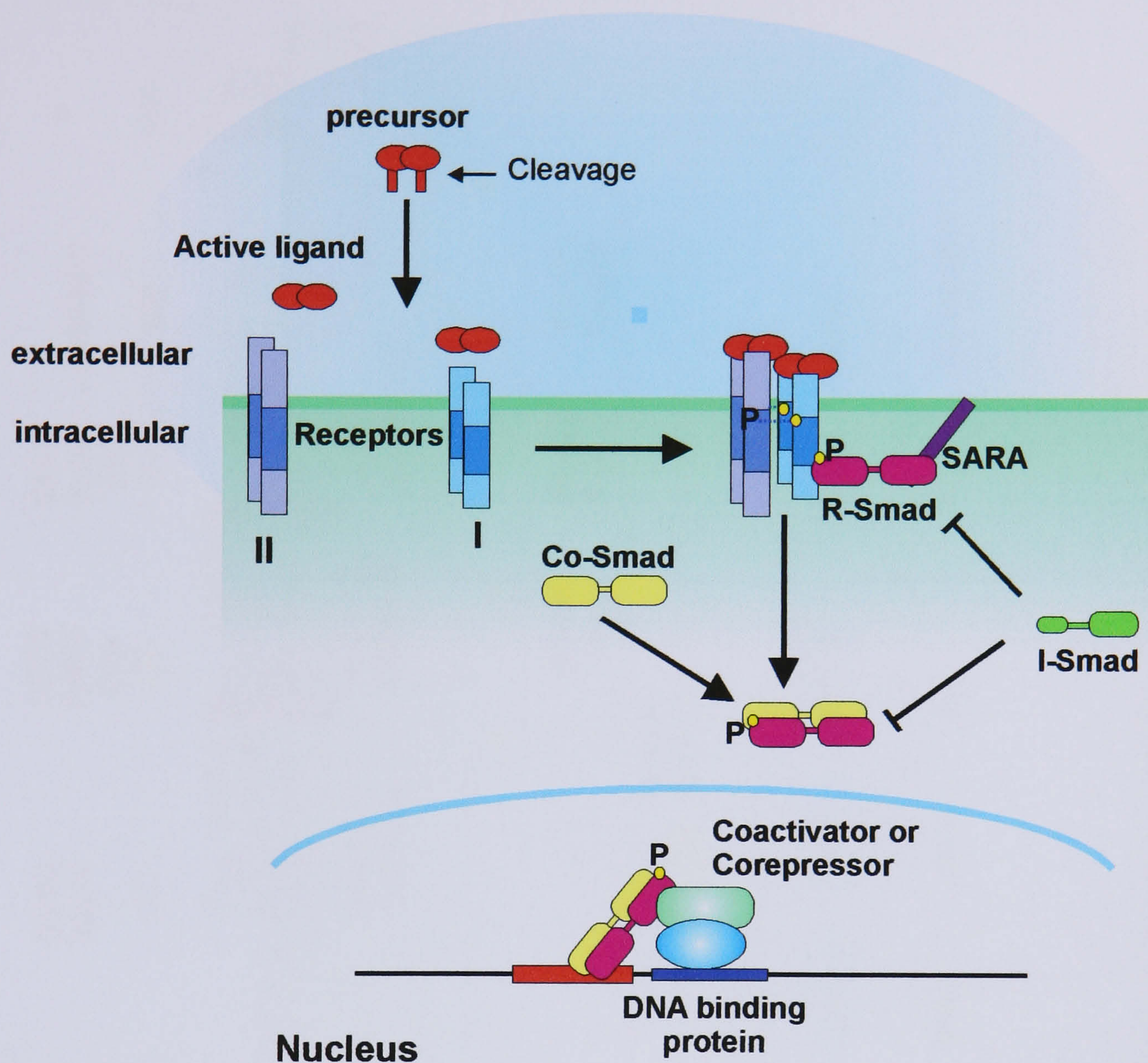

Figure 4.1.1
TGF-β signalling molecules are expressed as inactive soluble extracellular precursor dimers. Upon cleavage the ligand becomes active and able to bind to type II and type I receptors. Binding to type II receptors induces recruitment of type I receptors and the formation of a receptor complex. Type II receptors then activate type I receptors by phosorylation. Activated receptors can phosphorylate R-Smad proteins which in turn form a complex with Co-Smads. I-Smads can inhibit TGF-β signalling either at the receptor complex by inhibiting R-Smads or by preventing R-Smad/CoSmad complexes, which would normally enter the nucleus to bind to DNA and transcription factors. Together with co-activators and repressor molecules Smads can alter gene transcription.

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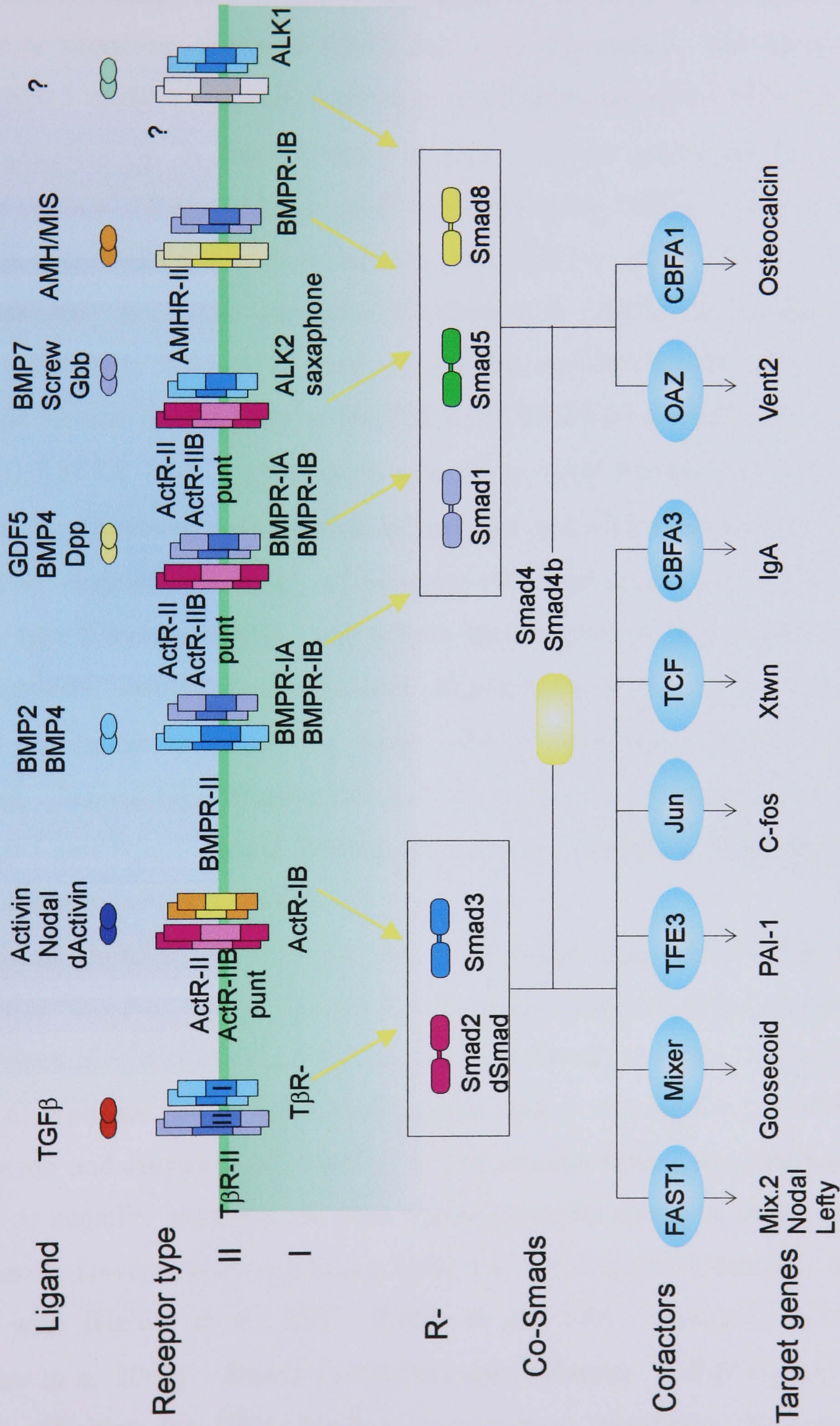

Figure 4.1.2
Possible receptor complexes and the corresponding ligand binding specificity, and R-Smad interactions.

thought that this enables a ligand gradient to be established in developing tissues where local signalling may overlap into other tissues, and signal specificity is essential (Massague et al., 2000, Miyazono et al., 2000, Hoffmann and Gross, 2001). As with many other areas of research, simultaneous identification of receptors by independent laboratories has meant that receptors are called by different names according to the cell type or signalling system in which they were discovered. For simplicity I will refer to type I receptors as ALK (activin receptor-like kinases) 1-7 (See figure 4.1.2). TGF-β receptors can be classed into 3 groups. ALKs 1 and 2 are very similar in structure yet bind different ligands (Hoffmann and Gross, 2001). ALK1 binds TGF-β ligands in osteoblasts and endothelial cells, and ALK2 to BMPs 7 and 2, the ligands most commonly associated with bone (Valcourt et al., 2002). ALK3 and ALK6 are also very similar in structure and activity, binding members of the BMP family (also BMP type IA and IB receptors or BMPRIA and BMPRIB respectively) (Valcourt et al., 2002). ALKs 4 and 5 (Activin receptor IB or ActR-IB and TGF-β receptor I or TβR-I respectively) are again similar in structure and where found to be the activin and TGF-β receptors in a variety of cell types (Valcourt et al., 2002). Of importance to bone, type I receptors ALK3 and 6 have been shown to play significant roles in chondrogenesis, osteoblastogenesis and adipogenesis (Chen et al., 1998). More recently additional receptors for some TGF-β superfamily ligands have been identified. Termed type III receptors proteins such as betaglycan and endoglin can alter type I and type II ligand binding and activity (Massague, 1998, Barbara et al., 1999, Hoffmann and Gross, 2001).

Smad proteins can transmit the intracellular signal of TGF-β signalling. Activated R-Smads associate with Co-Smads in the cytoplasm to induce translocation into the nucleus where these complexes can bind directly to DNA or co-operate with other transcriptional complexes or transcription factors (see fig 4.1.1a) (Wrana, 2000, Verschueren and Huylebroeck, 1999). In this context Smads can function as either positive or negative regulators of gene transcription by acting as transcriptional co-activators or co-repressors depending upon the transcriptional complex the Smads interact with (Heldin et al., 1997, Wrana et al., 2000, Miyazono, 2000a and b, Miyazono et a, 2001). Smads themselves can influence TGF-β signalling due to different affinities for DNA binding or sequence specificity (Verschueren and Huylebroeck, 1999). Amongst many others, Smad proteins have high affinity runt

homology transcription factors (for review see Miyazono et al., 2001). This family of proteins plays significant roles in regulating gene transcription of a number of different cell types discussed earlier (see section 1.2.1 Chapter 1). In particular, in response to TGF-β signalling, Cbfa1 an essential factor for osteoblasts and bone formation can associate with Smad proteins in the nucleus to induce transcription. In addition, a large number of binding sites for Smads have been identified in the promoter region for Cbfa-1 and osteocalcin. Smad signalling also has considerable cross-talk with other signalling pathways, and is capable of interacting with a number of other transcription factors that can regulate osteogenesis such as Lef/TCF transcription factors and β-catenin of the Wnt signalling pathway, VDR elements of vitamin D, and Hox genes (Miyazono et al., 2001). In many circumstances TGF-β signalling can also be regulated by other pathways, such at the MAPK and JAK/STAT pathways (see von Bubnoff and Cho, 2001 for review). Consequently, cross-talk between the PTH, Shh, Ihh, Wnt, and FGF signalling pathways have also been observed in bone both during development and in mature tissues (for review see Hoffmann and Gross, 2001, and von Bubnoff and Cho, 2001). Although only one of many signalling systems, it is clear that TGF-β signalling represents a complex and divergent regulator of bone remodelling.

4.1.1.2 FGF signalling

Fibroblast growth factors (FGFs) are another large family of secreted soluble growth factors that can interact with an equally large family of cell surface receptors (FGFRs) to regulate embryonic development and patterning, as well as cell proliferation, migration, chemotaxis, differentiation and fate determination in a large number of adult tissues (Pitaru et al., 1993, Goldfarb et al., 1996, Galzie et al., 1997, Debi et al., 1998, Scutt and Bertrom, 1999, McIntosh et al., 2000, Mansukhani et al., 2000, Ornitz and Marie, 2002). A significant number of mutations in FGF ligand or receptor expression and function have proved to be responsible for over 15 human disorders, a large number of which have profound skeletal phenotypes as the main symptom or reason for diagnosis (Muenke et al., 1998). To date, nearly all FGF ligands and FGFRs have been identified in bone, and roles in cell proliferation, differentiation and

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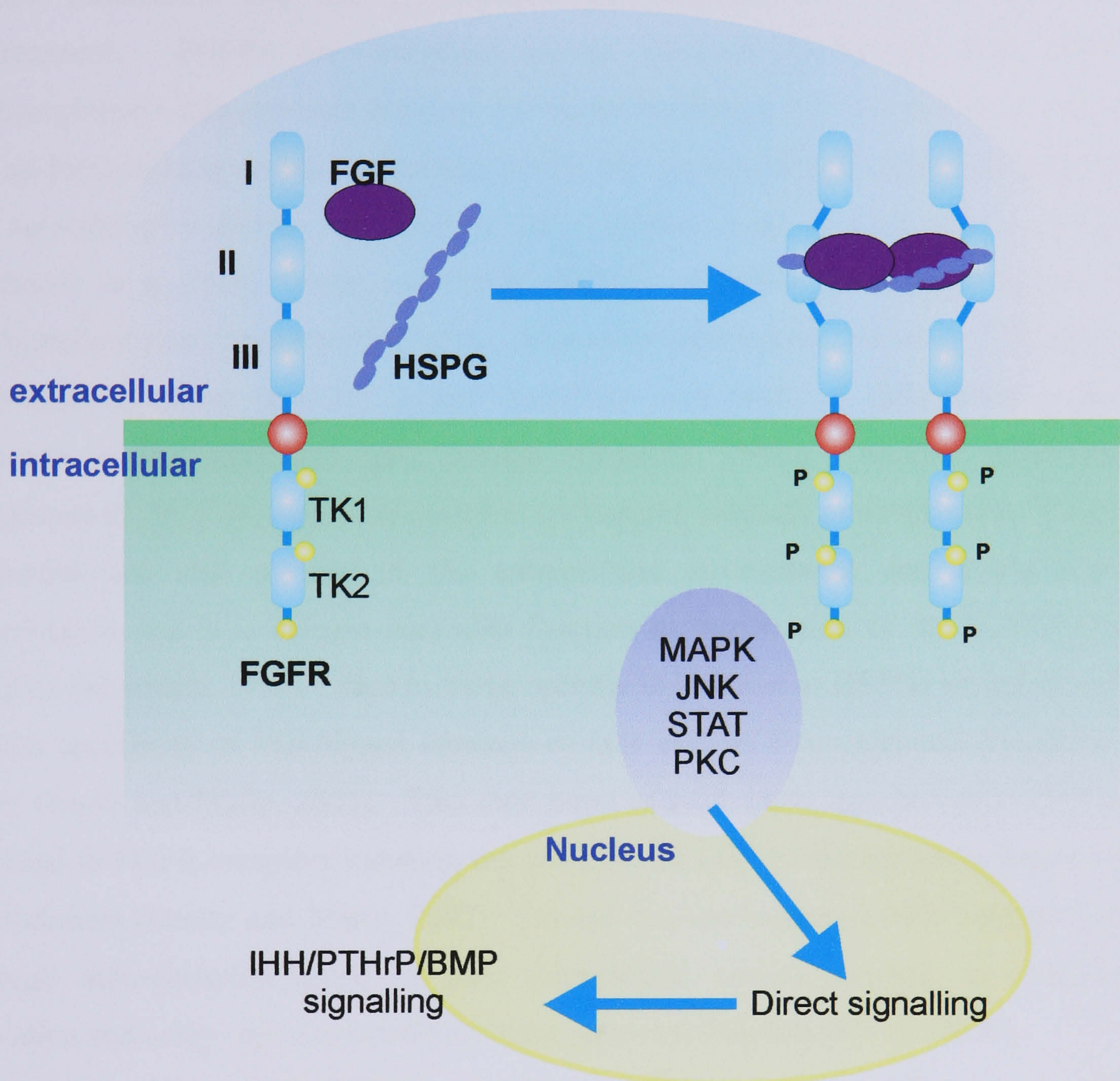

Figure 4.1.3
Extracellular soluble FGF monomers interact with heparan sulphate proteoglycans to form dimers that bind to two cell membrane associated FGFRs. Upon ligand binding receptors undergo autophosphorylation of two tyrosine kinase domains found in the intracellular domain to induce activation and signalling through RAS/MAPK, JNK/ERK, STAT, and PKC. This can induce a direct effect upon osteoblast function, proliferation, differentiation and apoptosis, and secondly upregulation and activation of IHH/PTHrP and BMP signalling pathways.

apoptosis have been suggested (Pitaru et al.,1993, Debi et al.,1998, Scutt and Bertrom, 1999, Mansukhani et al.,2000).

22 different FGF genes have been identified, divided into 6 subfamilies of FGFs based on sequence homologies, producing up to 18 diverse FGFs (McIntosh et al., 2000, Ornitz and Itosh, 2001, Ornitz and Marie, 2002). FGFs are expressed as soluble monomers that are glycosylated and secreted into the extracellular environment. FGFRs are composed of an extracellular domain with three immunoglobulin-like domains required for ligand binding, a transmembrane domain, and an intra-cellular domain composed of the two tyrosine-kinase domains required for auto-phosphorylation of receptors upon ligand binding (See figure 4.1.3) (McIntosh et al.,2000, Ornitz and Marie, 2002). 4 FGFR genes provides for 7 functionally distinct receptors for FGFs. Alternative splicing of 3 of the FGFR genes contributes to tissue restricted ligand specificity as a result of differences in the sequence of the innermost IgIII domain (Goldfarb, 1996). Another important component of the FGF signalling complex are heparan sulphate proteoglycans. These molecules are also present in the extracellular environment and allow FGF dimerisation, and it is thought may also function in stabilisation of the FGF/FGFR complex (Goldfarb, 1996). Due to tissue specific expression of HSPGs an impact on cellular specificity of FGF/ligand interactions may also be a consequence (Goldfarb, 1996, Ornitz and Marie, 2002). Two monomers of FGF come together with HSPGs and bind to FGFR receptors inducing the assembly of FGFR receptor homodimers or heterodimers (Ornitz and Marie, 2002). Ligand binding induces FGFR receptors to undergo auto-phosphorylation of their intracellular portion leading to receptor activation and action upon downstream target pathways (McIntosh et al., 2000).

The major target pathway of FGF signalling in many cell types is the RAS/MAPK pathway (Goldfarb, 1996). FGF signalling has a dual effect on osteoblast proliferation and differentiation. FGFs activate FGFRs in osteoblasts leading to formation of Grb2/FRs2/Shp2 complex and activation of MAPK. Shc and FRS2 have also been implicated in FGF signalling, acting as adapter proteins that target signalling molecules to the plasma membrane to link receptor activation with MAPK and other signalling pathways (Blaikie et al., 1994, Kouhara et al., 1997, Xu et al., 1998). However FGF signalling is also known to impact upon other intracellular downstream pathways, and considerable cross-talk between these intracellular pathways has been observed. FGF-2 has been found to induce phosphorylation of p44/p42 MAPK to

induce VEGF in MC3T3-E1 cells (Tokuda et al., 2003). However, FGF-2 activated p38 MAPK negatively regulates VEGF release. FGF also induces up regulation of and or phosphorylation of SAPK/JNK, moreover inhibition of SAPK/JNK reduces FGF-2 induced VEGF release (Tokunda et al., 2003). MAPK is important for activation and phosphorylation of Cbfa1 (Xiao et al., 2000). Significantly FGF signalling has proved to regulate Cbfa1 expression and activity via a number of different pathways. FGF signalling induces Cbfa1 expression, FGF-2, 4 and constitutively active FGFR2 stimulate Cbfa1 expression and binding to osteocalcin promoter (Xiao et al., 2002). PKC has also been linked to FGF signalling. Blocking PKC-delta completely inhibited FGF-2 induced Cbfa1 expression. PKC-delta also modified Cbfa1 transcriptional activity via posttranslational modification (Kim et al., 2003). In contrast, FGF-2 induces phosphorylation of ERK1/2, whilst ERK1/2 inhibition blocks FGF-2 stimulated Cbfa1 phosphorylation and binding to osteocalcin promoter. Moreover the C-terminal PST domain of Cbfa1 was found to be required for FGF-2 effects (Xiao et al., 2002). Immature osteoblasts respond to FGF with proliferation, however in differentiating cells, FGF does not induce DNA synthesis but causes apoptosis, by a reduction in AKT phosphorylation along with increased Bax levels and a delay in Bcl2 accumulation, along with no activation of STAT1 (Mansukhani et al., 2000).

Considerable cross-talk between FGF signalling and other signalling pathways have also been observed. This is clearly demonstrated during development where FGF signalling has cross-talk with Ihh, Shh, PTHrP pathways, through direct activation and upregulation of these genes (Ornitz and Marie, 2002). This is important for co-ordinated regulation of longitudinal bone growth and maturation as well as in skull expansion and co-ordination with brain growth (see next chapter for further discussion). Cross-talk with other signalling pathways also play an important role in mature tissues. For example TGF-β 0.1-10ng increased all FGF-2 mRNA transcripts but did not significantly alter FGFR1 or 2 in osteoblasts. Also TGF-β induced FGF-2 mRNA was markedly reduced by PKA inhibition (Sobue et al., 2002). In addition, mechanical loading increased bFGF expression also induced ERK1/2 phosphorylation and localisation to the nucleus (Hatton et al., 2003).

In chondrocytes, FGF signalling down-regulated proliferation through STAT1 phosphorylation and translocation to the nucleus (Sahni et al., 1999). The diversity of

FGF signalling molecules and receptors has provided for disparity on the effect of FGF signalling on endochondral bone formation. FGF-2 appears to inhibit longitudinal bone growth; in contrast FGF-2 also stimulated proliferation of chondrocytes (Mancilla et al., 1998). It is thought that FGF signalling either through differing molecules and receptors complexes, or even the same molecule may exert differential effects upon the chondrocytes at different stages of maturation. This is highly important in developing bones and longitudinal bone growth that occurs at the growth plate (see Chapter 1.4.3).

In addition to the tyrosine-kinase activity of FGF receptors some evidence exists to suggest a function in phophatidylinositol breakdown by serving as a SH2 domain for phopholipase C-γ1 (Mohammadi et al., 1992, Goldfarb, 1996, McIntosh et al., 2000). The functional significance of this in bone development is yet to be fully explored, and little is known as to the effect on other tissues.

The complexity of growth factor regulation of osteoblast function and bone formation is demonstrated by inter-regulation and cross-talk observed between many of the well established signalling systems. To add to this, physical regulation of bone mass through mechanical strains received during movement also has a fundamental role on osteoblast function, and thus must either regulate or interact with signalling systems to influence bone formation.

4.1.2. Mechanical Strain

The adaptation of bone through bone remodelling is essential to enable the skeleton to respond to the environmental changes in lifestyle and physical activity. Therefore responses to mechanical loading of bones are potent inducers of bone remodelling. Strain 'responsive' cells such as osteocytes located deep within the bone microarchitecture are subjected to strains as a result of physical activity via a number of mechanisms. Firstly, it is thought that the physical distortion of bones as a result of loading may result in a 'stretching' of these cells which in turn will activate 'stretch' induced channels found within the cell membrane (Hert et al., 1971, Chamay et al., 1972, Lanyon and Baggott, 1976, Frost, 1983, Carter, 1987, Burr et al., 1989, Knothe Tate and Knothe, 2000). Secondly, the distortion of the bone forces the movement of fluid through the bone microenvironment (fluid flow), and in turn the fluid passing by these same cells will also induce a strain on the cell membranes known as 'shear

stress' that will subsequently activate membrane-associated channels (Cowin et al., 1991, Turner et al., 1994, Duncan and Turner, 1995, Burr et al., 2002). It is known through in vitro studies that both these mechanisms (mechanical loading and fluid flow) stimulate bone formation (Klein-Nulend et al., 1995, Rawlinson et al., 1995, Banes et al., 1995, Knothe Tate and Knothe, 2000, Zaman et al., 2000). Fluid flow can induce a strain on cells that is comparable to the weight-induced strain on bones (Jacobs et al., 1998, Knothe Tate and Knothe, 2000, Burr et al., 2002). In vivo, analysis of mechanical loading has also established that mechanical strain is a 'programmed' system, which can adapt its responses. Bone cell sensitivity to mechanical strain can reach its maximum rapidly, after which time further strain, or increased strain will elicit no further effect (Turner, 1998, Burr et al., 2002). A period of rest is required to allow basal conditions to return, and make cells capable of responding to strains again (Turner, 1998, Huiskes et al., 2000). It was thus noted that a cyclical strain was required to elicit a sustainable effect of physical loading (Turner et al., 1998, Burr et al., 2002). In vivo, this may occur through the alternate strain of bones during exercise and a consequential change of fluid flow direction that would in turn induce a cyclical effect upon the resident cells (Huiskes et al., 2000). In this way, short periods of high levels of activity could produce a greater effect upon bones than longer sustain periods of low level exercise.

The exact mechanism by which bones interpret mechanical strains at the cellular level and relate this toward altered bone remodelling is still not fully understood, and is proving to be a highly complex system. However, it is known that the ultimate consequence of mechanical loading is increased bone remodelling and as a result increased bone mass. Therefore mechanical loading must have a direct influence on a great many of the processes that regulate bone cells. Indeed, mechanical loading has been shown to induce osteoblast proliferation, differentiation, apoptosis, matrix synthesis and secretion, and mineralisation both in vitro and in vivo (Raab-Cullen et al., 1994, Rawlinson et al., 1995, Rawlinson et al., 1996, Cheng et al., 1997, Webb et al., 1997, Burger and Klein-Nulen, 1999, Rawlinson et al., 2000, Nomura and Takano-Yamamoto, 2000, Zaman et al., 2000, Kaspar et al., 2000, Huiskes et al., 2000, Wetys et al., 2003). Therefore demonstration of regulated expression patterns of any protein as a result of mechanical strains or other established regulators of bone formation would suggest a significant role for such proteins in bone remodelling.

4.1.3. AChE adhesive properties

Demonstration of regulated AChE expression and secretion in osteoblasts suggests a role for AChE in osteoblast function. Moreover, localisation of AChE expression specifically to osteoblasts and osteoid at sites of new bone formation could suggest a role for AChE as a matrix protein. In light of these observations, and the concensus opinion of matrix protein function, it is possible AChE may mediate osteoblast function through adhesive interactions with the matrix. In neuronal tissues, numerous studies have demonstrated roles for AChE in mediating cellular interactions, migration, axon formation and differentiation via a non-cholinergic mechanism (Drews et al., 1974, Greenfield, 1991, Umezu et al., 1993, Layer and Willbold, 1994, Bataille et al., 1998, Grifman et al., 1998; Simon et al., 1999, Johnson and Moore, 2000). A subfamily of 'esterase-like' proteins, which are involved in cell-cell and cell-matrix interactions, share significant sequence homology to the AChE core catalytic domain but lack any catalytic activity (de la Escalera et al., 1990, Darboux et al., 1996, Botti et al., 1998, see chapter 1.5.2. and fig 1.9.). The drosophilia 'esterase-like' domain of neurotactin is known to mediate neuronal-epithelial interactions during development. Mutation analysis revealed that replacement of the neurotactin 'esterase-like' domain with the corresponding domain of acetylcholinesterase had no effect upon adhesion suggesting AChE may also possess adhesive properties (Darboux et al., 1996). Included in this esterase-like family is neuroligin-1, the ligand for neurexin-1β, involved in the formation of heterophilic cell-cell interactions during neuritogenesis (Ichtchenko et al., 1996, Nguyen and Sudhof, 1997, Song et al., 1999). Transgenic mice over-expressing AChE demonstrated significantly suppressed neurexin-1β mRNA specifically during embryonic development (Andres et al., 1997). In vitro, AChE antisense oligonucleotides application also induced a significant reduction in neurexin-1α expression, which could however be rescued by neuroligin-1 overexpression, indicating a linked role between AChE and neuroligin-1 (Grifman et al., 1998). Members of the 'esterase-like' family are all transmembrane proteins making it possible that intracellular signalling may be mediated through binding to counterparts found on adjacent cells or in the matrix, akin to the dual functions of integrin adhesion and signalling. As described in chapter 1, analysis of the molecular structure of AChE has identified a secondary substrate-binding site, termed the

peripheral anionic site, which is responsible for modifying catalytic activity and mediating inhibitor interactions (Friboulet et al., 1990). In addition, specific inhibitors or functional blockade of this site significantly reduced AChE-mediated adhesion in vitro, identifying a possible requirement for the peripheral anionic site in the adhesive function of AChE (Jones et al., 1994, Small et al., 1995, Bataille et al., 1998, Johnson and Moore, 1999 and Simon et al., 1999). More recently structural analysis of the AChE molecule and comparison with the other structurally related 'esterase-like' proteins has identified a highly-charged electrostatic region in the vicinity of the PAS in AChE, and in the esterase-like domain of related proteins (Botti et al., 1998). As electrostatic charge has proved to be of functional significance in the formation of protein-protein interactions and complex formation in solution these proteins were consequently termed the 'electrotactins' (Botti et al., 1998). In support of this, electrostatic interactions were identified between AChE and common matrix components such as collagen IV and lamanin-1 via the PAS (Johnson and Moore, 2003). AChE was also found to interact with amyloid in solution and induce aggregation in vitro, and is commonly associated with the plaques developed in the brains of Alzheimer's disease patients (Sberna et al., 1998, Bartolini et al., 2003, Piazzi et al., 2003). It is clear AChE possesses many qualities that could promote cellular interactions with both the matrix and other cells.

In this chapter, the effects of osteogenic stimuli, including TGF-β, FGF and mechanical loading in vitro and in vivo on osteoblastic AChE expression were determined. In addition, the functional effects of inhibiting AChE on osteoblast activity were identified. These data support the hypothesis that AChE acts as an osteoblast derived adhesion molecule in bone.

4.2 Materials and methods

4.2.1 Effects of osteotrophic factors on AChE expression

4.2.1.1 Transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (bFGF)

MG63, SaOS-2, MC3T3-E1, and primary rat and human osteoblasts were used in these studies. All cultures were serum-starved for 24 hours prior to growth factor treatment. Cells were then plated and cultured in serum free medium supplemented with 2% bovine serum albumin (BSA) and either TGF-β1 (0.5ng/ml – 2ng/ml), or bFGF (0.5ng/ml – 5ng/ml). Primary rat osteoblasts were cultured in osteogenic medium (as described previously, see section 2.1.2.2) until day 3 when they were serum starved and exposed to growth factors on day 4 as described above. Whole cell lysates were taken at 24, 48 and 72 hours after treatment and AChE expression determined by western blot analysis following the protocol described in Chapter 2 (see sections 2.1.4.1 and 2.1.4.3).

4.2.2 Effects of mechanical stimulus on AChE expression

4.2.2.1 Mechanical strain in vitro

TE85 human osteosarcoma cells were plated onto type I collagen coated FlexerCell dishes. Using the FlexerCell FX-3000 system, a cyclical strain of 4,500με (peak) for 10 minutes at 1Hz was applied to confluent cultures. Whole cell lysates taken at 1, 4, 16, and 48 hours after loading were analysed for AChE expression by western blot analysis and compared to unloaded control cultures as detailed in Chapter 2 (see sections 2.1.4.1 and 2.1.4.3).

4.2.2.2 Mechanical strain in vivo

We determined expression of AChE during in vivo loading events. Tissues were taken from loading experiments performed by Dr. Alex Brabbs, University of York. Anaesthetised mature male Wistar rat right ulnae were subjected to mechanical strain of ~4000με, cyclically for 3.3 minutes at 2Hz on 5 consecutive days using a modified Instron materials testing device as described previously (Hillam and Skerry, 1995). Animals were then killed 1 week after loading experiments; loaded rat ulnae and

unloaded control left ulnae were removed immediately. Transverse sections of the proximal ulnae were taken for analysis by immuno/enzyme histochemistry on parallel sections as described in Chapter 2 (see section 2.1.6.2).

4.2.3 Functional role for AChE in osteoblast adhesion

4.2.3.1 Adhesion assays

Osteoblast adhesion was analysed using an adhesion assay system. Firstly MG63, SaOS-2, and MC3T3-E1 osteoblast like cells or primary rat osteoblasts were cultured overnight in serum free medium supplemented with 2% BSA. Cells were trypsinised and plated at high density (6 x 10^4 cell/cm^2) in 96 well plates. After removal of media and non-adherent cells, surface bound cells were fixed in 95% ethanol for 5 minutes before staining for 30 minutes with 0.5% crystal violet in 95% ethanol (Sigma). Surplus crystal violet was removed by gentle washing, and wells allowed to dry for 4 hours. Crystal violet was eluted from cells using acidic isopropanol with gentle agitation and absorbance read at 570nm using a Dynex microplate reader. To determine the optimal time for adhesion of osteoblasts, pilot studies using a variety of time points were carried out as above, all subsequent adhesion assays were for 4-6 hours.

The effect of AChE (torpedo electric organ, Sigma) on osteoblast adhesion was compared to that of collagen type I (rat tail, Sigma) or a related cholinesterase, BChE (human blood, Sigma). Solutions of these substrates (100μg/ml) were prepared in medium and used to coat tissue culture plates at 4°C overnight. 1 hour prior to use, coated wells were rinsed with PBS and then blocked in 0.5% BSA to prevent non-specific binding. MG63 cells were plated as above and adhesion was analysed after 4 hours.

4.2.3.2 Effect of AChE inhibitors on osteoblast adhesion

The effect of AChE inhibitors on osteoblast adhesion was determined by plating MC3T3-E1 cells in the presence of DFP, BW284C51 or the BChE inhibitor iso-OMPA (10^{-4}M –10^{-7}M) and assayed for adhesion after 6 hrs. Cell viability was tested by MTT assay.

4.2.3.3 Inhibition of AChE expression and osteoblast adhesion

Antisense studies were performed to determine the effect of inhibition of AChE expression on osteoblast adhesion. Antisense oligonuceotides (oligos), 20 nucleotides in length and phosphorothioated on their last three 3' internucleotide linkages to prevent nucleolytic degradation were used. Oligo and transfection vehicle concentrations were optimised for use with MC3T3-E1 and SaOS-2 osteoblast-like cells, using concentrations between 0.1μM and 100μM for 24, 48 and 72 hours. The effect on protein expression was determined by western blot analysis using an antibody directed to the core domain of AChE (BD Transduction labs). For adhesion assay experiments mRNA oligos targeted to the core AChE domain (exon 2 and exon 3, Grifman and Soreq, 1997) and control oligos directed at BChE, or inverse to AChE oligos (see table 4.1 for oligos and positions) were used. SaOS-2 osteosarcoma cells were pretreated with 5μM oligos in a water vehicle for 24 hrs and then replated in the presence of oligos and assayed for adhesion after 6 hrs. Efficiency of oligos to reduce AChE expression was again determined by western blot of whole cell lysates. Cell viability was tested by MTT assay.

4.2.3.4 MTT assay

Mitochondrial dehydrogenases in viable cells convert yellow 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide Thiazolyl blue (MTT) into a water insoluble purple coloured formazan product by the cleavage of the terazolium ring. To test for toxicity of antisense oligonucleotides and AChE inhibitors MTT assays were carried out in parallel to adhesion assays. Cells plated as above were incubated with 5mg/ml MTT (Sigma M 5655) in sterile PBS for 4 hours under standard culture conditions. Converted salt product was eluted by cell lysis with acidic 0.5% SDS or acidic isopropanol and absorbance read at 405nm on a Dynex microplate reader as detailed in Chapter 2 (see section 2.1.3.1).

4.2.3.5 Statistical analysis

All MTT and adhesion assay experiments contaianed 10 samples per treatment and were carried out in triplicate. To determine the statistical significance of adhesion and MTT

assays multiple anova and paired T-tests and appropriate post hoc tests were carried out using SPSS version 10.

4.3 Results

4.3.1 Growth factor regulation of AChE expression in osteoblasts

4.3.1.1 Effect of TGF-β1 and bFGF on AChE expression by osteoblasts

Primary rat osteoblasts were treated with TGF-β1 (0.5ng/ml, 1ng/ml, or 2ng/ml) for up to 48 hrs. Western blot analysis of whole cell lysates taken at 24 and 48 hrs after treatment revealed increased AChE expression and the appearance of a second AChE band after 2ng/ml treatment that was not present in untreated control cultures (Figure 4.3.1). Whole cell lysates taken from MG63 osteosarcoma cells treated with 0.5ng/ml, 1ng/ml and 5ng/ml bFGF, were analysed by western blotting, demonstrating increased AChE expression in cultures treated with >1ng/ml bFGF after 24 hrs (Figure 4.3.2).

4.3.2 Regulation of AChE expression by mechanical stimulus.

4.3.2.1 In vitro

TE85 human osteosarcoma cells were subjected to a cyclical strain of approximately 4,500με (peak) for 10 minutes at 1Hz, western blot analysis of whole cell lysates taken at different time points after loading. No significant short term (2-60 minutes) effect on AChE expression was observed (Figure 4.3.3A). At later time points (1-48 hours) an increase in AChE expression was identified 4 hrs after loading, which was maintained for a further 48 hrs (Figure 4.3.3B).

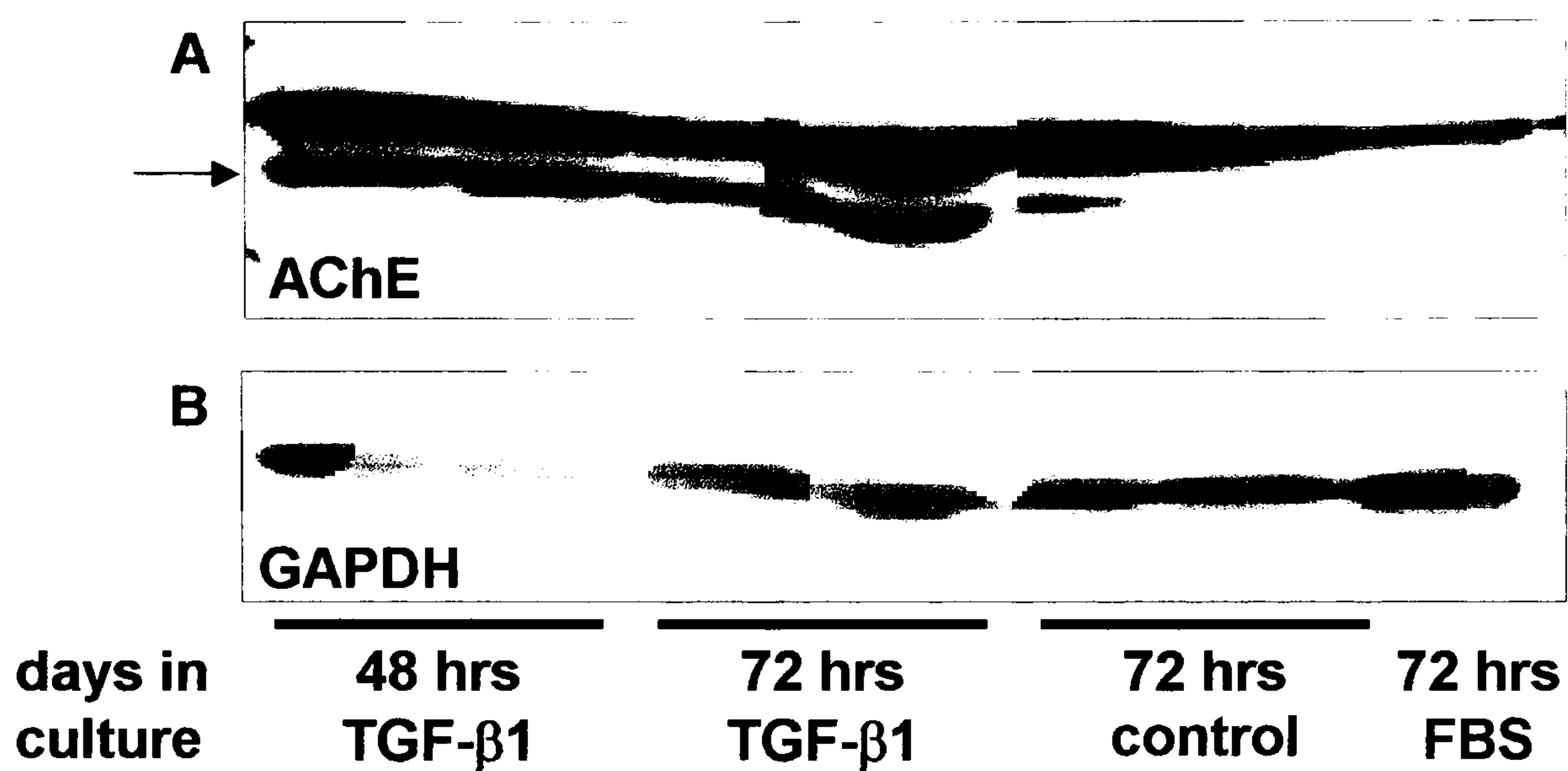

Figure 4.3.1
Western blot analysis for AChE expression in whole cell lysates of primary rat osteoblasts treated with 2ng/ml TGF-β1 for 48 and 72 hrs. (A) Expression of a 68 kDa species was observed in all cultures. Treatment with TGF-β1 induced expression of a second AChE species of ~55 kDa (A arrow) not present in untreated control cultures, or cells grown in the presence of FBS. (B) GAPDH expression on same blot.

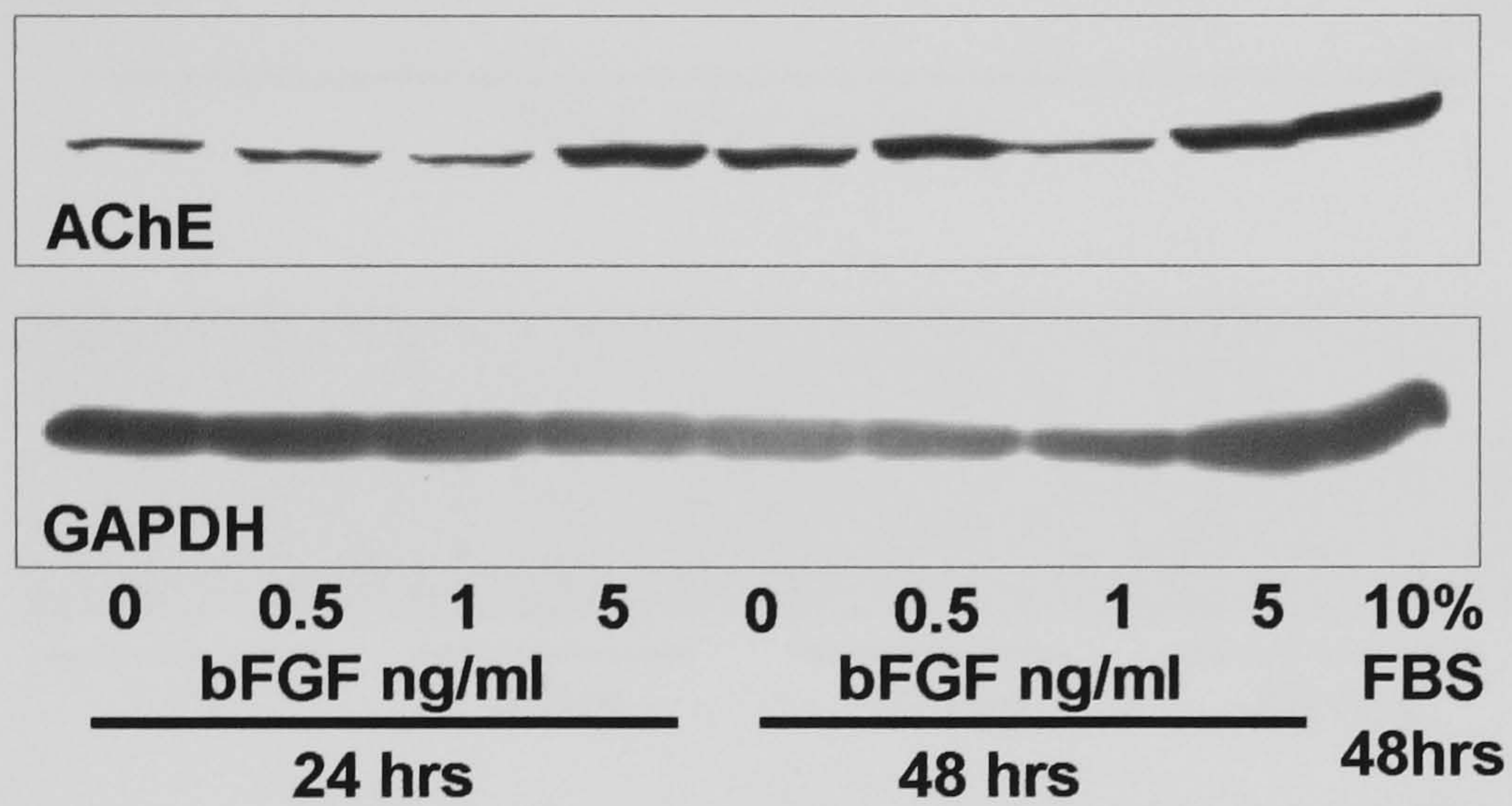

Figure 4.3.2
Whole cell lysates of MG63 osteosarcoma cells treated with 0.5, 1 and 5ng/ml bFGF for 24 and 48 hours were analysed by western blotting. Cells treated with >1ng/ml bFGF after 24 hours increased AChE expression compared to untreated controls.

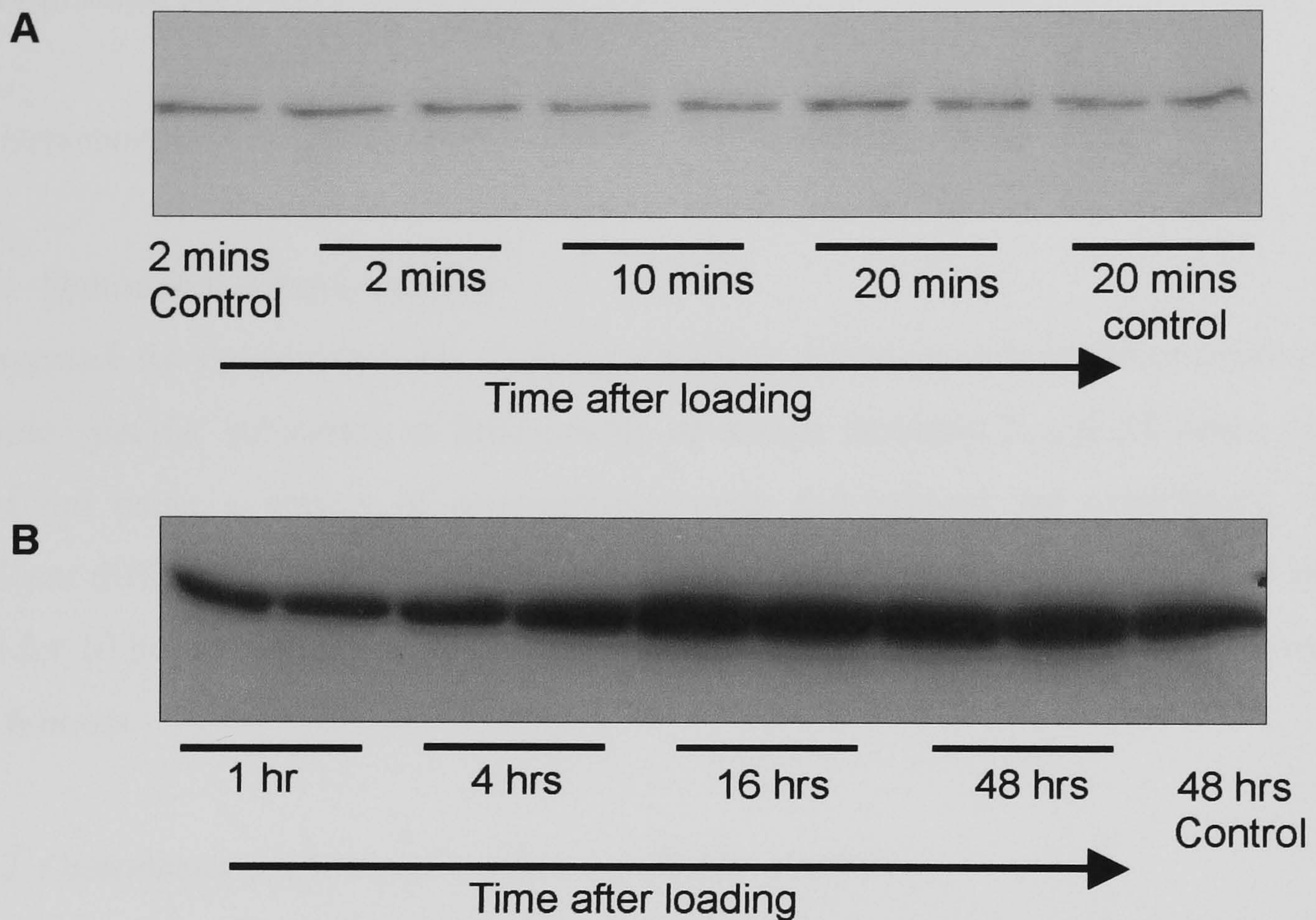

Figure 4.3.3
A) TE85 cells loaded at 4500 με for 10 minutes at 1Hz using a Flexercell FX300 system. Western blot analysis of whole cell lysates taken 2, 10 and 20 minutes after loading revealed no apparent effect on AChE expression at these time points after loading.

B) Western blot analysis of TE85 human osteoblast like cells loaded for 10 minutes at 1Hz to induce a strain of 4500με, whole cell lysates taken at 1hr, 4hrs 16hrs and 48hrs after loading. A significant increase in AChE expression was observed after 4hrs, which is maintained through to 48 hrs after loading.

4.3.2.2 In vivo

AChE expression was also increased during in vivo loading events. Immunolocalisation for AChE on cryosections from loaded ulnae revealed elevated AChE expression when compared to unloaded controls, especially at sites of induced bone formation following mechanical loading. This corresponded to increased levels of alkaline phosphatase activity in serial sections (Figure 4.3.4).

4.3.3 Determination of a functional role for AChE in osteoblast adhesion

4.3.3.1 Optimising adhesion assays

To determine the optimal time for assaying osteoblast adhesion with inhibitor treatments and onto specific substrates, a time course of assays between 2 and 24 hours were carried out using a variety of osteosarcoma cells and primary rat osteoblasts. No significant differences in adhesion were observed when cells (controls and treated) were plated for 10 hours or greater. All subsequent assays were therefore carried out between 4 and 6 hours.

4.3.3.2 Osteoblasts preferentially adhere to AChE substrates

To determine the effect of AChE substrate on osteoblast adhesion, MG63 and SaOS-2 osteosarcoma cells were plated onto wells precoated with AChE (100μg/ml), and compared to that of cells plated onto the related cholinesterase, BChE (100μg/ml), or collagen type I (100μg/ml). Cells adhered to and spread onto AChE substrates in a way similar to cells grown on collagen type I, however they retained a more rounded morphology (Figure 4.3.5). In contrast, cells plated onto BChE did not adhere successfully when compared to adhesion on collagen type I and AChE substrates, and shared a similar morphology to cells plated directly onto tissue culture plastic (Figure 4.3.5).

4.3.3.3 The effect of AChE inhibitors on osteoblast adhesion

The effect of AChE inhibitors on osteoblast adhesion was determined by plating MC3T3-E1 cells in the presence of DFP, BW284C51 (AChE inhibitors), or iso-OMPA (BChE inhibitor) for 6 hours. A concentration dependent-decrease in osteoblast

Original in colour

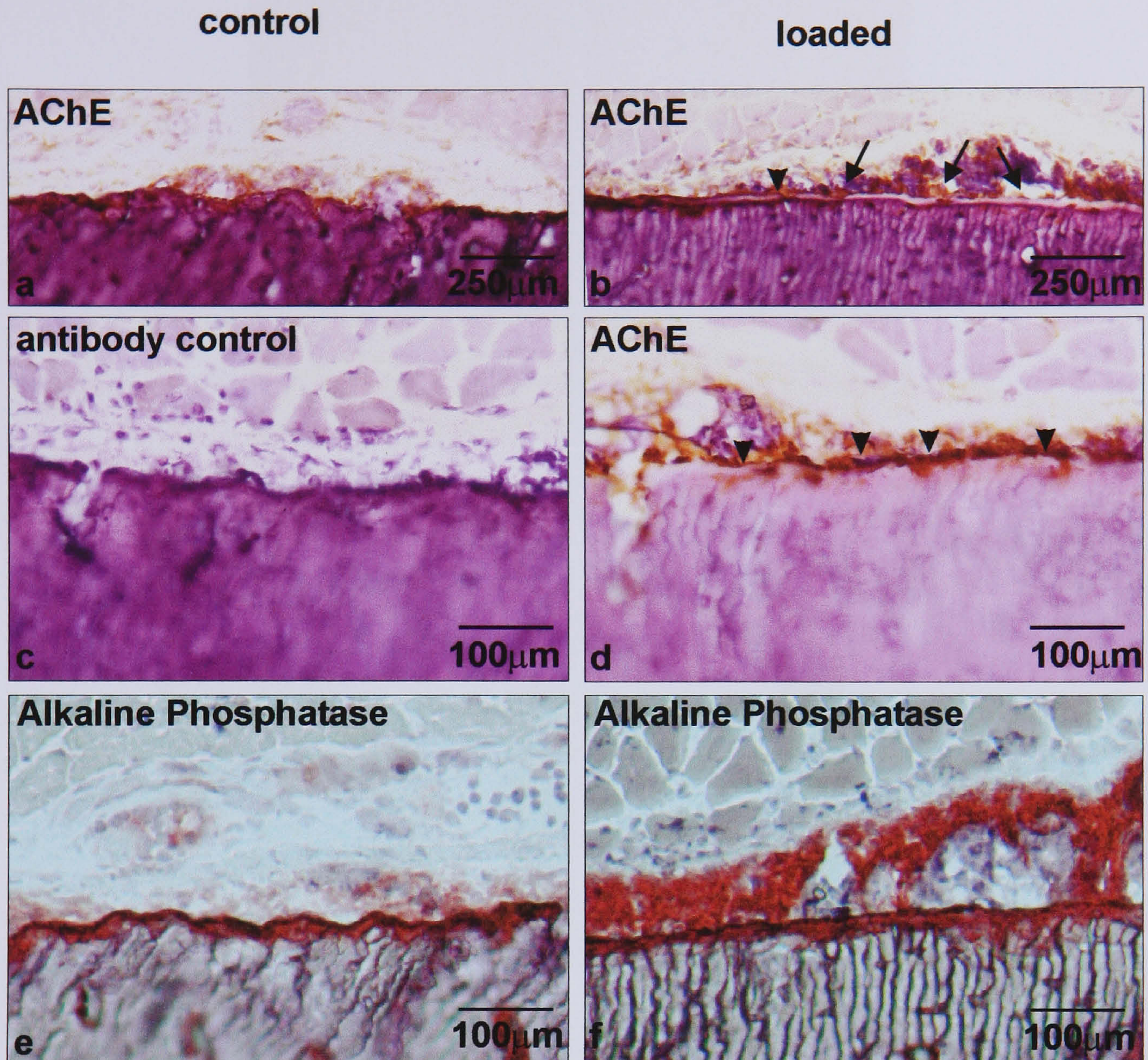

Figure 4.3.4
(a) Low level AChE expression was localised on the quiescent medial periosteal surfaces of unloaded ulnae. (b) Following the osteogenic response to mechanical stimulus, enhanced AChE expression was observed in recruited osteoblasts (b arrows, brown staining), in the newly formed osteoid layer (d arrowheads, brown staining). (c) antibody control, (e and f) alkaline phosphatase activity on serial sections.

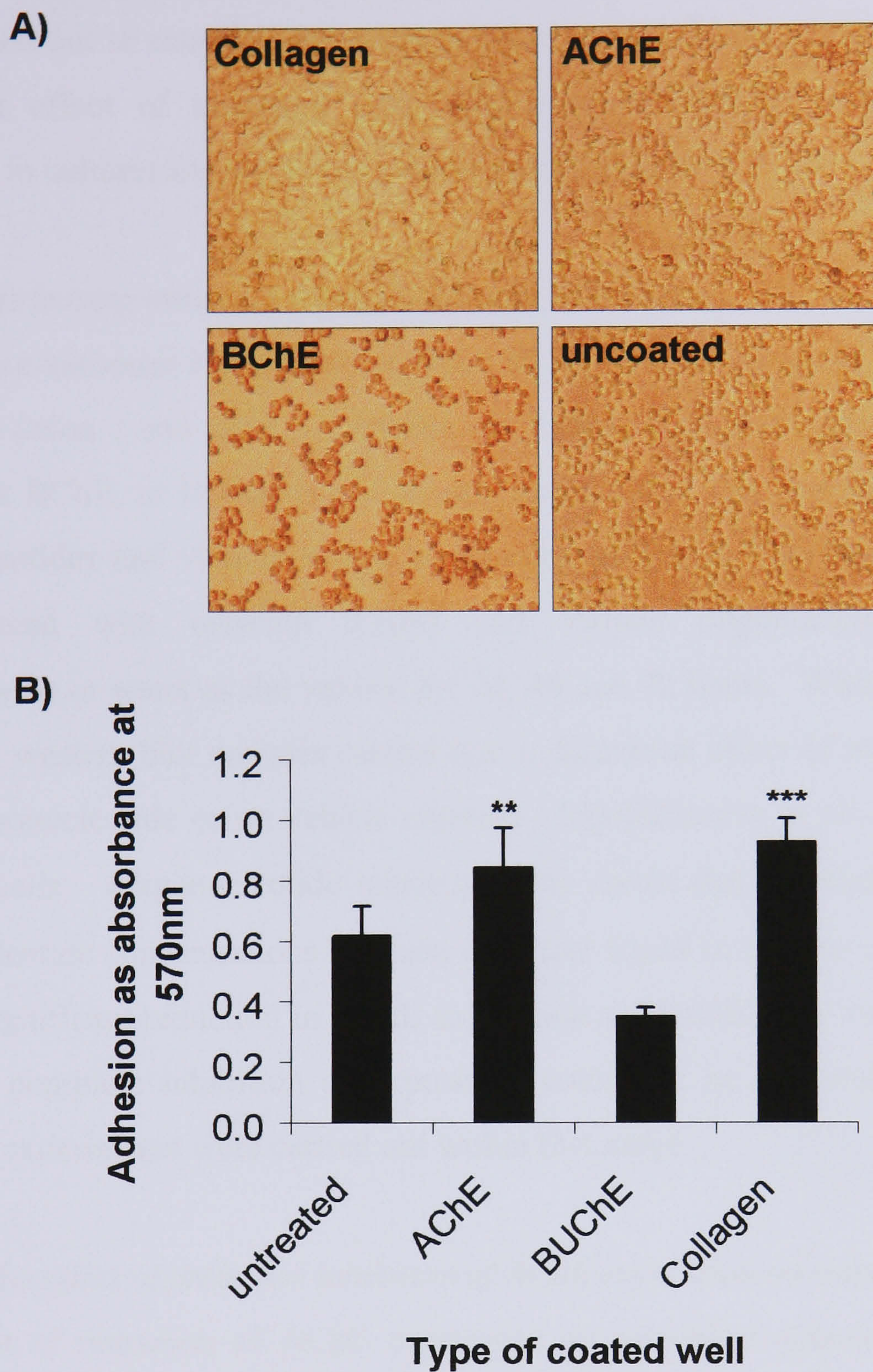

Figure 4.3.5
SaOS-2 osteosarcoma cells plated to 100μg/ml torpedo AChE, human BChE, or collagen type I coated wells and assayed for adhesion after 4 hours. (A) Cells plated to AChE substrates adhered and spread to a similar level to cells on collagen type I, albeit with a more rounded morphology. Cells plated onto BChE and uncoated well remained rounded after 4 hours.
(B) A significant increase in cell adhesion was observed in cells plated on AChE (n=10, ** $p<0.01$) and collagen type I substrates (n=10, ***$p<0.001$), compared to uncoated control wells or cells plated on BChE.

adhesion was observed in cells plated with AChE inhibitors. Cells plated in the presence of iso-OMPA had no significant effect (Figure 4.3.6). Parallel MTT assays were carried out to establish the effect of these treatments on viable cell numbers. No significant effect of treatment with cholinesterase inhibitors was observed when compared to cultures treated with a vehicle control (Figure 4.3.6).

4.3.3.4 Optimising antisense transfer and concentration

I carried out antisense studies using mRNA oligonucleotides targeted to the core domain of AChE (exon 2 and exon 3, Grifman and Soreq, 1997) and control oligonucleotides directed at BChE, or inverse to AChE oligonucleotides. To optimise concentration of oligonucleotides and vehicle to use in adhesion assays, SaOS-2 and MC3T3-E1 cells were treated with between 0.1μM and 100μM oligonucleotides with either lipofectamine or water as the vehicle for 24, 48 and 72 hours. Whole cell lysates were taken and western blot analysis carried out to determine effect of treatments compared to no oligonucleotide or no vehicle controls. Lipofectamine at all concentrations was toxic to cells. Oligonucleotide concentrations above that of 50μM were also toxic. Oligonucleotide concentrations between 1μM and 10μM in a water vehicle did however cause a significant reduction in AChE expression as identified by western blot analysis. However complete inhibition of expression could not be achieved. All subsequent antisense experiments were carried out within this range.

4.3.3.5 The effect of antisense inhibition of AChE expression on osteoblast adhesion

The effect of reduction of AChE expression on osteoblast adhesion was carried out using the above antisense oligonucleotides. SaOS-2 osteosarcoma cells, pre-treated and plated with 5μM oligonucleotides in a water vehicle, were assayed for adhesion after 6 hours. Efficiency of oligonucleotides to reduce AChE expression was determined by western blot analysis of whole cell lysates. A significant reduction in adhesion was observed in cells with combined exposure to AS1 and AS4 oligonucleotides (see table 3.1 for details), adhesion could be rescued by plating these cells onto AChE substrates. However there was no significant effect of adding soluble AChE to the culture medium. Cultures treated with antisense oligonucleotides were tested for cell viability using an MTT assay. Antisense oligonucleotide treatment did not appear to reduce viable cell number (Figure 4.3.7).

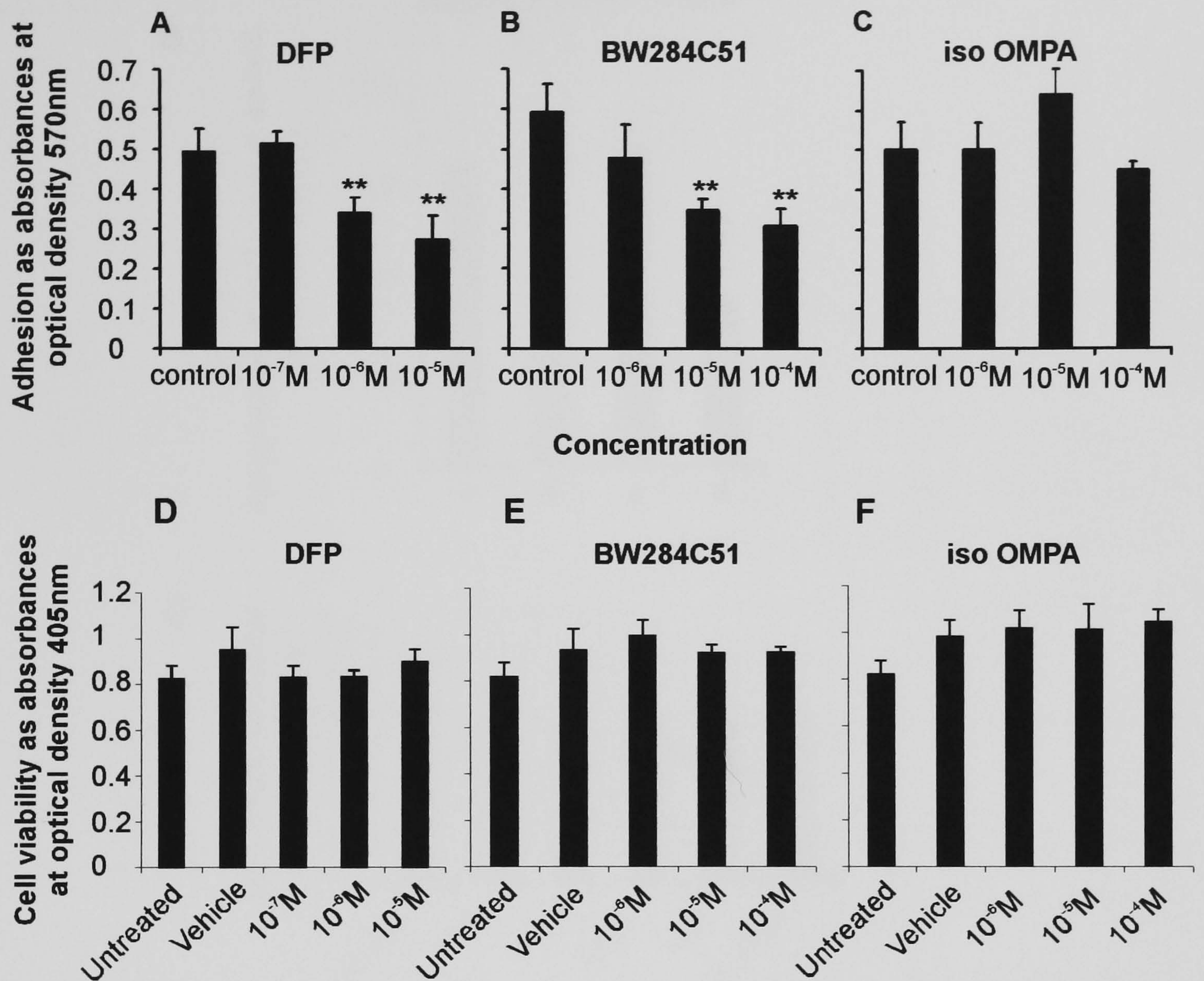

Figure 4.3.6
MC3T3-E1 osteoblast like cells plated in the presence of AChE inhibitors DFP (A), BW284C51 (B), or BChE inhibitor iso-OMPA (C). After 6 hrs a concentration-dependant decrease in MC3T3-E1 cell adhesion was observed in both DFP and BW284C51 treated cells when compared to vehicle controls (n=10, ** $p < 0.01$). Iso-OMPA had no significant effect. MTT assay for revealed no significant effect of inhibitor treatments on cell viability (D, E and F).

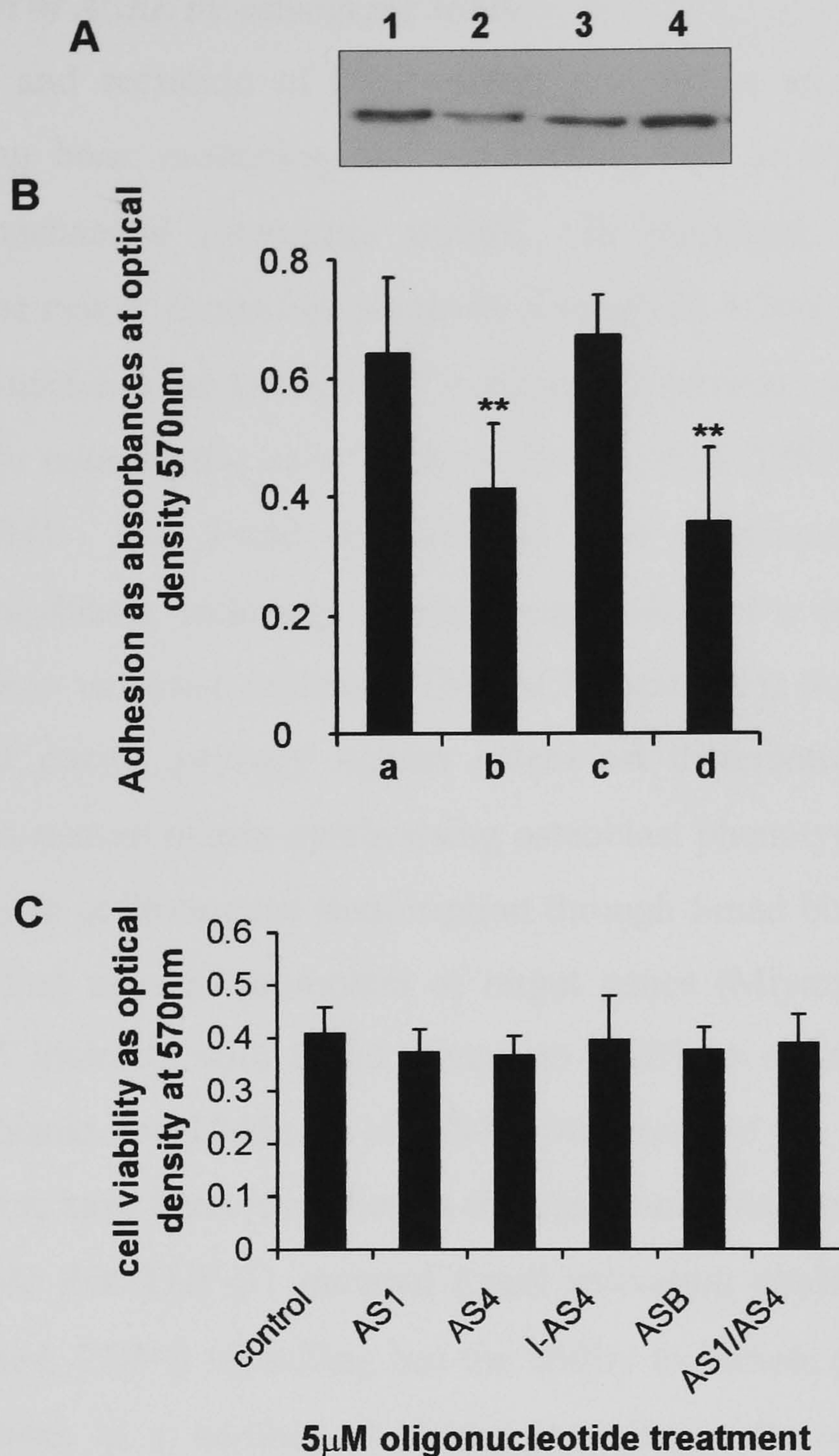

Figure 4.3.7
(A) Western blot analysis demonstrating AChE expression in untreated cultures (lane 1), cultures treated with antisense RNA oligonucleotides AS1 and AS4 against AChE (lane 2), against BChE (lane 3), or inversed sequence of AS4 oligonucleotide (lane 4). A reduction in AChE expression was observed in cultures treated with antisense against AChE.
(B) A significant reduction in adhesion was observed in SaOS-2 cells treated with antisense oligonucleotides against AChE for 6 hours ($n=10$, ** $p< 0.01$) (b), when compared to controls (a). Adhesion was rescued by plating these cells on AChE substrates (c), but not by the addition of soluble AChE to the culture medium (d).
(C)Using MTT assays of viable cell numbers, it was demonstrated that antisense oligonucleotide treatment for 24 hrs had no significant effect compared to untreated control cells.

4.4 Discussion

4.4.1. Regulation of AChE by osteogenic stimuli

The expression and secretion of bone matrix proteins is an essential function of osteoblasts during bone modelling and remodelling that is regulated by numerous chemical and mechanical osteogenic stimuli. In particular, TGF-β1 is a potent stimulator of bone matrix formation, primarily through its action on osteoblasts. TGF-β1 significantly increases the synthesis of bone matrix proteins such as type I collagen and tenascin-C in osteoblastic cells (Gehron Robey et al.,1987, Mackie et al.,1998, Harris et al.,1994). We found that TGF-β1 also significantly increased AChE expression in osteoblasts, inducing abundant expression of a second AChE species, which was absent in untreated cultures. This AChE species is the same size (~55kDa) to that observed during primary human osteoblast differentiation and may be a characteristic of a mature matrix-synthesising osteoblast phenotype. TGF-β signalling has the capability to activate gene transcription through Smad binding of transcription factors, and binding sites in promoters of target genes (Miyazono et al.,2001). In particular Smad5 interacts with Cbfa1 bound to OSE1 to regulate osteocalcin gene expression (Yoshianki and Heldin et al., 1997, Miyazono et al., 2003). As numerous Cbfa1 binding sites have been identified in the upstream promoter region of the AChE gene it is possible that TGF-β1 induced Smad activation could initiate AChE gene expression. Indeed, TGF-β signalling has the ability to initiate gene transcription via other Runx proteins in a number of tissues including cells of the haematopoietic lineage in which AChE plays a significant non-cholinergic role (Miyazono et al.,2001). Moreover, aberrations in normal TGF-β signalling via Runx proteins, p300, CBP and Evi-1 can lead to diseases that have been associated with abnormal AChE expression i.e. familial platelet disorders, acute myeloid leukaemia (Grisaru et al.,2000, Massague et al.,2000, Miyazono et al.,2001). Characteristically, due to the significant effect of TGF-β signalling in bone, aberration of this signalling system can also result in bone disorders such as cleidocranial displasia (CCD), hereditary chondroplasia and osteoporosis (Francis-West et al.,1999, Massague et al.,2000, Miyazono et al.,2001). Moreover, TGF-β1 knock out mice have a significantly reduced bone mass and bone elasticity due to perturbations in the bone matrix (Geiser et al.,1998). This suggests that the bone matrix and bone matrix proteins like AChE are targets of signalling by

TGF-β1. However, the effects on osteoclast function and their contribution to the phenotype must also be taken into consideration. In addition, TGF-β1 is just one member of the TGF-β super family that exerts effects on osteoblasts. Indeed, other members have proved to be potent stimulators of osteoblast differentiation and function including members of the BMP family BMPs 2, 7 and 4. Cross-talk, regulation or synergism between TGF-β1 and other TGF-β1 signalling molecules has been observed in other tissues (Hoffmann and Gross, 2001, and von Bubnoff and Cho, 2001). It is therefore possible that the effects of TGF-β1 on AChE expression we observed only represent a small component of the role of TGF-β signalling. Differential effects or regulation of AChE expression may be observed when other members of the TGF-β super-family are present in a manner similar to the in vivo state.

Signalling by fibroblast growth factors (FGFs) is well established as a regulator of bone remodelling and development. A large number of skeletal dysplasias are associated with mutations in FGF signalling components, in particular receptors FGFR2 and 3, develop cranio-facial and limb abnormalities as a result of disrupted functioning of osteoblast and chondrogenic cells (Wang et al.,1999, Mansukhani et al.,2000, Isaac et al.,2000, for reviews see Goldfarb, 1996, McIntosh et al.,2000, Ornitz and Marie, 2002). Analysis of bone samples taken from thanotorphoric dysplasic patients, a disorder caused by mutations in the FGFR3 receptor (that in most cases is lethal), revealed reduced levels of AChE expression and activity, as well as a severe reduction in a number of other bone matrix proteins (Grisaru et al.,1999). Here it was demonstrated that a concentration-dependent upregulation in AChE expression was observed in MG63 osteosarcoma cells after exposure to bFGF for 48 hours. This could implicate AChE as a downstream target molecule for FGF signalling, and lends further support to a role for AChE as a matrix protein. FGF signalling is known to activate the MAPK pathway. MAPK has been implicated in the phosphorylation of Cbfa1 resulting in increased transcriptional activity. As there are significant numbers of Cbfa1 binding sites present in the AChE promoter region it is possible that FGF signalling could target AChE expression via the MAPK pathway (Goldfarb, 1996, Xiao et al., 2000, Xiao et al., 2002). In osteoblasts, FGF signalling has been linked with increased proliferation. The increased levels of AChE expression observed in this

experiment may well be associated with increased proliferation and as a consequence increased cell number and availability of protein. However the absence of fluctuations in GAPDH on the same blot indicate equal levels of protein loading. In addition, it was observed that TGF-β1 treatment induced expression of a second AChE isoform, which was not observed in the FGF treated cells. This could be a consequence of differences in cell type (cell line versus primary cells), but may also be a reflection of the different effects of these signalling systems on AChE protein expression. Certainly, in osteoblasts FGF signalling is most commonly associated with proliferation and TGF-β with differentiation (Mansukhani et al.,2000, Miyazono et al., 2001). It is possible that AChE may act differently at distinct points in osteoblast maturation, this is supported by our previous observations of regulated AChE secretion during osteoblast differentiation which was discussed in Chapter 3 (see section 3.3.4). Under differentiating conditions, FGF treatment has also been shown to induce osteoblast apoptosis (Mansukhani et al.,2000). However, as our treatments were carried out under non-differentiating osteoblastic conditions it is unlikely this may be a factor. Further analysis of the effect of FGF signalling on AChE expression through osteoblast differentiation using primary cell types and more specific FGF molecules may increase our understanding of the regulation of AChE by FGFs.

4.4.2. Regulation of AChE expression by mechanical loading

Mechanical loading is one of the most potent stimulators of bone formation, acting on cells of the osteoblast lineage to induce expression and secretion of bone matrix proteins. Studies have indicated that mechanical stimulation in vitro can increase AChE expression and secretion by muscle cells (Hubatsch and Jasmin, 1997). Here it was demonstrated that AChE expression by osteoblasts increased in response to mechanical loading both in vitro and in vivo. Observations of a rapid (4hrs) yet long lasting (48 hrs) induction in vitro, and specific localisation of AChE expression to newly formed osteoid and osteoblasts at sites of load-induced bone formation in vivo strongly suggest that AChE is acting as a bone matrix protein. Characterisation of the molecular events resulting from mechanical loads indicate an immediate rapid rise in intracellular calcium. As discussed in Chapter 3 increased intracellular calcium levels induce up regulation in AChE secretion and a subsequent up regulation in AChE expression to replenish the intracellular stores of AChE (see section 3.4.5). In

addition, it is thought that one mechanism by which mechanical loading in bone induces its effects is by increasing fluid flow received by osteocytes located in bone, which induces a 'shear stress' (Burr et al., 2002). As a result, shear stress response elements have been identified and studied in bone. Factors such as Cox2 and iNOS are known to induce and regulate the expression of mitogenic factors such as c-fos/AP1 and cAMP. Numerous c-fos/AP1 binding sites are present in the immediate promoter of AChE, and AP1 is a common regulator of AChE gene expression in a number of different cell types (Aziz Aloya et al., 1993, Getman et al., 1995, Mutero et al., 1995). cAMP response elements (CRE) are also present in the immediate and upstream promoter region of AChE. cAMP is also known to induce AChE expression in muscle cells, another mechanically responsive cell type (Mutero et al., 1995, Wan et al., 2000, Choi et al., 2001, Siow et al., 2002). In addition, studies of the responses to mechanical stimulus in periosteal cell expression profiles have indicated an initial transient production of mitogenic and growth factors such as c-fos (2hrs), and later TGF-β1 and IGF-1 (4hrs) (Raab-Cullen et al., 1994). This supports our observations of induced AChE expression by growth factor treatments and could suggest that AChE expression is a downstream effect of the initial responses to mechanical loading. Cbfa1 has been identified as a target of mechanical loading in vitro, with signalling through the MAPK/ERK pathway causing increased activity and inducing activation of Cbfa1 DNA binding and expression (Ziros et al., 2002). This mechanism may modulate AChE expression in response to mechanical loading, as the upstream promoter region of AChE contains a number of Cbfa1 binding sites (see section 4.4.1).

In other tissues, stress responses similar to those induced by mechanical stimuli are known to induce overexpression and modulation of AChE isoforms that results in neurodeterioration and haematopoietic alterations (Beeri et al., 1995, Stephenson et al., 1996, Grifman et al., 1998, Sternfeld et al., 2000a, Grisaru et al., 2001, Meshorer et al., 2002). In addition, study of a chromosomal region immediately upstream to the ACHE gene revealed a large locus containing many binding sites for a variety of stress responsive elements (Grant et al., 2001). It is thought that AChE could well be a stress response element, as many neurological conditions in which AChE expression is altered show considerable similarity in aetiology and symptoms to some stress related conditions including Gulf war syndrome, manic depression and chronic fatigue syndrome (for review see Soreq and Seidman, 2001, see section 1.6.5 chapter 1).

Under stress conditions in the brain (forced swim for example) there is a rapid yet long lasting switch in AChE alternative splicing resulting in an over expression of the AChE-R isoform (Meshorer et al., 2002, Kaufer et al., 1999). Recently it was identified that the AChE-R isoform has the ability to interact with the scaffolding molecule RACK1, which is involved in the PKC signalling pathway (Birikh et al., 2003). PKCβII is capable of mediating stress responses in the brain, and activity and expression is unregulated in neurodegenerative disorders (Ono et al., 1987, Paola et al., 2000, Pakaski et al., 2001). Furthermore AChE-R transgenic mice that display fear induced behavioural inhibition associated with stress responses have significantly increased RACK1 and PKCβII co-localised to stress responsive brain regions (Birikh et al., 2003). In addition, expression of PKCβII has been associated with oxidative stress in the brain as a response to stress conditions (Paola et al., 2000). PKC is also thought to play a significant role in the processing of APP into β-amyloid, a characteristic of Alzheimer's disease, a disorder where AChE is well established in the disease aetiology (Nitsch et al., 1992). More recently, observations that AChE inhibitors lead to a change in APP processing were attributed to increased PKC activity and expression (Pakaski et al., 2001). As catalytic inhibition of AChE can lead to an over expression of AChE-R similar to that observed under stress conditions, it may be possible that a complex regulation of stress pathways through AChE gene expression and PKC exists (Sternfeld et al., 2000). Indeed, gene expression regulation via modulation of AChE expression has been observed previously (see section 1.6.3 and 1.6.5 of Chapter 1). APP induced increases in AChE activity in Alzheimer's disease were recently linked to the oxidative stress pathway (Melo et al., 2003). As oxidative stress is a well established mediator of the effects of mechanical loading in bone (Van'T Hof and Ralston, 2001), it could be possible that in bone AChE-R specifically may be a target of, or play a role in mechanical loading responses. Further research is required to determine the effect of mechanical loading on isoform specific expression of AChE in bone. Also an immediate upregulation of AChE expression, contrasting with the observations of stress responses in neuronal tissues was observed. This may be due to the lesser requirement of an immediate increase in AChE activity in bone, but rather a requirement for a matrix production and deposition. Indeed, discrepancies in regulation of signalling systems in response to mechanical loading when compared to stress responses in the brain have been observed for signalling systems such as

glutamate, FGF, Wnt, and TGF-β (Coyle and Puttfarcken, 1993, Agostinho et al., 1997, Schwarzschild et al., 1999, Martin-Blanco, 2000, Grotewold and Ruther, 2002, Chen et al., 2003, Franceschi and Xiao, 2003). Our findings indicate that AChE expression in osteoblasts is regulated by osteogenic stimuli in a similar way to that of other well characterised bone matrix proteins (Raab-Cullen et al., 1994, Webb et al., 1997, Mackie et al., 1998, Ornitz and Marie, 2002).

4.4.3. AChE as an adhesion molecule in bone?

AChE has been shown to possess adhesive properties and displays significant sequence homology to a number of neuronal adhesion molecules (Jones et al., 1995, Bataille et al., 1998, Sternfeld et al., 1998). Previously, it has been shown that osteoblasts preferentially adhere to AChE substrates at a level similar to collagen type I (Genever et al., 1999). Using cholinesterase inhibitors, we demonstrated a concentration-dependent decrease in osteoblast adhesion in cultures treated with specific AChE inhibitors, suggesting that inactivation of endogenous AChE is sufficient to impair osteoblast adhesive interactions. It is possible that inhibitors may cause changes in AChE structure, or bind to and obscure possible adhesive sites on the AChE molecule. Studies have implicated the peripheral anionic substrate-binding site in the adhesive role of AChE using site-specific blocking antibodies (Johnson and Moore, 1999, Simon et al., 1999). BW284C51, a specific inhibitor of AChE, is known to obscure the peripheral anionic site, however DFP an irreversible inhibitor of AChE and BChE 'ages' the protein at the active site (Bataille et al., 1998, Jones et al., 1994). Although it is not certain that other changes to the AChE molecule occur due to DFP inhibition, it is possible this may be the cause of the reduction in osteoblast adhesion. However it is also possible that downstream effects on AChE expression may be a factor. Regulation of AChE expression is affected by inhibition of AChE catalytic activity in other tissues, where a transcriptional feedback response induces rapid changes in AChE isoform expression from the membrane bound synaptic form to over expression of the soluble read through form (Grifman et al., 1999, Kaufer et al., 1999, Meshorer et al., 2002 as discussed above). This transcriptional feedback response also affected expression of other cholinesterase-like proteins such as neurexin1β and neuroligin 1 that have been linked to cell adhesion and cell-cell contact (Song et al., 1999, Grifman et al., 1998, Kaufer et al., 1999, Meshorer et al., 2002) (as already

discussed in section 1.6.3, and section 4.4.2 of this chapter). This suggests a functional overlap and compensatory overexpression or 'functional redundancy' between AChE and neuroligin-1 in the brain, and provides a direct functional link bewteen AChE and cell-cell contact. We identified neurexins 1α and β and neuroligins 1 and 2 in primary rat osteoblasts by RT-PCR. Although these proteins have significant roles that are independent of AChE, and numerous proteins that are also capable of interacting with neurexins and neuroligins are expressed in bone (including. PDZ domain proteins NMDA accessory proteins) it is possible that an adhesion system similar to that of brain exists in bone. As already discussed in the previous chapter, expression of other neural cell adhesion molecules (NCAMs) has already proved to be of vital significance in bone development and osteoblast adhesion.

To clarify if reduction in osteoblast adhesion was a direct result of AChE, we inhibited AChE expression in SaOS-2 osteosarcoma cells. The use of two antisense oligonucleotides directed to exons 2 and 3 of the human AChE gene induced a significant reduction in AChE expression. This reduction in AChE expression caused a significant reduction in osteoblast adhesion compared to cultures treated with oligonucleotides directed at BChE or random sites. Furthermore, plating the cells onto AChE substrates rescued the antisense mediated reduction in osteoblast adhesion, returning adhesion to levels of untreated control cultures. However, the addition of soluble AChE to the culture medium had little effect. This suggests strongly that AChE can act as an adhesion molecule in osteoblast cultures, and could indicate that it acts as part of the bone matrix not as a free soluble factor. This could also be indicative of a polarised adhesive function for AChE in osteoblasts, an important factor for directed matrix deposition, which is consistent with the observation of AChE present in osteoid of newly formed bone. In support of this, recent studies have demonstrated strong electrostatic interactions of soluble AChE with a number of common matrix components such as collagen and laminin (Johnson and Moore, 2003). Neurexins are thought to mediate some of their adhesive properties through an extracellular region homologous to Lamanin A (Darboux et al., 1996). Furthermore, AChE has been demonstrated to interact directly with matrix components such as amyloid, and cell membrane proteins such as RACK1 in brain both in vitro and in vivo (see section 4.4.3). RACK1 has the ability to bind to a number of elements to induce intracellular signalling. Certainly, integrins can induce intracellular signals when bound to the cell

matrix through RACK1. Although much investigation into the effects of AChE-matrix and AChE-cell interactions is required to establish their exact function, these data lends support to the idea that AChE may function in cell-cell, cell-matrix interactions, and may induce more complex effects than just upon cell adhesion.

Further to observations of regulated AChE expression and secretion in osteoblasts, and observations of AChE localised to the bone matrix, the demonstration of AChE mediated osteoblast adhesion provides clear functional data supporting a role for AChE as a matrix protein in bone. However the demonstration of isoform specific differences in expression in bone could suggest fundamental differences in the mechanism of AChE isoform function, and it remains unclear as to what is the overall effect of AChE expression in the in vivo state.

Chapter 5 :

Isoform Specific Functions for AChE During Normal Bone Development

5.1 Introduction

Considering the distribution patterns of AChE expression observed in bone, it was important to determine how isoform-specific AChE expression influenced bone cell function, development and remodelling. As previously discussed there is a multitude of data that implicate non-cholinergic roles for AChE, but the specific effects of the different AChE isoforms are still poorly understood. Tissue-specific expression of AChE isoforms is better defined, and may indicate distinct roles for the differing AChE isoforms. Moreover, isoform-specific differences of C-terminal structures and sequences provide for divergent AChE variants with distinct differences in capacity for cellular localisation and interaction with other proteins.

5.1.1 Non-cholinergic isoform specific roles for AChE

5.1.1.1 AChE-E

It is clear from the accumulating data that AChE is a multifunctional protein. One of the strongest arguments for AChE possessing non-cholinergic roles is the fact that AChE is expressed in multiple molecular species that display a tissue-specific regulated expression profile (See section 1.5.4 chapter 1 for full description of AChE isoforms). AChE is known to play a role during the development of a variety of tissues; expression profiles in these tissues is widely different to that in mature tissues and in many cases isoform-specific expression patterns have not been established. The one AChE isoform that appears to have restricted cellular distribution is the AChE-E isoform, which when expressed in adult tissues appears to be limited to cells of the erythrocytic lineage. Although terminal differentiation of some haematopoietic cell types is accompanied by dramatic increases in AChE expression, activity and secretion, very few studies have focused on an isoform-specific role for this AChE-E (Samuels et al., 1967, Lawson and Barr, 1987, Barr and Keokebakker, 1990, Paoletti et al., 1992, Soreq et al., 1994, Chan et al., 1998). However, a number of studies analysing the glycosylation and sialyation of AChE in the blood and erythrocytes with relation to its involvement in circulatory clearance rates have identified a significant regulation of erythrocyte AChE (Saxena et al., 1997, Chitlaru et al., 2002). It is thought that blood AChE acts as scavenging molecules to clear up possible inhibitory factors absorbed into the blood stream that

may upset normal neuronal and muscle cholinergic signalling (Appleyard, 1994). However this is still under significant debate, and there is no direct evidence that AChE-E on red blood cells or in the blood stream acts to remove toxins from the blood stream.

5.1.1.2 AChE-S

In most other tissues studied with detail in relation to a role for AChE, where both the AChE-S and the AChE-R isoforms are expressed, specific roles for these isoform have been suggested. AChE-S is by far the most versatile isoform of AChE and can be oligomersied in a variety of forms and bound to the cell membrane via anchors and electrostatic interactions, as well as immobilised in the basement membrane via attachment to a collagen-like tail that has the capacity to bind to heparin sulphate proteoglycans (please refer to section 1.5.5). The AChE-S isoform is predominantly expressed at the neuromuscular junction where a clear role for AChE in cholinergic signalling is present. However, AChE-S is expressed in a number of other cell types and tissues of neuronal origin, and multiple functional roles have been identified. Firstly, a general neurogenic role for AChE is well established. In neuronal tissues AChE expression or AChE addition can induce neurite extension and outgrowth, axon formation, and organisation of the neuromuscular junction (Bataille et al., 1998, Grifman et al., 1998; Simon et al., 1999, Johnson and Moore, 2000). A significant body of evidence suggests that AChE has a role in cell adhesion (See section 1.6.3 chapter 1). However, many of these studies do not determine whether or not this role has an isoform-specific function. Many of the earlier studies identified AChE-S as the prototype isoform, simply because no in vivo role for AChE-R had been established at that time. It would be easy to assume that the adhesive function of AChE is fulfilled by the PRiMA membrane bound AChE-S isoform as this is the most abundantly expressed isoform in neuronal tissue. Dominant overexpression of the variant AChE isoforms in glioma cells demonstrated significant differences in isoform effects in vitro. The AChE-S isoform induced process extensions whereas the AChE-R isoform causes cell rounding (Karpel et al., 1996). In vivo, significant differences between the effect of AChE-S and AChE-R on cell process and neuromuscular organisation have been observed (Shapira et al., 1994, Seidman et al., 1995, Andres et al., 1997, Andres et al., 1998, Sternfeld et al., 1998). The collagen-tailed variant of AChE-S is well characterised, and known to be essential for the

normal development and functioning of the neuromuscular junction (see section 1.5.4 chapter 1). Although the ColQ collagen-like tail is encoded by a gene distinct to ACHE, a specific non-cholinergic role for this AChE-S variant has not as yet been identified. However, this variant does have the potential to possess specific non-cholinergic activities. Attachment to the basement membrane via the ColQ can be achieved through a number of heparan-sulphate proteoglycans such as perlecan and dystroglycan (Peng et al., 1999, Arikawa-Hirasawa et al., 2002). These heparan sulphate proteoglycans have multiple roles in other tissues and therefore interaction with AChE in such other tissues, including bone, and could enable the exertion of AChE effect.

5.1.1.3 AChE-R

AChE-R was the last of the AChE isoforms to be identified, though this finding has been the subject of much scepticism and criticism due to the unconventional intron inclusion, and the initial lack of direct in vivo evidence of existence. However significant data now suggests that the 'read-through' AChE isoform is a stress response molecule with complex transcriptional regulation (see section 1.6.5 chapter 1). In a number of tissues non-cholinergic roles related to stress responses have been established for AChE-R. For example, a novel function for the AChE-R isoform was identified in the testes (Mor et al., 2001). Overexpression of AChE-R in mature male mice caused significant aberrations in tissue morphology, sperm count and motility (Mor et al., 2001). This was thought to be as a result of reduced germ cell (progenitor cell) proliferation and expansion. Moreover, stress of mature male mice intensified the expression of AChE-R in the testes and spermatozoa. In contrast the levels of AChE-S were largely unaffected, suggesting that AChE-R may specifically modulate the effects of stress on male fertility (Mor et al., 2001). It is now thought that the AChE-R isoform, expressed as a soluble monomer, could be the 'carcinogenic-embryonic' AChE described by Drews in 1975. Indeed, significant evidence exists to suggest that AChE plays a role in carcinogenesis, and many other proteins involved in embryogenesis are associated with cancers (Greenfield, 1996, Layer and Willbold, 1994, Perry et al., 2002). More recently, the AChE-R isoform has been implicated in leukaemias and brain tumours. AChE-R expression parallels tumour progression and aggressiveness (Perry et al., 2002). Furthermore, AChE-R expression levels were also found to parallel Runx1/AML1 expression patterns and

levels, and a splicing shift favouring AChE-R over AChE-S expression was observed. Overexpression of AChE-R was also found to induce in vitro cell proliferation of U87MG glioblastoma cells (Perry et al., 2002). Elevated levels of AChE-R were also detected in blast cells of leukaemia (AML) bone marrow. A specific function for the AChE-R isoform has also been identified in the differentiation of megakaryoblasts into megakaryocytes (Grisaru et al., 2001, Deutsch et al., 2002). Although AChE was already known to be involved in the terminal differentiation of this cell lineage, the AChE-R isoform displays regulated expression and is localised to the nucleus (see section 1.6.4) (Lev Lehman et al., 1997, Grisaru et al., 2001, Deutsch et al., 2002). Moreover, in mice overexpressing the AChE-R isoform, a disrupted blood and bone marrow cell composition has been described (Grisaru et al., 2001, Deutsch et al., 2002). AChE-R was also found to induce CD34+ haematopoietic progenitor cell expansion in vitro (Deutsch et al., 2002). The R isoform of AChE was also found to be dramatically increased in blood cells exposed to stress both in vitro and in vivo (Grisaru et al., 2001). In addition, the C-terminal peptide of AChE-R, ARP, can induce progenitor cell expansion and proliferation of haematopoietic cultures (see section 1.6.4 and 5.1.2.4).

5.1.1.4 C-terminal effects of AChE

The functional differences in the AChE isoforms is predominantly attributable to the C-terminal differences produced by alternative splicing. The differences in C-terminal peptides composition are instrumental in the establishment of possible secondary interactions. However more recent data corroborates that these peptides alone have functional roles in vitro and in vivo. Putative cleavage sites found at the C-terminus of AChE can give rise to C-terminal peptides differing in composition depending upon the AChE isoform from which it is derived. As already indicated, the peptide of AChE-R (APR) is detected in stressed mice and can induce haematopoietic alterations both in vitro and in vivo. The peptide of the AChE-S isoform was analysed and found to possess a secondary structure that was capable of binding to certain transcriptional regulatory proteins, and was localised to the nucleus in cancerous cells (Perry et al., 2002). However, these peptide have not yet been identified under normal conditions in vivo. It may be that altered gene expression such as that occurring under stress conditions and in tumours could create

an environment where cleavage may occur. Certainly, MMPs might have the capacity to cleave AChE to generate such peptides, and MMP activity is increased in tumours in association with tumour progression (for review see Itoh et al., 2002). These newly identified regions of AChE could represent yet another novel function of AChE.

It is clear that some isoform-specific roles for AChE exist. However in most tissues where AChE expression has been identified, expression of both AChE-S and R was apparent. So do these isoforms play specific roles, or are their differing properties involved in a more complex regulation of AChE expression and function It has been suggested that AChE-R can counteract the effects of AChE-S and vice versa. A specific example of such an occurrence would be the transcriptional feedback response where the switch of expression from AChE-S to the AChE-R isoform can lead to aberrations in the neuromuscular junction and neurodeterioration (Sternfeld et al., 2000, Meshorer et al., 2002). Such transcriptional regulation would indeed account for many of the circumstance discussed above. A recent study of the pathophysiology of myasthenia gravis, a syndrome characterised by mutation or absence of components of the cholinergic machinery, most notably acetylcholine receptors, has revealed increased levels of extracellular AChE-R (Brenner at al, 2003). In this condition AChE-R can in fact compete with membrane associated AChE-S to attenuate the effects of the already reduced cholinergic signalling by reducing the levels of ACh at the synapse. In addition, antisense treatment targeted to AChE-R relieved at least some of the conditions symptoms (Brenner et al., 2003). If the AChE isoforms can exert such differences, and competition in cholinergic systems it is equally likely AChE can act in a similar way for non-cholinergic roles in other tissues.

5.1.2 AChE transgenic mice

Due to the imperative cholinergic capacity of AChE, production of AChE knock out mice has been both difficult and lengthy. However, mice lacking in AChE expression have been produced and used to provide a greater understanding of the complex regulation of cholinergic signalling and AChE function. Although viable mice can be produced they have significant abnormalities. A definitive way to establish isoform-specific functions for any protein is to analyses each isoform in isolation from the other. With this aim in mind, transgenic animals overexpressing

the differing AChE isoforms have been developed and characterised in terms of their cholinergic capacity and other non-cholinergic functions in a variety of tissues. These animals have been instrumental in the considerable advances in the identification of functions in non-cholinergic tissues. Work presented in this chapter has exploited these transgenic animals to determine the in vivo effects of manipulating AChE expression on skeletal development, in particular, craniofacial organisation.

5.1.3 Control of suture patency

Craniofacial development is a complex, poorly understood process that involves both endochondral and intramembraneous ossification of skeletal elements derived from a number of embryonic sources (See section 1.4.1, Chapter 1). It is therefore not surprising that craniofacial deformities are a common birth defect found in approximately 1 in 2,500 live births in the western world (McIntosh et al., 2000). Severe craniofacial deformities such as exencephaly, cleft palate, and craniosynostosis can be fatal in the absence of clinical intervention, often resulting in numerous dangerous operations during the early years of life. A large number of craniofacial deformities are a result of aberrations in suture patency and development. Sutures form an integral part of craniofacial development, especially in the development of the cranial vault. During embryogenesis the mesenchymal condensations that form the anlagen of the future skull bones (calvaria) develop ossification centres at the centre of the future bones, the bone fronts of which are separated by large gaps that eventually form the calvarial sutures (Wilkie and Morriss-Kay, 2001, Morriss-Kay, 2002). Most mammalian skulls have four sutures important to postnatal skull expansion; two running vertically (the midline sutures) across the skull, and two running horizontally (Opperman, 2000, Wilkie and Morriss-Kay, 2001, Morriss-Kay, 2002, and Mao, 2002). The metopic or interfrontal suture separates the two frontal bones running from the front of the centre of the skull (Opperman, 2000, Wilkie and Morriss-Kay, 2001). The sagittal suture, also set vertical, separates the two parietal bones running from the centre to the back of the skull (Opperman, 2000). Running horizontally is the coronal suture separating the two frontal bones from the two parietal bones (centre), and the lambdoid suture separating the two parietal bones from the occipital bone (back) (Opperman, 2000) (for representation see figure 5.1.1). Midline and horizontal sutures differ in their

Original in colour

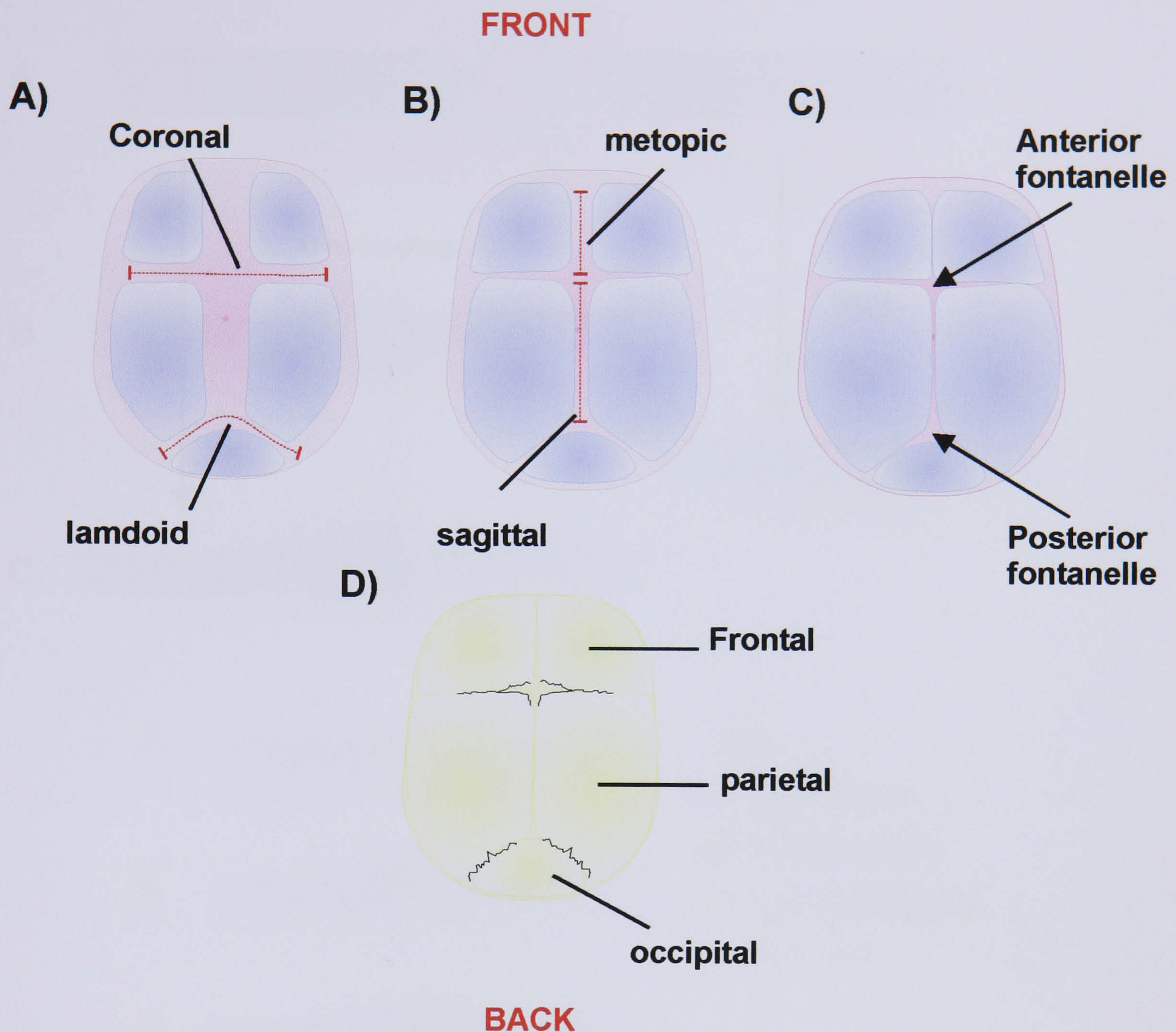

Figure 5.1.1
The developing cranial vault. The cranial bones (2 frontal, 2 pareital, and 1 occipital) are separated by sutures running horizontally (A) and vertically (B) across the skull, ossifying from the centre outwards. Where the sutures cross open unossified areas form known as the anterior and posterior fontanelle (C). The bones grow by the addition of bone at the osteogenic fronts which are immediately adjacent to the sutures that separate the bones. Once the brain is fully developed the sutures fuse to unify the bones of the skull (D).

Origional in colour

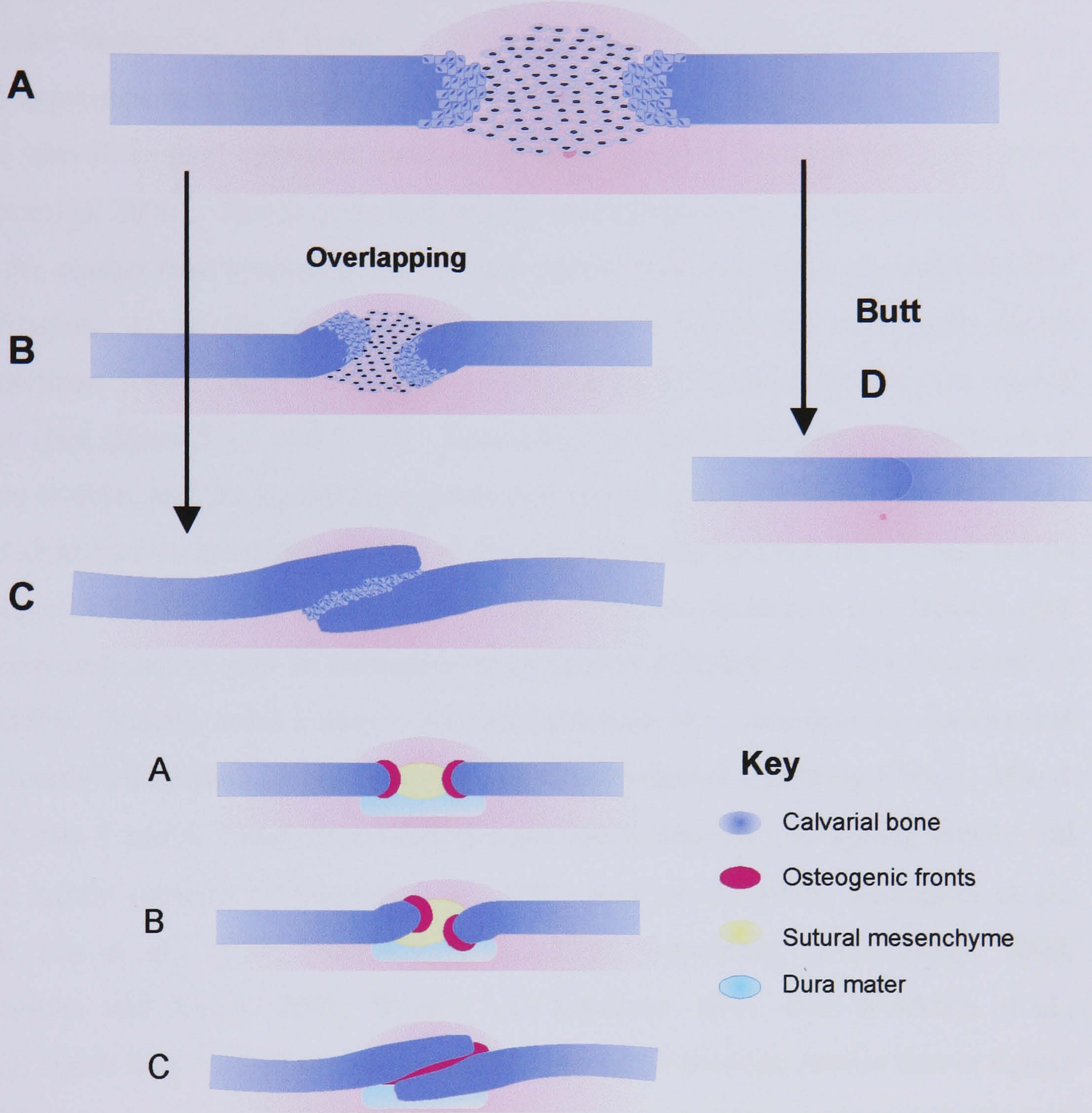

Figure 5.1.2
Suture formation and fusion. Cranial bone are separated by a mesenchymal membrane called sutures (A). During skull development, sutures follow a programmed pattern of development and fusion and can either overlap before fusion (B and C), or directly fuse (D). At each point in development of the sutural tissue, a specific pattern of gene expression is found in the bone, bone fronts, mis-sutural mesenchyme and dura mater (see key and figure 5.1.3).

development and patency, osteogenic fronts of midline sutures remain flush to each other during patency and directly fuse, however coronal and lamdoid sutures overlap during development and remain patent before fusion (see figure 5.1.2) (Opperman, 2000, Wilkie and Morriss-Kay, 2001). The points at which these sutures cross form wide opened unossified areas apparent in the newborn known as the anterior and posterior fontanelles (see figure 5.1.1) (Wilkie and Morriss-Kay, 2001). Postnatal skull expansion in line with the further development of the brain is heavily reliant on these sites remaining open and maintaining their size until development is complete (Opperman, 2000). This is done both via the osteogenic fronts of the calvarial bones and the cranial base sychondroses. As the cranial base lengthens via endochondral ossification, so do the cranial bones by adding bone at the osteogenic fronts (Opperman, 2000). Once the brain is mature, sutures are ossified to unify the cranial bones (See figure 5.1.1 and 5.1.2). Knowledge of the transcriptional regulation of suture closure, and the signalling systems that control transcriptional regulation have been identified by genetical analysis of disorders that display premature suture fusion or craniosynostosis such as Apert syndrome, Crouzon syndrome and Boston type craniosynostosis, as well as transgenic mice models (Opperman, 2000, McIntosh et al., 2000). Specific roles in suture patency have now been identified for factors that are already established as regulators of osteoblast function including Cbfa-1, Msx-1 and 2, Dlx 5 and 6, Twist, FGF, TGF-β superfamily members, hedgehog family and some matrix proteins (Winograd et al., 1997, Kim et al., 1998, Acampora et al., 1999, Liu et al., 1999, Davideau et al., 1999, Opperman, 2000, Ducy, 2000, Candeliere and Aubin, 2001, Wagner and Karsenty, 2001, Blin-Wakkach et al., 2001). Such factors display a clear spatio-temporal expression profile that is tightly regulated, and play multiple roles at different stages in suture patency (see Figures 5.1.3 and 5.1.4) (Kim et al., 1998, Opperman, 2000, Holleville et al., 2003, Greenwald et al., 2003).

In addition to transcriptional control, postnatal cranial development is influenced by a number of chemical and physical factors. Essential to suture patency is the dura mater that lies between the brain and the developing cranial bones. Both in vitro and in vivo experiments have demonstrated that an absence of dura mater tissue can result in premature suture fusion (Greenwald et al., 2000). Moreover, the type of dura mater tissue is specific to the suture type, and therefore must display a

Original in colour

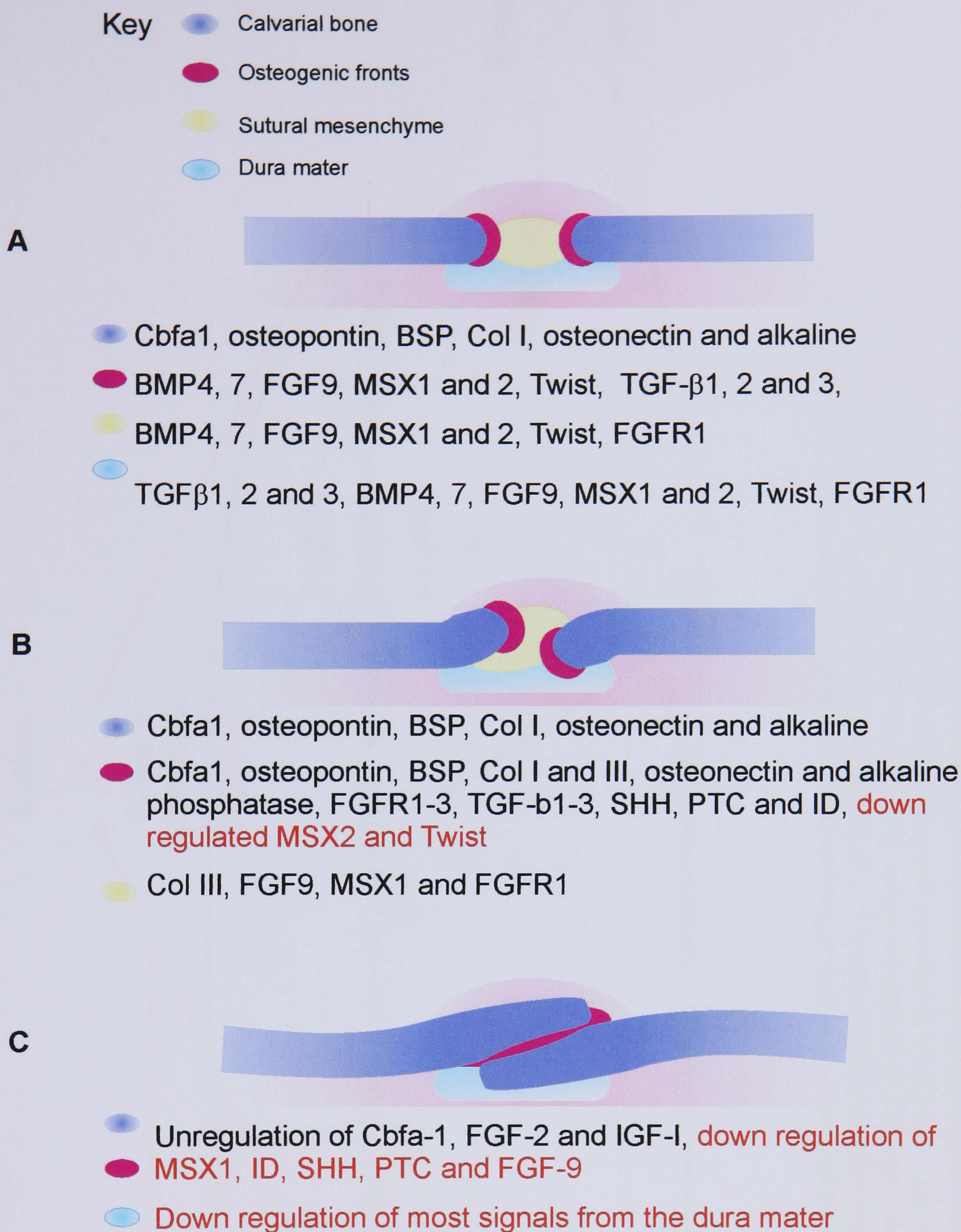

Figure 5.1.3
Distribution of transcription factors, growth factors and matrix proteins in the developing suture.
A) in the newly formed patent suture the dura mater plays an essential role providing signals to the undifferentiated sutural mesenchyme that determine suture patterning and patency.
B) as the suture begins to overlap, the signals from the dura mater are changed and downregulated. The sutural mesenchyme starts to specialise its matrix and express more osteogenic factors.
C) in the fusing suture, dura mater signals are less important and signals from the osteogenic bone fronts cause the sutural mesenchyme to differentiate and start to fuse.

Origional in colour

Delay suture fusion /suture patency

regulation

Dlx5

regulation

BMP 4

Msx2

FGF 2

TWIST

FGFR1

FGFR2

FGFR3

Cbfa1

Suture fusion

TGF-β3

shh ihh

FGF 9

FGF 4

TGF-β2

Figure 5.1.4

Regulation of suture closure and patency. A variety of factors have been found to effect suture closure, or induce downstream effects of factors that regulated suture patency. Upregulation in factors such as BMP4, Msx2 and FGFRs can lead to upregulation of the Cbfa1 gene resulting in premature suture fusion. Alternately down regulation in factors such as Msx2 can have a negative effect upon Dlx5 and Cbfa1 expression resulting in prolonged suture patency and delayed suture closure. A number of other signalling systems such as TGF-β superfamily and hedgehog family members are also know to interact with the factors that regulate suture patency, abberations in these genes can also impact upon suture closure.

suture specific spatio-temporal expression profile during suture development and patency (see figure 5.1.2). In mice the lambdoid suture remains patent until later life, and closes much later than the coronal suture in humans (Warren and Longaker, 2001). Exchange of dura mater tissue between the fusing coronal suture and the patent lambdoid suture resulted in early closure of the lambdoidal suture and prolonged patency of the coronal suture (Opperman, 2000, Greenwald et al.,2003, Helms and Schneider, 2003). It is thought the difference in the dura mater from these regions reflects the specific changes in the underlying brain regions (Greenwald et al.,2003). The brain is also known to influence suture patency through mechanical load received to the sutural mesenchyme as the brain grows (Mao, 2002, Kopher and Mao, 2003). Mechanical loads to the sutures are also received during mastication, which would only occur later in infancy after tooth eruption (Mao, 2002, Kopher and Mao, 2003).

Considering the AChE localisation pattern observed in developing calvarial bones (see chapter 3, section 3.3.1.1) work presented in this chapter describes the effects of AChE isoform overexpression and gene ablation on craniofacial development in transgenic mice. Further analyses of whole skeletons, long bones and ex vivo cultures provide evidence that AChE has a significant influence on bone development in vivo.

5.2 Materials and methods

5.2.1 Transgenic AChE mice

5.2.1.1 Maintenance and genotyping

Five different lines of AChE transgenic mice were used in these studies. Transgenic lines of mice with constitutively dominant over expression of the synaptic form of AChE (AChE-S mice) or the 'readthrough' (AChE-R mice), dominant expression of an inactive form of AChE (inactive mice), or expressing antisense against AChE (antisense mice) have been developed and characterised at the Hebrew University of Jerusalem, Israel (see refs for details – Beeri et al, 1995, Beeri et al, 1997, Andres et al, 1997, Andres et al, 1998, Sternfeld et al, 1998, Sternfeld et al, 2000, Lev-Lehman et al, 2000, Grisaru et al, 2001, Mor et al, 2001 Erb et al, 2001 and Farchi et al, 2003). AChE-R, AChE-S, inactive, and antisense mice lines were bred and maintained at the Hebrew University of Jerusalem by Tamah Evron, Liat Ben-Moyal and Al Grant. Day 3 newborn pups were killed and tail tips were taken for genotyping before fixation, storage and shipment in 95% ethanol (procedure kindly carried out by Tamah Evron).

Mice lacking in expression of AChE (AChE $^{-/-}$) were developed by Professor Oksana Lockridge and Dr. Weihua Xie, and characterised by Dr Ellen G Duysen, Epply Institute, University of Nebraska Medical Centre, Omaha. Briefly, homozygous knockouts were obtained by stable transfection of embryonic stem cells with an AChE knockout gene targeting vector and microinjection into blastocytes, followed by homologous recombination of heterozygous mice (see Xie et al, 1999, and Duysen et al, 2002 for detailed methods). Due to weakening of the muscles, suckling was impaired and therefore litters of nullizgous mice were weaned at postnatal day 14 and maintained on a high glucose/fat/calorie liquid diet of Ensure® (Abott laboratories , Ross Product Division, Columbus, OH). Mice genotyping carried was out by RT-PCR was kindly performed by Dr Ellen Duysen. Animal skeletons were cleared by African flesh eating beetles. Bone samples and newborn pups were kindly collected by Dr Ellen Duysen and Dr Paul Genever before shipment to the UK.

5.2.1.2 Whole mount skeletal staining

AChE-R, AChE-S, inactive, antisense and knock-out AChE lines were analysed for bone and cartilage using alizarin red and alcian blue as previously described (Dingerkus

and Uhler, 1977, Rosa-Moliner et al, 1999). Postnatal day 0 and 3 (P0 and P3) pups of all transgenic mice, and embryonic day 19 (E19) and 21 (E21) knock-out mice only were skinned and eviscerated before fixation in 95% ethanol for at least 5 days, and acetone for 4 days. Staining with 0.3% alcian blue and 0.1% alizarin red was followed by clearing of the non-skeletal tissue with 0.1M potassium hydroxide (KOH) in a 20% glycerol solution until the stained skeleton was visible. KOH solution was then replaced with increasing concentrations of glycerol.

5.2.1.3 Ex-vivo culture of whole marrow extracted from transgenic AChE lines.

Whole marrow extracted from mature male mice of the AChE-S, AChE-R, inactive and antisense AChE transgenic mice were culture as described for rat marrow in chapter two (see section 2.1.2.4). Analysis of surface bound alkaline phosphatase activity was carried out either at end point day 16, or at 3-day intervals during the culture period also as previously described in chapter two (see section 2.1.3.1).

5.2.1.4 Statistical analysis of ex-vivo cultures

Alkaline phosphatase assays were carried out in repeats of 10 samples per treatment/sample origin. Each sample was anylsed in duplicate and normalised to total protein concentration carried out as indicated in section 2.1.4.1. Samples were then analysed using paired t-tests carried using SPSS version 10.

5.2.1.5 Contact x-ray analysis

Postnatal day zero (P0) AChE knock-out ($AChE^{-/-}$), heterozygote ($AChE^{+/-}$) or wildtype (+/+) mice pups were fixed in 95% ethanol, and dried skeletal samples cleared of flesh by African flesh eating beetles were subjected to transmission x-ray analysis using a Faxitron 120KV x-ray machine, at the Department of Archaeology, University of York. Briefly, pups or skeletons were arranged in a variety of positions on blackout cassettes containing Kodak x-ray film. KV exposure and time were optimised to between 20-30 kV for 2-3 minutes. X-ray films were developed by hand using a traditional method, and finished using a film drier. X-rays were then visualised with the aid of a light box and digital images taken using a digital camera.

5.2.1.6 Histology

The mice hind and fore limbs of postnatal day zero (P0) AChE knock out, heterozygote and wild type mice were decalcified and paraffin wax embedded prior to cutting 10µm longitudinal sections of the proximal tibiae and fibiae using a microtome as described in chapter two (section 2.1.6.2a.) Postnatal day 18, 21, 36, 72, 181 calvariae were processed as above using varying times for decalcification for traverse sectioning across the sagittal suture. After deparaffinisation in xylene and rehydration through graded ethanols, histological staining with haematoxylin and eosin was performed. Tissue morphology was observed by standard light microscopy using DMLA upright microscope (Leica).

5.2.1.7 Immunolocalisation

Immunolocalisation of AChE and Msx-2 (see Chapter 2, table 2.1 for description of antibodies used) was carried out on paraffin sections of $AChE^{-/-}$, $AChE^{+/-}$ or wild type calvariae essentially as described in chapter two (see section 2.1.5.2b).

5.2.1.8 Dual energy x-ray absorptiometry (DXA) analysis

Age and sex matched -/- (n=10), +/- (n=10), and +/+ (n=10) were weaned early at day 14 on a controlled diet of Ensure, kindly carried out by Dr Ellen Duysen. Mice were sacrificed on day 21 and perfused with formalin before removal of some organs and brain tissue. Complete skeletons were then post fixed in 95% ethanol before shipping to the UK. Whole skeleton DXA analysis was kindly performed by Dr Mark Perry, University of Bristol, Bristol, UK.

5.2.1.9 Statistical analysis of DXA

Analysis of variance using multiple t-tests, one–way ANOVA and Bartletts and Tukey's post hoc tests were carried out using SPSS (version 10) statistics programme.

5.3 Results

5.3.1 Skeletal phenotype of AChE transgenic animals

5.3.1.1 Isoform-specific effects of AChE transgenesis on craniofacial development in vivo

The skeletal phenotype of transgenic mice over specifically expressing AChE-R, AChE-S, expressing antisense oligonucleotides against AChE or expressing an inactive form of AChE was characterised using neonatal day 3 mice pups stained for bone and cartilage using alizarin red and alcian blue. Although there was an apparent reduction in body size of mice overexpressing AChE-R or antisense against AChE, no overt skeletal abnormalities were apparent in the long bones of the fore or hind limbs, or the bones of the rib cage (Figure 5.3.1). However, significant differences in the calvarial bones of AChE-R and antisense transgenic lines in comparison to other lines and wild type littermate controls were observed (Figure 5.3.1). A reduction in alizarin red staining in the cranial bones was apparent in mice specifically overexpressing the AChE-R isoform, and in mice expressing antisense against AChE. An absence of staining in the parietal foramen region of the skull also in the AChE-R and antisense AChE mice could suggest a widening of the posterior cranial fontanel and possibly developmental delay of the sagittal and lambodial sutures.

5.3.1.2 Isoform-specific effects of AChE transgenesis on osteoblast differentiation ex vivo

Whole bone marrow stromal cells were extracted from mature male AChE transgenic mice and cultured in the presence of osteogenic supplements (Malaval et al, 1994). In all cultures overexpressing AChE, a marked increase in alkaline phosphatase activity was observed after 16 days in culture, most significantly in cells overexpressing the AChE-R isoform when compared to wild type controls (Figure 5.3.2). To characterise this further, cultures were analysed for alkaline phosphatase activity at different time points in culture up to day 15. Alkaline phosphatase activity of AChE overexpressing cultures followed a similar pattern to that of wild type cultures, however a progressive increase in alkaline phosphatase activity was observed in AChE-R and AChE antisense cultures when compared to wild type controls at later time points in culture (Figure 5.3.3).

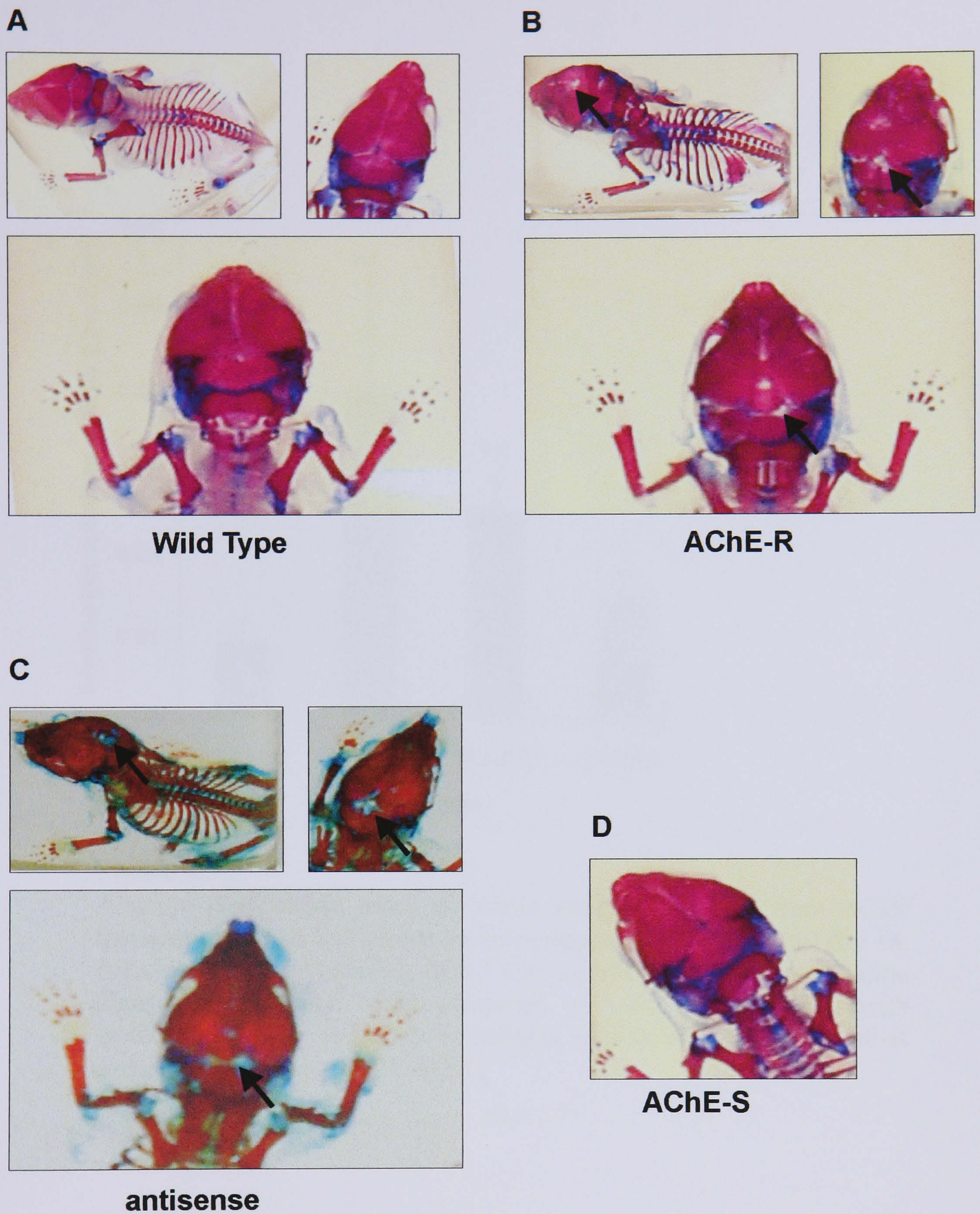

Figure 5.3.1
Whole mount skeletal staining using alizarin red (bone) and alcian blue (cartilage), of 3 day old transgenic mice. Absence of staining in the parietal foramen region of the calvaria was identified in AChE-R (B arrows) and antisense transgenic mice (C arrows) in comparison to wild type controls (A), indicative of delayed closure of the sagittal and lambodial sutures. (D) Cranial vault of AChE-S overexpressing mice.

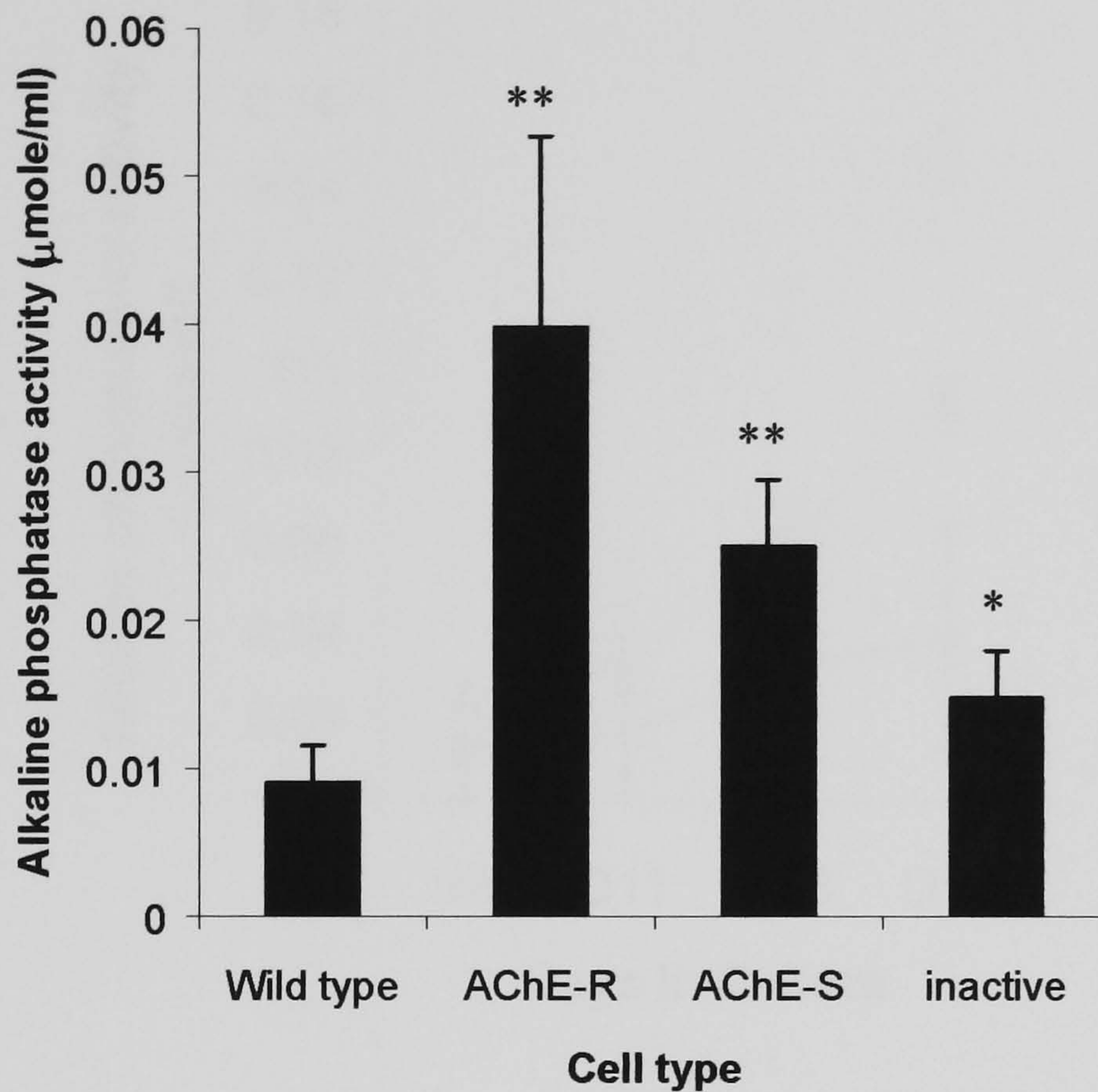

Figure 5.3.2
Alkaline phosphatase assay of whole marrow extracted from AChE transgenic animals and grown under osteogenic conditions until day 16. All cultures overexpressing AChE had significantly increased alkaline phosphatase activity when compared to wild type controls, with greatest increases observed in cultures overexpressing the AChE-R isoform (n=6, ** $p<0.01$, * <0.05).

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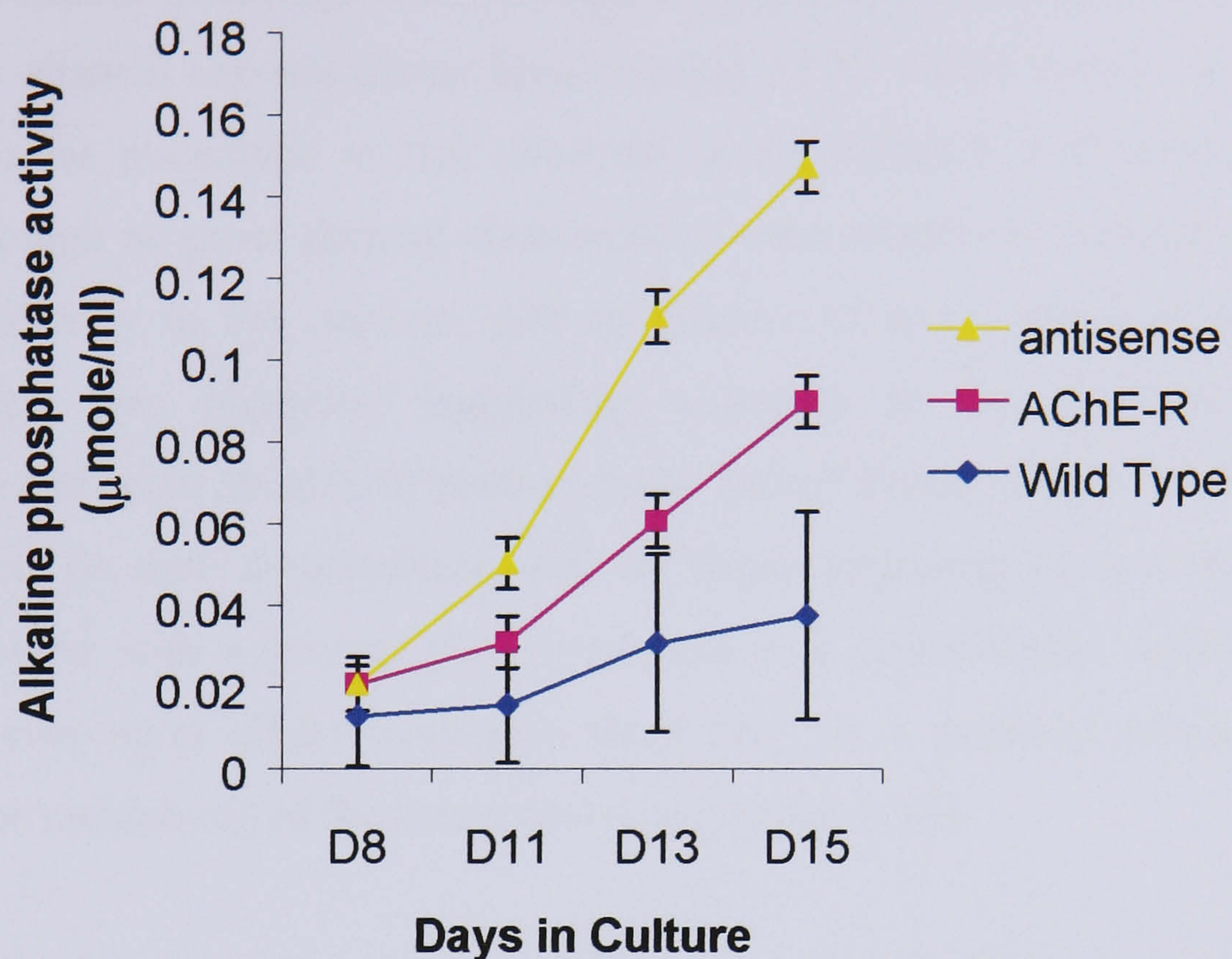

Figure 5.3.3
Alkaline phosphatase activity of whole marrow extracted from AChE transgenic mice overexpressing AChE-R or antisense against AChE, and wild type animals was determined at different time intervals in culture until day 15. A significant and progressive increase in alkaline phosphatase activity was observed in AChE-R cultures when compared to wild type control cultures. AChE antisense cultures constantly displayed significantly increased alkaline phosphatase activity when compared to both AChE-R and wild type control cultures.

5.3.2 Skeletal phenotype of AChE$^{-/-}$ mice

5.3.2.1 Effect of AChE knock out on skeletal development

AChE knock out mice samples were kindly provided for analysis of skeletal phenotype by Professor Oksana Lockridge and Dr Ellen Duysen, University of Nebraska, USA. Whole mount alizarin red and alcian blue staining of P0 AChE knock out skeletons revealed a similar phenotype to that observed in the AChE-R and antisense AChE animals. Although no gross skeletal abnormalities were observed, reduced alizarin red staining, specifically in the cranium, and an absense of any staining at the parietal foramen region was identified suggesting widening of the posterior fontanel. Haemotoxylin and eosin staining of sections taken from P0 hind and fore limbs revealed no overt effects on limb development, with all bones appearing to have developed a normal phenotype with a growth plate organised in a conventional manner (Figure 5.3.4). However upon closer inspection there may be a possible reduced cortical thickness at the metaphysis of the bones analysed (Figure 5.3.4).

5.3.2.2 *Specific effect of knock out of AChE on craniofacial developmental*

Haemotoxylin and eosin staining of calvaria taken from AChE knock out mice at days 11 and 18 after birth revealed a significant reduction in thickness and development (Figure 5.3.5), which was also observed when skulls taken from AChE knock out mice were compared to sex matched wild type samples under back illumination (Figure 5.3.6). Immunolocalisation for Msx-2, a regulator of calvarial development and suture patency, in AChE knock out mice calvaria did not reveal significant differences in expression levels or localisation compared to wild type controls (Figure 5.3.7).

5.3.2.3 Reduced bone mass in AChE$^{-/-}$ animals

Whole P0 AChE$^{-/-}$ and wild type mice were compared by x-ray analysis, an increased radiolucency of the AChE$^{-/-}$ skeletons compared to wild-type controls, indicative of generalised osteopenia was observed (Figure 5.3.8). This was particularly marked in the skull bones of older AChE$^{-/-}$ mice. Reduction in bone mass was confirmed by DXA analysis of sex-matched day 21 mice which revealed a significantly reduced bone

Original in colour

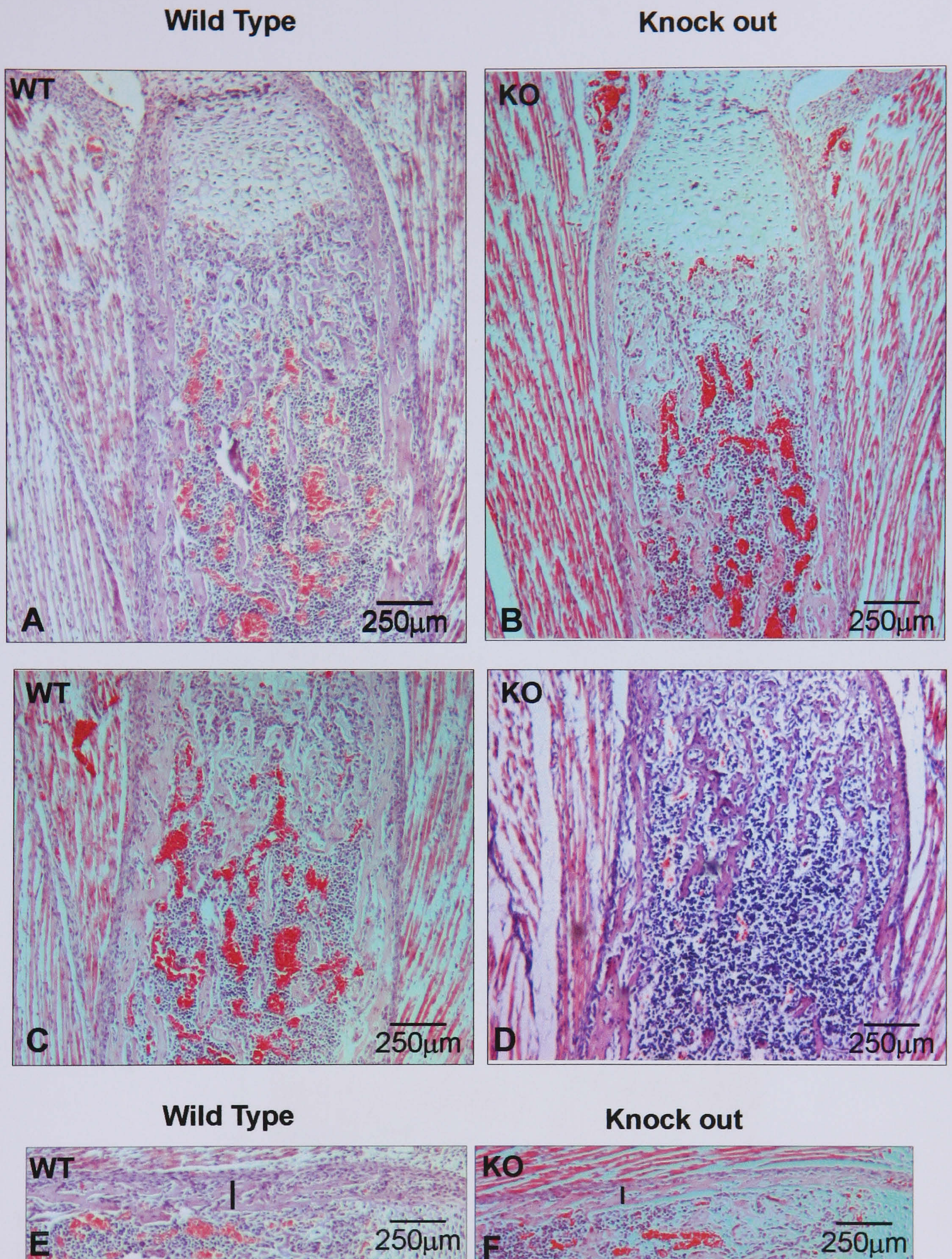

Figure 5.3.4
Haematoxylin and eosin staining of paraffin sections taken from P0 wild type and AChE knock out mice lower limbs. Anatomically, bones of wild type (A, C and E) and AChE knock out mice (B, D and F) appear very similar with fully developed growth plate, established trabecular bone, and marrow cavity. Close inspection and comparison of the compact bone of the diaphysis of the bones suggests a reduced thickness in the knock out mice (E) when compared to wild type controls (F).

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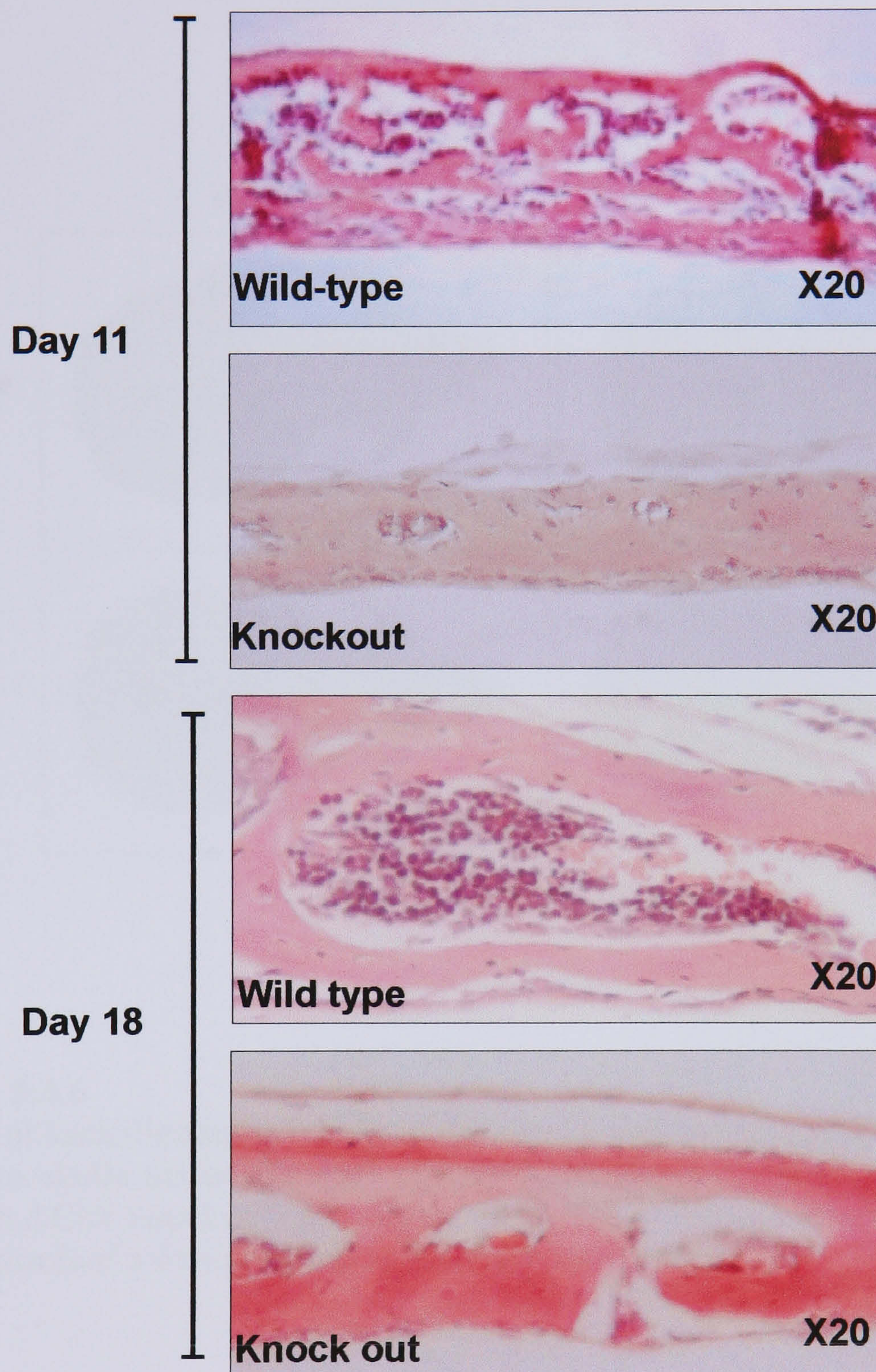

Figure 5.3.5
Haemotoxylin and eosin staining of paraffin wax sections taken at similar anatomical locations from postnatal day 11 and day 18 wild-type and AChE knockout mice. A reduction in calvarial width and vascularisation was observed in AChE knock out mice when compared to wild type control mice.

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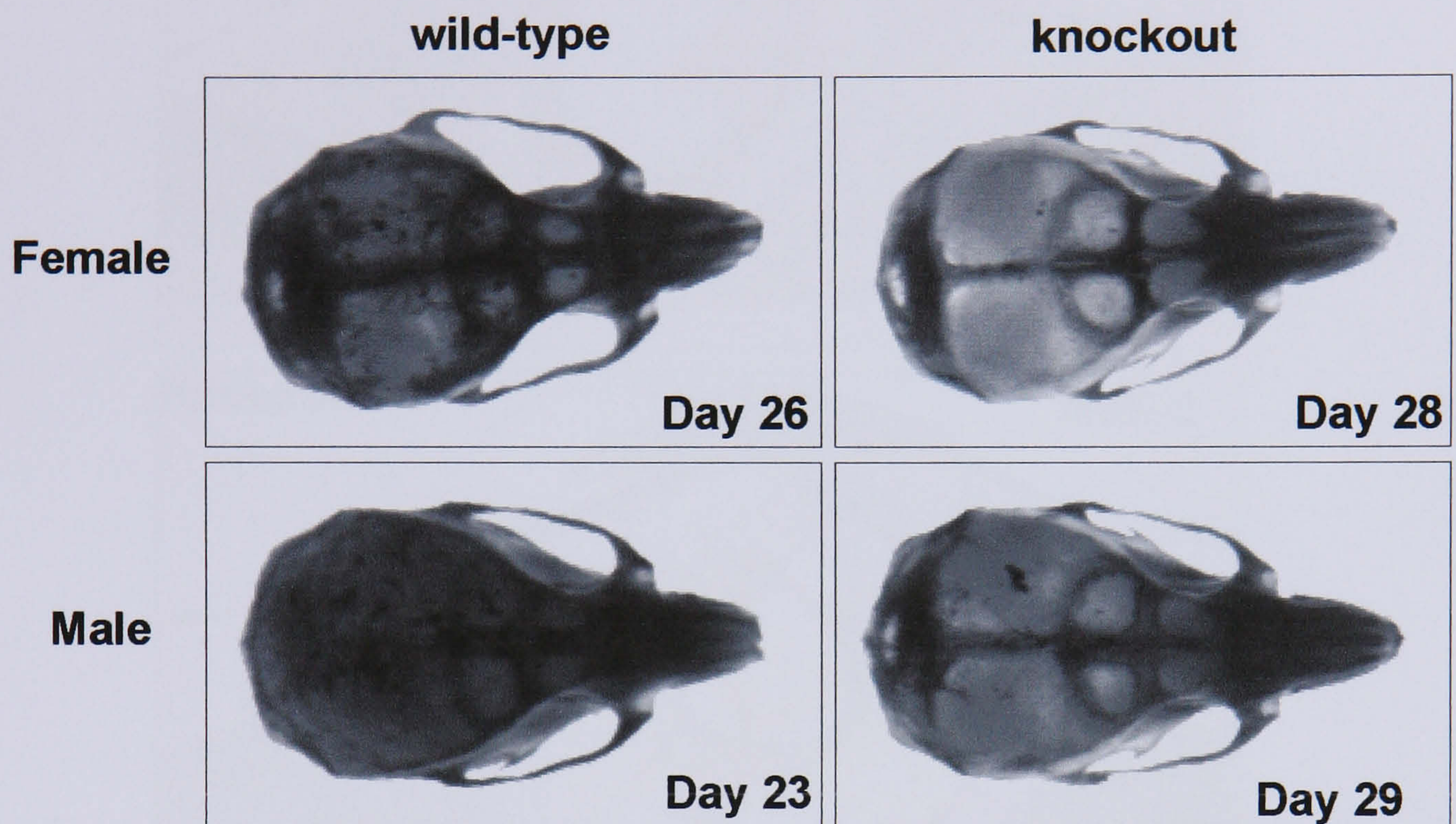

Figure 5.3.6
Images of back illuminated skulls of male and female, wild type and AChE knock out mice skulls under identical exposure conditions. Thinning of the parietal bones in AChE knockout mice can be seen when compared to sex matched wild type controls of a similar age under identical exposures.

Original in colour

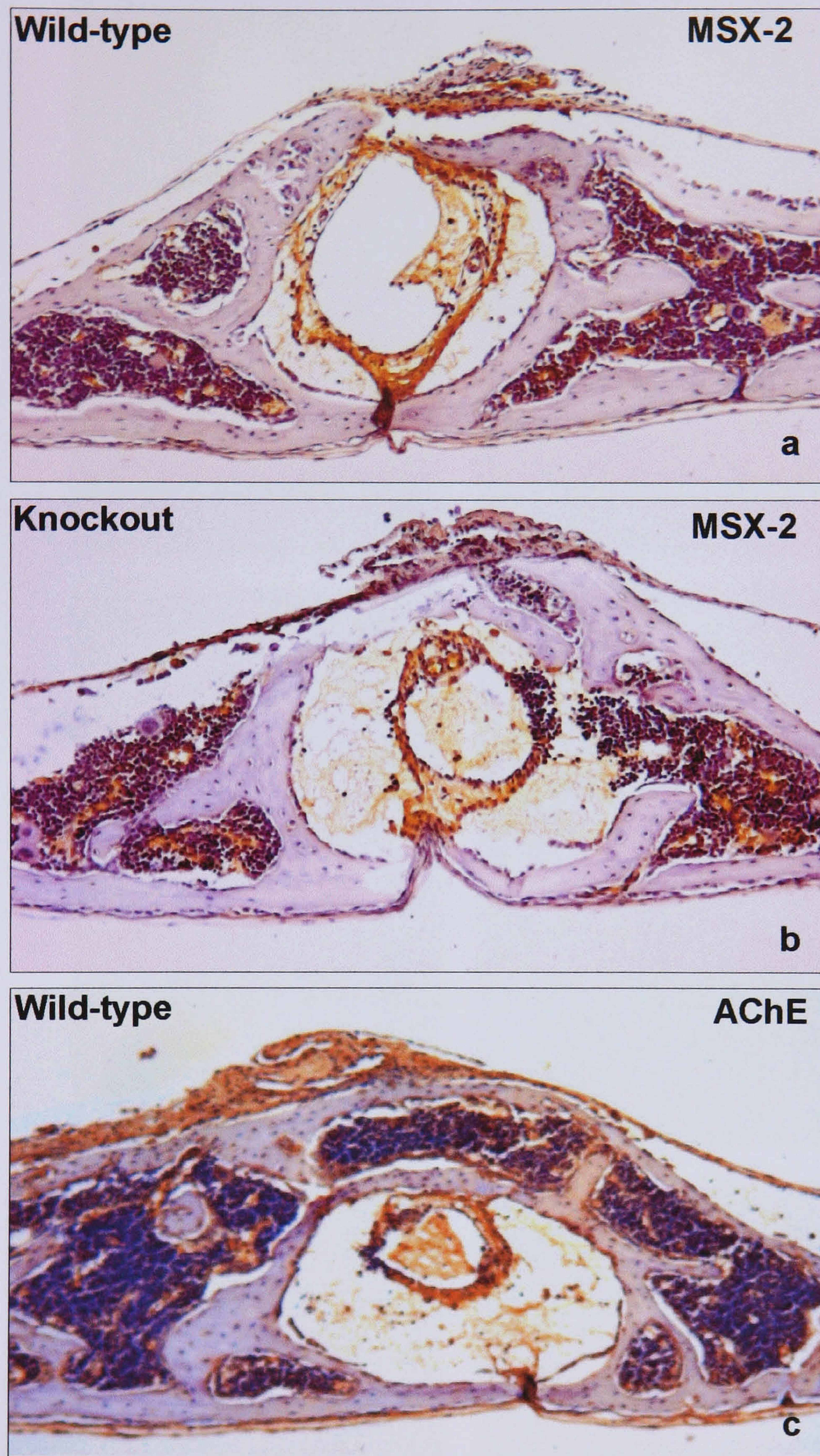

Figure 5.3.7
Immunolocalisation Msx2 (brown staining) on paraffin wax sections of postnatal day 18 wild type (a) and AChE knockout calvaria (b). Expression of AChE (brown staining) in wild type calvaria (c).

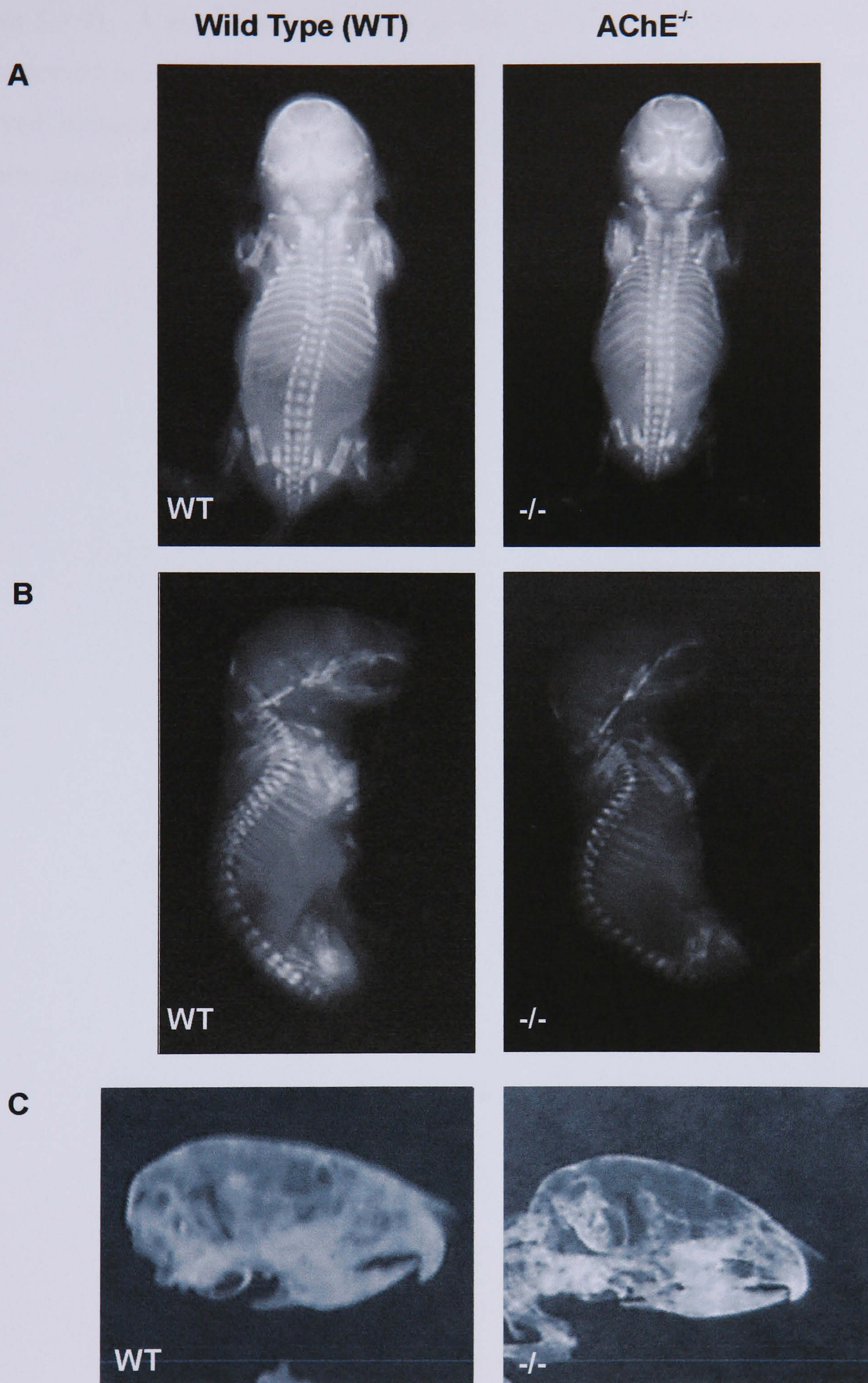

Figure 5.3.8
X-ray analysis of wild type (WT) and AChE$^{-/-}$ neonatal mice pups, demonstrating a reduced bone mass in AChE$^{-/-}$ (A and B) when compared to wild type littermate controls. Images where taken using identical exposure times
C) X-ray analysis of wild type (WT) and AChE$^{-/-}$ postnatal day 23 skulls revealing a continued reduction in bone mass seen most specifically in the cranium. Images were taken using identical exposures.

mineral density (BMD) ($p<0.001$), and bone mineral content (BMC) ($p<0.001$) and bone area ($p<0.001$) in AChE$^{-/-}$ mice when compared to wildtype and AChE$^{+/-}$ mice (Figure 5.3.9). A significant reduction in BMC ($p<0.05$) and bone area ($p<0.05$) was also observed in AChE$^{+/-}$ mice. A reduced overall body mass in AChE$^{-/-}$ samples was observed, however the percentage body fat to lean tissue of AChE$^{-/-}$ samples was within the same range as wild type and AChE$^{+/-}$ mice (Figure 5.3.10).

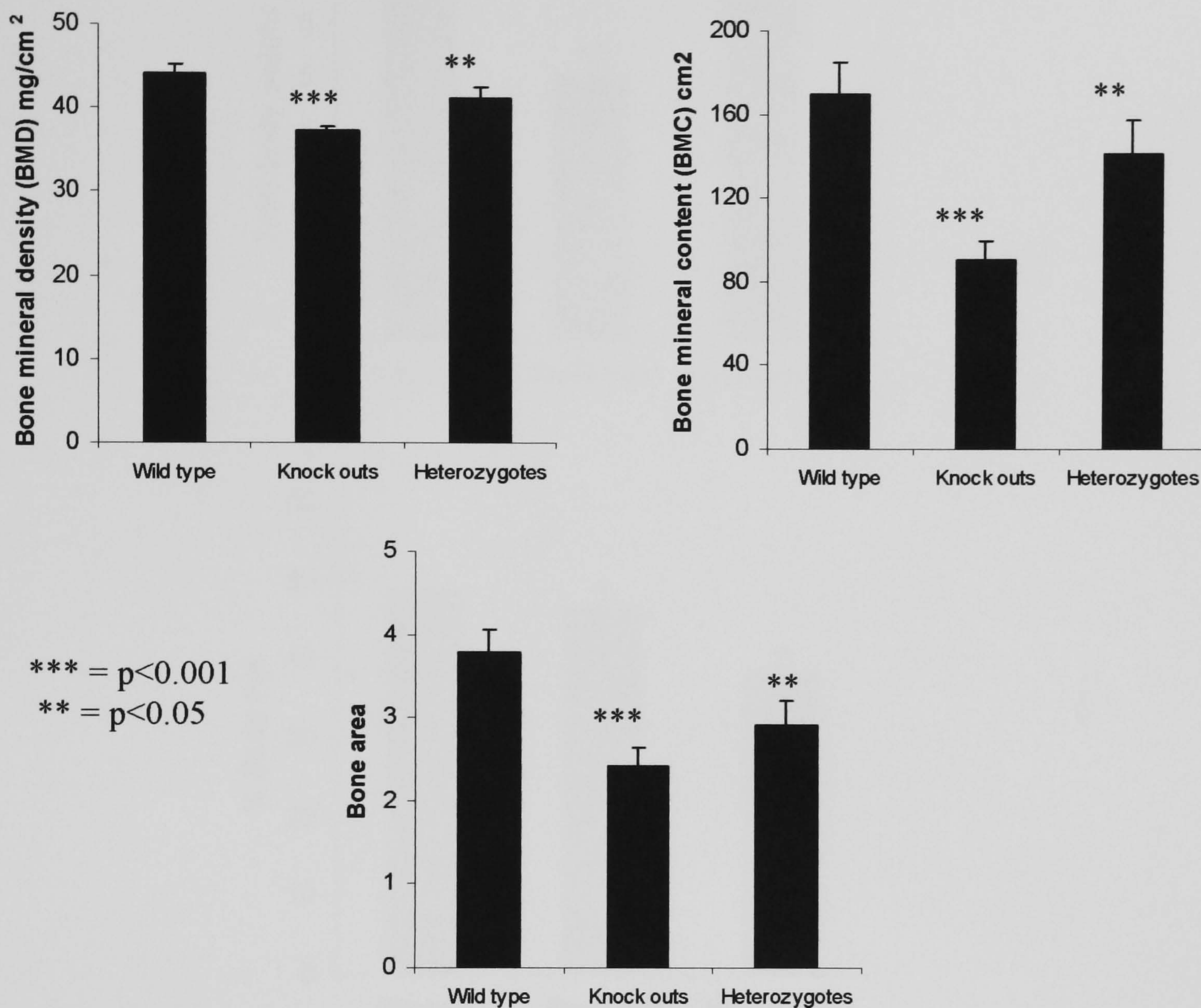

Figure 5.3.9
DXA analysis of day 21 AChE knock out, heterozygotes and wild type mice. Significantly reduced bone mineral content (BMC) and area was observed in knock out and heterozygous mice when compared to wild type controls. Bone mineral density (BMD) was also significantly decreased in $AChE^{-/-}$ mice compared to wild type controls (n=10, ** $p<0.05$, *** $p<0.001$).

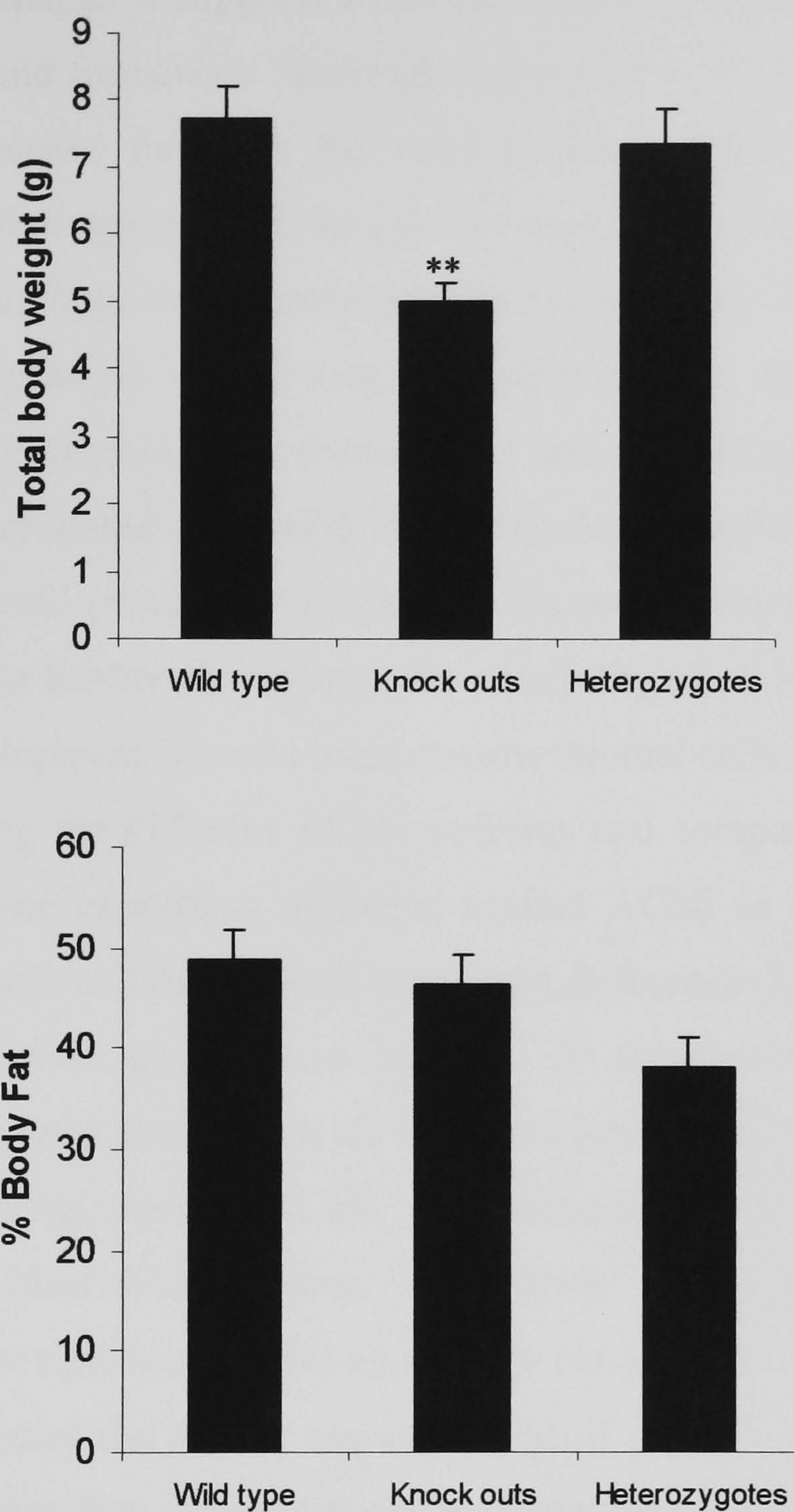

Figure 5.3.10
DXA analysis of day 21 $AChE^{-/-}$, $AChE^{+/-}$ and wild type mice. AChE knock out mice have a significantly reduced body mass when compared to wild type controls. However no significant differences in the percentage of body fat to lean tissue were observed (n=10, **P<0.05).

5.4 Discussion

5.4.1 Isoform specific roles for AChE in bone formation and development.

Data presented in chapter 4 suggests a role for AChE as a matrix protein, involved in bone development and formation. Although expression of all AChE isoforms had been established, the specific functions for each isoform during osteogenesis was still unclear. Here, the findings suggest that specific expression of different AChE isoforms is required for normal bone development in vitro and in vivo. Characterisation of AChE isoform specific expression during long bone development revealed overlapping and specific patterns of expression (see section 3.3.1 and 3.4.3 Chapter 3). This suggested that the temporal and spatial expression of specific AChE isoforms is important to bone development and could provide an insight into the precise roles of the different AChE isoforms in bone. To further characterise the possibility of an isoform-specific role for AChE in bone development we used bone marrow stromal cells derived from transgenic mice over-expressing the different AChE isoforms and compared them to cultures of cells taken from mice expressing antisense against AChE or expressing catalytically inactive AChE. These cultures showed significant differences in osteogenic capacity as determined by alkaline phosphatase activity. A significant increase in alkaline phosphatase activity was observed in all cultures over-expressing AChE, including the cultures over-expressing inactive AChE. This suggests that overexpression of AChE can influence osteoblast differentiation, in a manner that is independent of catalytic activity. As the most significant effect on alkaline phosphatase activity was observed in cultures over-expressing the soluble secreted AChE-R isoform, it could be that secreted AChE influences osteoblast differentiation more specifically than membrane-associated isoforms. These data would support previous observations that AChE secretion was regulated during osteoblast differentiation, and that AChE matrices supported osteoblast adhesion (see section 4.5.1). Accordingly, it may also explain why a partial effect of overexpression of AChE-S was observed, as only a small ratio of the AChE-S produced by the cell is secreted (Rossi and Rotundo, 1992, Belbeoch et al., 2003). Further analysis of the exact cellular localisation of the AChE isoforms is important to determine the role of AChE in osteoblast function and the establishment of any isoform-specific effects. A similar, yet more substantial increase in alkaline phosphatase activity was also observed in cultures expressing antisense oligonucleotides against AChE. The dramatic increases in alkaline phosphatase activity observed in cultures overexpressing

AChE-R or antisense against AChE at later time points in differentiation could be indicative of aberrations in osteoblast differentiation. As alkaline phosphatase activity typically reaches peak activity during the matrix production and mineralisation phase of osteoblast differentiation it is possible that increased alkaline phosphatase activity observed in the AChE-R and antisense cultures is a result of delayed osteoblast maturation, or even increased proliferation resulting in a larger population of cells. Although alkaline phosphatase activity is a widely excepted marker of osteoblast differentiation it is highly important to establish isoform-specific effects of AChE on other markers of osteoblast phenotype such as osteocalcin, osteonectin, osteopontin and Cbfa1. Moreover, whole marrow cultures can be used to analyse osteoprogenitor cell frequency if analysed for bone nodule formation in addition to alkaline phosphatase activity and calcium content.

It may seem counter intuitive that over-expression of AChE and inhibition of expression appear to have similar effects upon osteoblast differentiation. However similar effects have been previously observed in bone marrow. Recent publications indicate that the AChE-R C-terminal peptide (ARP) can act as a growth-promoting agent in stem cells of haematopoietic origin (Grisaru et al., 2001, Deutsch et al., 2002). In addition, in vivo application of AChE antisense oligonucleotides has also been shown to induce progenitor cell expansion in ex vivo haematopoietic bone marrow cell cultures (Soreq et al., 1994). It is therefore also possible that enhanced alkaline phosphatase activity may be a result of an increased incidence of osteoprogenitor cells. This could indicate that AChE expression plays a role early in osteoblast differentiation and in vitro bone formation. It is possible that overexpression or inhibition of AChE expression renders osteoprogenitors at a definite stage in maturation. Numerous independent investigators have suggested functional redundancy of AChE with other molecules, in that compensatory mechanisms exist that operate in the absence of AChE expression or activity (Sternfeld et al., 1999, Meshorer et al., 2002, Xie et al., 2000). For example AChE inhibition or antisense suppression can invoke a transcriptional compensatory response resulting in AChE-R over-expression (Grifman et al., 1998). AChE knock out animals also appear to have a functional compensatory overexpression of BChE in the brain and muscle (Li et al., 2000). It is possible that a functional redundancy of AChE with other molecules exists in osteoblasts, which could explain parallels in the effects of inhibition and overexpression observed. If distinct isoform-specific roles for AChE are apparent in bone, it may be that the switch of expression from one AChE isoform to

another AChE isoform, or a similar substitute molecule could exert substantial effects on osteoblast function and maturation. Further work along these lines is warranted.

5.4.2 A role for AChE in suture patency

Analysis of the craniofacial phenotype of AChE transgenic mice also indicated a possible isoform-specific role for AChE in bone development. Specifically the AChE-R, antisense, and knock-out AChE transgenic mice exhibited a widened posterior fontanel indicative of delayed closure or developmental delay of the lambodoid and saggital sutures in the region of the parietal foramen. This suggests that expression of AChE-R during suture development could be concentration-dependent, or could indicate that a secretable AChE isoform plays a role in suture patency. In addition, the data suggest a specific function for AChE-R, or properties possessed by AChE-R (i.e. ARP), in the differentiation of early mesenchymal tissues. The exact nature of suture patency is still unresolved. Analyses of cytokine, receptor and matrix protein distribution patterns in the developing cranial bones suggest that a specific spatio-temporal expression profile(s) is required. The identification of AChE in the developing calvaria prior to significant levels of alkaline phosphatase activity suggest AChE does play an important role in early mesenchymal tissues (see sections 3.3.1 and 3.4.1 Chapter 3). AChE expression displays a diffuse profile throughout the sutural mesenchyme, but is also prominently expressed in periosteal tissues and at osteogenic fronts. This could suggest multiple functions for AChE during suture development and patency, with roles both in ossification and plasticity of the mid-sutural mesenchymal tissue. Although many factors have been identified in the patency of sutures, two signalling pathways, FGFs and the TGF-β super-family, have received most attention (Ornitz and Marie, 2002, Massague et al., 2000). Not only do these two signalling families have a large repertoire of effects on osteoblast differentiation and bone formation, but significant numbers of craniofacial malformations are a result of mutations in members of these signalling systems. Moreover, there are data detailing a complex inter-regulation of suture patency through these signalling cascades (Kim et al., 1998, Greenwald et al., 2000, Opperman, 2000). In support of a role for AChE in suture patency, it was demonstrated that AChE expression by osteoblastic cells of a

calvarial origin was regulated by both these signalling systems (see chapter 4, sections 4.3.1 and 4.4.1).

Before any isoform-specific role for AChE may be concluded from these results it is important not to assume that the effects observed are directly attributable to a role of AChE in bone or suture patency. Many other factors also impact on suture patency, such as surrounding tissue development including the brain and other cranio-facial skeletal elements. Co-ordination between skull expansion and brain development is maintained via the sutures. As AChE is thought to play significant roles in brain development it may be that as a result of disruption in AChE expression a change in the way signals to the sutures have occurred (Layer and Willbold et al., 1994). Mechano-transductional signals are also thought to influence suture patency; disturbed brain development as a result of altered AChE expression may have an effect on such a mechanism, rendering sutures in a hypoplastic state (Kopher and Mao, 2003). However, this may also fit with the observations that AChE expression is regulated in response to mechanical loading (see chapter 4 sections and 4.3.2 and 4.4.2). The in vivo loading model in particular could be likened to the mechanical loads received by sutural mesenchyme in response to the growing brain. The dura mater lying between the developing cranium and the brain is also thought to aid in maintaining co-ordinated brain-skull growth (Greenwald et al., 2000). Alterations in AChE expression may also result in a disruption in signalling between the dura mater, the brain, and the developing cranium. Innervation of the dura mater via a catecholaminergic mechanism has been suggested, however not studied in depth and is based on analysis of expression patterns, using AChE activity as the basis of evidence for cholinergic innervation (Artico and Cavallotti, 2001). While this must not be dismissed, a cholinergic role for AChE in the dura mater is still to be substantiated.

Although the cranium is primarily developed by intramembraneous ossification, the skull base and a number of other cranio-facial elements are developed through endochondral ossification. Disrupted AChE expression may exert effects upon these skeletal elements resulting in knock-on effects on the developing calvarial bones. It is likely that as a result of disrupted AChE expression a combination of effects caused the developmental delays in cranial development observed. Nonetheless, taking all these points into consideration, an isoform-specific

phenotype is apparent and may be instrumental in understanding the role of AChE in bone.

A great many skeletal malformations caused by genetic mutations have been identified, the majority displaying significant craniofacial abnormalities. Largely, abnormalities in suture closure are observed as a result of premature closure or craniosynostosis. However more recently, a small number of disorders with aberrations in suture patency that results in open fontanelles have been identified (Opperman, 2000, Ornitz and Marie, 2002). Cleidocranial dysplasia (CCD) is a skeletal disorder characterised by absent or delayed suture closure. This disorder is thought to be caused by mutations in the osteoblast specific transcription factor Cbfa-1/RUNX2 gene (Otto et al., 1997, Lee et al., 1997). Although full knock out of Cbfa-1 produces a lethal phenotype with normal bone pattern formation but complete absence of mineralisation and lack of osteoblasts, partial knock out found in the heterozygotes generates a severe bone phenotype displaying similarities to CDD. Transgenic Cbfa-1 heterozygous mice also have a cranial phenotype similar to that reported here for the AChE-R and antisense AChE animals, however widening and patency of all sutures was identified in Cbfa-1 heterozygotes. As various bone-related elements, including Cbfa-1 binding motifs, have been identified in the extended AChE promoter region (Grisaru et al., 1999), AChE may act downstream of Cbfa-1 in suture development. Congenital parietal foramina (CPF) is another human disorder resulting in delayed suture closure and reduced membraneous mineralisation in the cranium (Winograd et al., 1997, Wilkie et al., 1999, Liu et al., 1999). This disorder displays most similarity to the phenotype observed in AChE transgenic animals (Wilkie et al., 1999). Loss of function mutations in the Msx2 gene have been identified in patients with this disorder and Msx2 knock-out mice present with multiple craniofacial abnormalities, as well as delayed suture closure. Msx2 plays a fundamental role in osteoblast differentiation and function (see section 1.2.3). After suture closure, Msx2 expression is still apparent in proliferating and expanding populations of osteoblasts. Using immunolocalisations, there was no apparent effect of AChE knock-out upon Msx2 levels in the region of the coronal suture, which undergoes most active postnatal closure in the first few weeks of life. These findings suggest that AChE might play a role downstream to Msx2. Another rare, but severe bone disorder, cranium bifidium, also results in gaps between the

skull bones (Wilkie et al., 1999). This phenotype is caused by duplication of the Twist gene in patients. Twist plays an early developmental role in osteoblast differentiation, with expression progressively decreasing as cells become more mature (Opperman, 2000). It seems that aberrations in the factors that control early osteoblast maturation result in developmental effects on suture patency and skull bone development. It could therefore be suggested from our results that AChE may play an early role in osteoblast differentiation.

5.4.3 A role for AChE in intramembraneous bone formation and mineralisation

Although an obvious craniofacial phenotype was observed in the AChE transgenic mice, initial inspection of the overall skeletal phenotype revealed no overt deformities although more detailed histomorphometric studies are required. However, further characterisation of the skeletons yielded data suggesting a role for AChE in bone development. Skeletons of AChE$^{-/-}$ mice are reduced in density by visual inspection and handling. Whole mice analysed by DXA and x-ray also displayed a significant reduction in BMD and BMC. Such reductions could be attributable to a number of factors. Firstly, as already suggested, AChE$^{-/-}$ may impact upon osteoblast maturation and proliferation (see 5.4.1 and 5.4.2). Many other knock-out mice that present with reduced bone mass, also have a significant reduction in osteoblast number (including mice lacking Msx-2, Dlx5, TGF-β1, cadherin-11, osteonectin, Cbfa-1) (Otto et al., 1997, Lee et al., 1997, Acompora et al., 1999, Kawaguchi et al., 2001). TGF-β1 knock out mice for example have a reduced bone mass, reduced osteoblast number, and in addition, a reduced functional activity in ex-vivo osteoblastic cultures. Analysis of osteoblast number and functionality in AChE$^{-/-}$ mice could clarify such factors. Reductions in osteoblast number if present in AChE$^{-/-}$ mice could be as a result of functions for AChE that regulate osteoprogenitor and osteoblast differentiation, possibly mediated via cell-cell contact and cell-matrix interactions (see sections 3.4.1, 4.4.1, and 5.4.1). Secondly, reduction in AChE expression and deposition may impact upon matrix composition and calcification. As previously discussed, AChE possesses a calcium binding EF-hand motif similar to that of osteonectin, which may play a role in matrix mineralisation. Indeed, osteonectin knock-out mice present with an

osteopenic phenotype. Thirdly, it is possible that AChE could act as a survival factor.

Reductions in bone mass in AChE knock-out mice were most apparent in the skull region of the skeleton. Histological analysis of the long bones of $AChE^{-/-}$ mice also revealed a possible reduced cortical thickness though further studies are required to support this finding. Cortical thickness is achieved primarily through intramembraneous ossification and the ARF sequence, in a similar way to that of the calvarial bones. Therefore it would seem logical that reduction in bone density in membranous bones would be reflected in cortical bone thickness. A similar phenotype to the $AChE^{-/-}$ mice has been observed in cadherin-11 knockout mice, which also display an open posterior fontanelle. This would suggest that cell-matrix contact is important during intramembraneous bone deposition and development, and would support the data that indicate a role for AChE in osteoblast adhesion (see section 4.4.3). Histomorphometrical analysis of bones taken from AChE knockout mice at various other time points in maturation would provide a greater insight into the of effect of AChE knockout on bone remodelling. Other knockout models targeted to bone matrix proteins have provided a limited phenotype during early life, yet display severity as animals age (for example biglycan and thrombospondin knockout mice). Although further studies are required, taken together these data suggest that knockout of AChE expression has a significant effect upon bone development.

5.4.4 How reliable are transgenic and knock out mouse models

In recent years it has become common for knockout and transgenic animals to be used in the determination and analysis of protein function. In the bone field this technology has been extremely useful in defining the essential elements required for osteoblast function and differentiation, with a good example being the Cbfa-1 knock out mice. Generation of whole knockout animals for the analysis of protein function in specific tissues has also undoubtedly lead to the discovery of roles for proteins in tissues other than for their primary function, and in many cases secondary or multiple functions. In this way studies on AChE transgenic mice have complemented the data that suggest a role for AChE in bone development. However, analyses of such animals must be performed with some caution. Not only is it unknown if the effects are a direct result of the proteins studied, possible

downstream effects that may have contributed to the phenotype are impossible to extrapolate. Moreover, the possibility of functionally redundant proteins that are interchangeable and co-regulated with other similar proteins cannot be discounted, and may obscure the true function of the proteins analysed. A more direct approach to transgenic mice has been adopted in recent studies, using tissue or cell type-specific promoters to establish a tissue specific role for proteins and signalling systems whilst removing side effects produced on surrounding tissues. The effect upon embryonic development may also be overcome by using promoters of genes that are only activated upon birth or maturation of the tissue. This would enable the analysis of protein function specifically in mature tissues, which are often dissimilar to developmental roles. The effects of AChE knock-out and overexpression observed must therefore be considered with this in mind –the observations may not be a direct result of disruption in AChE expression, and may not identify the actions of AChE due to functional redundancy.

A particularly important factor in bone is the behavioural and physical effects of knock-out or overexpression. Firstly, behavioural changes as a result of disruption in neuronal and muscular signalling may impact on general exercise and movement. Increased or decreased exercise would have a significant effect on bone remodelling and may have consequence for bone mass (Goldspink, 1999, Robling and Turner, 2002). In addition, dietary changes can also affect bone deposition, mass and volume (Ducy et al., 2000, Paakkunainen et al., 2001, Murray et al., 2003). Due to poor suckling reflexes, $AChE^{-/-}$ mice are weaned early on a special diet high in glucose (Duysen et al., 2002). Although this may be compensated for by feeding control mice on the same diet as done with the DXA analysis of this chapter. As the insulin like growth factor (IGF) family are known to exert considerable effects upon bone remodelling, increased blood glucose could have an effect on bone mass as a result of a high glucose diet (D'Ercole, 1993, Cornish et al., 2002, Banu et al., 2003). However, it has not been established that there are increases in blood glucose in the $AChE^{-/-}$ mice, and without such a diet animals fail to survive to maturity. Similar to this, leptin is known to regulate bone mass, and diabetic mice have decreases in bone mass (Thomas and Burguera, 2002, Khosla, 2002, Takeda et al., 2003, Watanabe, 2003). All AChE transgenic mice, other than the knock-out strain survive on a normal diet and still display a similar bone phenotype to the knock-out animals. Further analysis of the bone mass and volume of AChE transgenic and

knock out mice would clarify such matters. Furthermore analysis of knock-out animals at birth before behavioural or dietary effect could have an impact on bone mass did reveal a bone phenotype, suggesting that AChE may play a role in the maintenance of bone mass.

Chapter 6 :

AChE - Non-cholinergic Roles as a Bone matrix protein.

Chapter 6

AChE : Non-cholinergic roles as a bone matrix protein

AChE is one of the fastest acting enzymes known to man. Which begs the question of why it should be expressed in a mineralised tissue in the absence of a relevant cholinergic signalling pathway? In addition to theories of novel signalling systems, or interactions with resident nervous or surrounding muscular tissues, there is a growing body of studies that implicate multiple non-cholinergic, non-catalytic roles for AChE. This thesis has focused upon non-cholinergic roles for AChE in bone, and indeed there is a remit for a great many of the proposed 'secondary' functions of AChE within this tissue. The challenge has not been to determine a non-cholinergic function for AChE in bone, rather to define which of the multifunctional properties of AChE lends most significance in bone. Although this project is still very much in its infancy, I would like to use this next chapter to discuss what conclusions could be drawn from current knowledge of AChE in bone.

Complex regulation of AChE isoform expression in bone

"Two paradoxes are better than one; they may even suggest a solution."
Edward Teller

Considering the essential role that AChE plays in cholinergic signalling, and consequently in the developing nervous system, it is surprising that AChE knock out mice survive. Moreover, in light of the accumulating data on non-cholinergic roles for AChE, the survival of AChE knock out mice could be seen as almost impossible. For AChE knock out animals to survive, it is most likely that a functional redundancy between AChE and other proteins must exist. Certainly, this has already been established in the brain, where continued cholinergic signalling in the absence of AChE has been attributed to a functional increase in BChE expression and activity (Li et al., 2000). In spite of this we have identified a significant skeletal phenotype in AChE knock out mice that not only supports a role for AChE in bone, but also contributes to the growing data on non-cholinergic roles for AChE. Furthermore, the skeletal

phenotype of AChE overexpressing transgenic mice presents yet another enigma, and indicates a complexity in the functioning of AChE in bone. Observations of an analogous skeletal phenotype in AChE transgenic mice both lacking and over-expressing specifically AChE-R would appear to contradict previous observations in vitro. It would appear that although AChE isoform expression shows considerable overlap in bone, distinct roles do exist. Exploration of isoform-specific functions may therefore be vital in the understanding of AChE function in bone. In a number of other tissues, overexpression of AChE-R shows considerable parallels to inhibition of expression or activity of AChE both in vitro and in vivo (Grifman et al., 1998, Grisaru et al., 2001, Deutsch et al., 2002). Taken together, transcriptional feedback and functional redundancy as demonstrated in neuronal tissues could provide a mechanism by which over expression may cause the same effect as knocking out the expression in bone (See Chapter 1 and chapter 5, and figures 6.1 and 6.2).

Isoform-specific differences in functional localisation of AChE could provide for competition in cell-cell, cell-matrix interactions, as will be discussed in further detail later in this chapter. Additionally, the AChE-R isoform can generate a functional C-terminal peptide, which is distinct from that of AChE-S (Grisaru et al., 2001, Deutsch et al., 2002, Perry et al., 2002). Hence, transcriptional feedback resulting in overexpression of AChE-R in bone may potentiate the normal function of AChE-R in bone. Two independent studies have already indicated that low-level exposure to AChE inhibitors that would induce transcriptional feedback in the brain, can cause severe bone loss in humans (Compston et al., 1999, Compston et al., 2003). If indeed a complex transcriptional regulation was present for AChE in bone, it could suggest that excessive AChE-R may in fact have deleterious effects on bone formation.

Analysis of the bone mineral density of AChE-R mice may perhaps provide support for this theory, as well as providing further support for isoform-specific functions in bone. As AChE inhibitors are currently used as treatment for a number of disorders, including Alzheimer's disease and myasthenia gravis, the significance of a compensatory mechanism should not be overlooked (Millard and Broomfield, 1995). Furthermore, additional stringent measures to avoid occupational and environmental exposure to these agents should be explored. It is clear that there are functional differences in the AChE isoforms in bone, and therefore a complex regulation of AChE

expression might be present in bone. If that is so how might this impact upon bone at the cellular level?

AChE a mediator of cell-cell and cell-matrix interactions in bone

Accumulating data from a variety of sources suggests that AChE can mediate cellular interactions with the matrix and other cells. In bone, cell-cell and cell-matrix interactions are essential for osteo/chondrogenesis (see section 3.1.1). Osteoblast adhesion and migration is also fundamental to the bone remodelling process. The effects of AChE on cell adhesion have been highlighted in other tissues and discussed in previous chapters (See sections 1.6.3 and 4.1.2, 4.4.3) (Jones et al., 1995, Darboux et al., 1996, Johnson and Moore, 1999, Bigbee et al., 1999). In neuronal tissues, AChE can induce cell adhesion, spreading, neurite extension, and axon guidance; moreover this can be inhibited by functional blockade of the adhesive site (Shapira et al., 1994, Seidman et al., 1995, Jones et al., 1995, Darboux et al., 1996, Andres et al., 1997, Andres et al., 1998, Sternfeld et al., 1998, Johnson and Moore, 1999, Bigbee et al., 1999). In vivo, AChE has also proved to be involved in the organisation of the neuromuscular junction, with aberrations in AChE expression resulting in disarray and disorganised morphology (Shapira et al., 1994, Seidman et al., 1995, Andres et al., 1998). In addition, AChE possesses a number of sites with potential adhesive or electrostatic properties that have proved to be functionally active (Darboux et al., 1996, Botti et al., 1998, Johnson and Moore, 2003). Most significantly however is the esterase-like family of proteins sharing homology to AChE in the esterase-like domain, many of which have roles in mediating cell-matrix, cell-cell interactions in vivo. Furthermore, a transcriptional inter-regulation and functional redundancy of AChE with other esterase-like proteins has been identified, suggesting AChE has the potential to interact with other proteins to mediate cellular interactions (Andres et al., 1997, Grifman et al., 1998).

I have provided data to suggest AChE can mediate osteoblast adhesion when present as a matrix component, but not if present as a soluble factor in the extracellular environment (See sections 4.3.3.2, 4.3.3.3, and 4.3.3.5). The previous chapter outlines that AChE in bone may also play an isoform-specific role both in development and osteoblast differentiation (see section 5.4.1 and 5.4.2). In particular, it is demonstrated that the AChE-R isoform is predominantly expressed in bone and can affect osteoblast

Original in colour

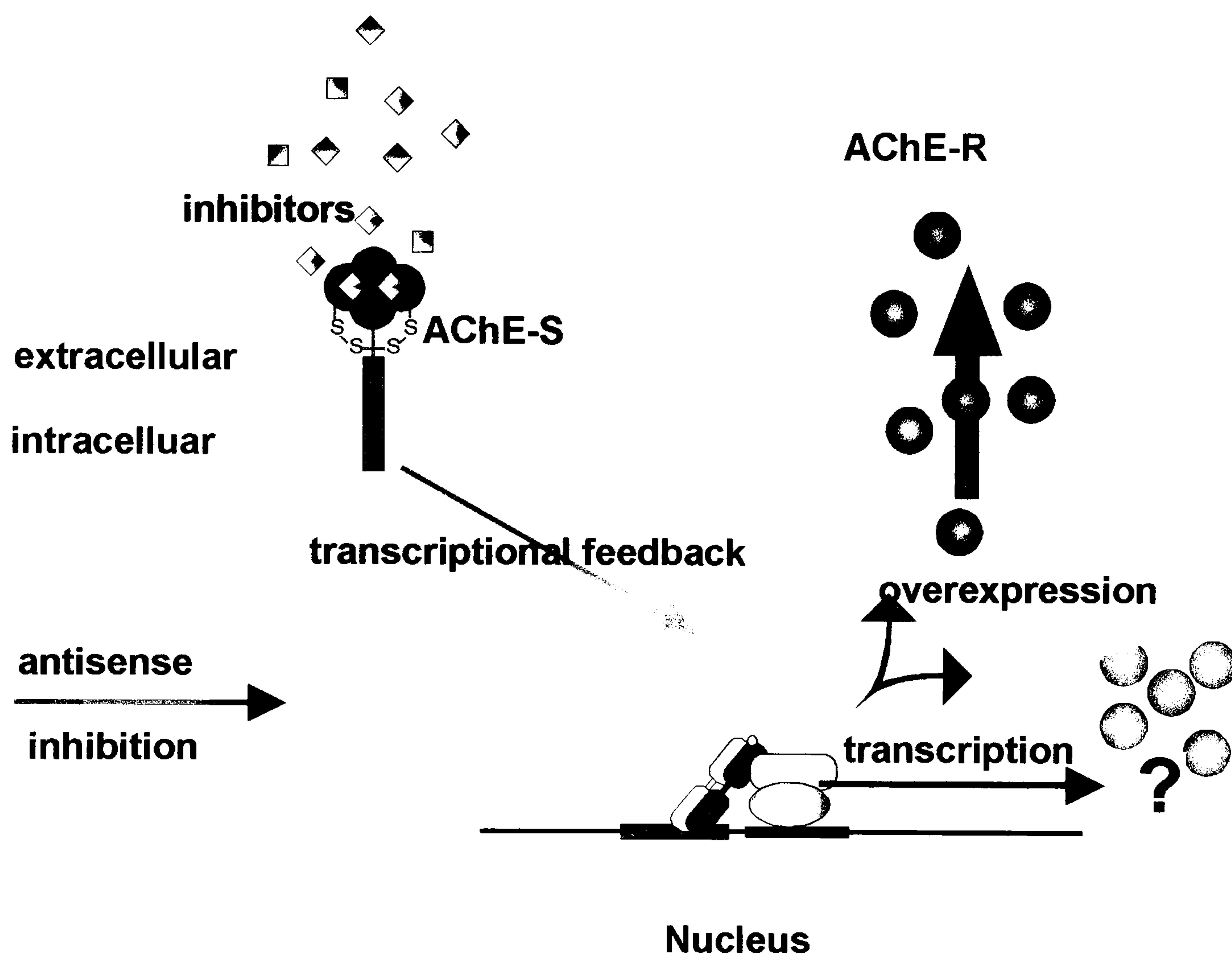

Figure 6.1
Inhibition of AChE catalytic activity, or reduction in AChE expression due to antisense inhibition results in a transcriptional feed back response leading to a fast but long lasting switch of expression of AChE-S to AChE-R, soluble monomer, or other homologous proteins such as BChE and neuroligin.

Original in color

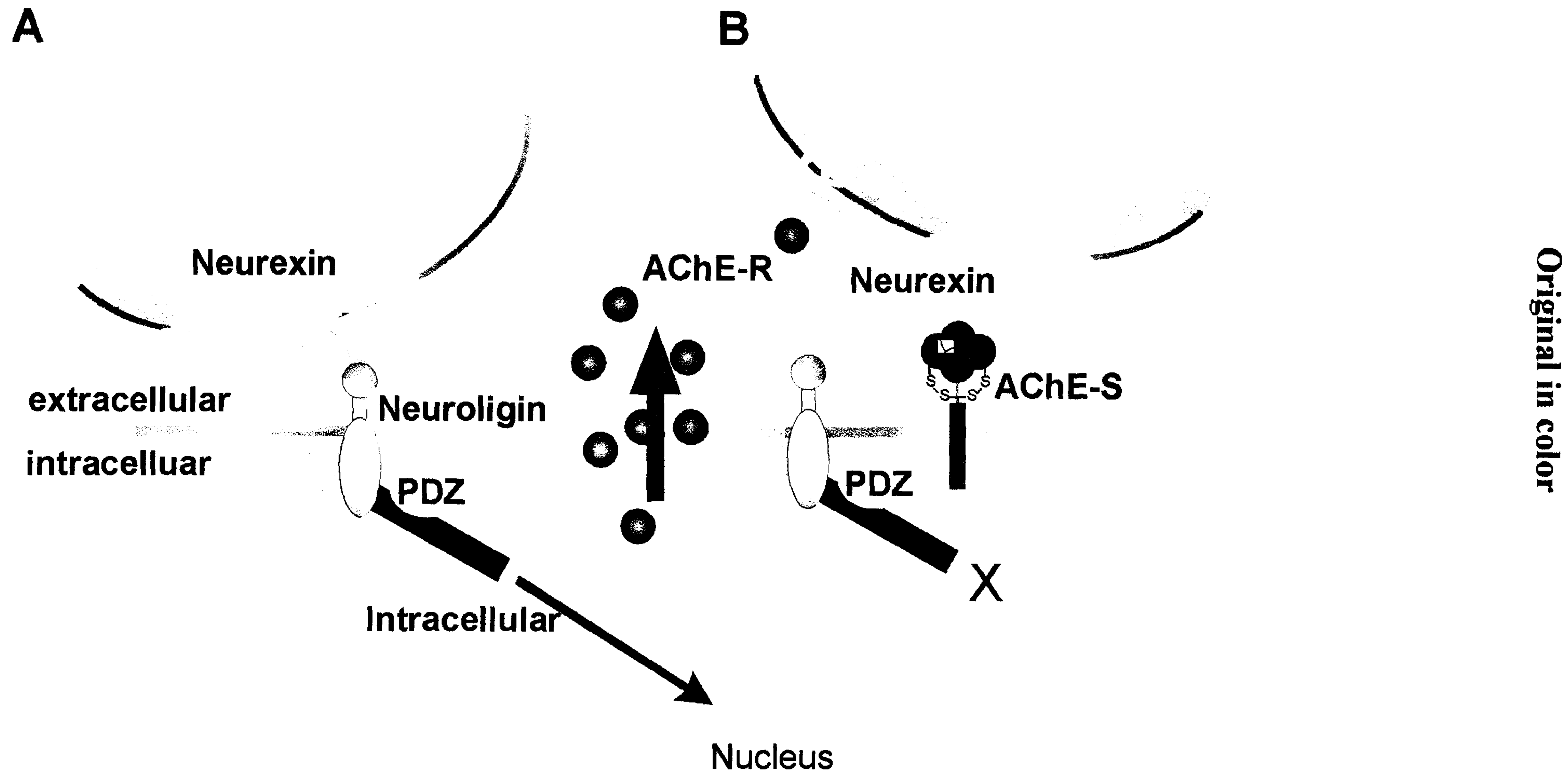

Figure 6.2
A) In the brain, cell surface expressed neurexins can bind to neuroligins expressed on adjacent cells to induce heterophilic cell-cell contact and synapse formation. It is thought that signalling through PDZ domain proteins may also induce intracellular signalling.

B) Neuroligin shows homology and functional redundancy with AChE it is possible that AChE may bind to neurexins in a similar fashion. Cell surface expressed AChE could suseqently also induce cell-cell contact. However soluble secreted AChE could prevent this interaction by blocking neurexin from binding to either neuroligin or cell surface AChE.

differentiation in vitro. This would fit with observations of regulated AChE secretion during osteoblast differentiation as the AChE-R isoform is secreted as a soluble monomer. However as the other AChE isoforms are also expressed in bone as well as ColQ, and considering previous evidence that AChE is also localised to the cell membrane in osteoblasts (Genever et al., 1998), there could be a number of mechanisms by which AChE might mediate osteoblast adhesion or cell-cell contact in bone

Firstly, AChE present on the cell surface of osteoblasts could attach to the bone matrix through its adhesive domain or through electrostatic interactions to induce cell adhesion (see figure 6.3). Adhesion through AChE could provide the cell-matrix proximity for the establishment of focal adhesions with more specific adhesion molecules found on the cell surface and in the matrix.

Secondly, secretion and deposition of AChE into the matrix would provide an AChE substrate for osteoblast adhesion. AChE could be localised in the matrix through electrostatic interactions with other matrix proteins, or through binding of ColQ to heparan sulphate proteoglycans in the matrix (Peng et al., 1999, Rotundo et al., 2002). Indeed, the collagen-like tail has proved essential in localisation of AChE in the basement membrane, for example perlecan knock out animals have an absence of AChE at the basement membrane (Rotundo et al., 2002). AChE molecules may also be localised in the matrix through interactions with as yet unidentified receptor molecule(s) or matrix proteins through its adhesive domain (see sections 1.5.2, 4.1.2 and figure 6.3). Osteoblasts could then bind to the AChE present in the matrix, again through a number of ways. It has been suggested that AChE may bind to other AChE molecules present on other cells or in the matrix using a mechanism similar to oligomerisation of multiple AChE molecules (Botti et al., 1998). AChE localised on the cell surface via the PRiMA subunit or through electrostatic interactions could therefore bind to AChE in the matrix causing cell-matrix adhesion. Conversely, AChE localised in the bone matrix could interact with receptor molecules found on the cell surface. Functional redundancy of AChE with neuroligin 1 has been demonstrated in neuronal tissues (Grifman et al., 1998, Sternfeld et al., 1999). Neurexin-neuroligin interactions are well established in the mediation of cell-cell interactions at synaptic sites (see figure 6.3) (Nguyen et al., 1997, Scheiffele et al., 2000). Expression of a number of neurexin and neuroligin partners has been demonstrated in bone (see section 4.3.5), therefore it is possible that AChE localised in the bone matrix could interact with neurexins or analogs thereof

Original in colour

Cell-matrix Adhesion

Prevention of cell-matrix interactions

A

B

receptor ligand interactions

Bone Matrix

Bone Matrix

C

D

electrostatic interactions

Bone Matrix

Bone Matrix

E

F

AChE-AChE binding/AChE -esterase-like interactions

Bone Matrix

Bone Matrix

AChE-S/COLQ

AChE-R

AChE receptor/ligand

AChE-S/PRiMA

Esterase-like molecule

Figure 6.3
Possible interactions of AChE as a matrix component to induce or inhibit ce adhesion/attachment.

1. AChE could induce cell-matrix adhesion through matrix immobilised AChE interacti with yet to be identified cell surface ligands (A), or inhibit adhesion through secret AChE blocking matrix bound receptor molecules from cell surface AChE (B).
2. Electrostatic interactions of cell surface expressed AChE could also induce cell-matr adhesion. Secreted AChE may also block and obscure electrostatic sites to prevent ce surface expressed AChE from binding to the matrix.
3. Esterase-like molecules and their binding partners may also enable cell-matr interactions. Secretion of esterase-like molecules and subsequent binding to matrix bou ligands may however block cell surface expressed AChE from binding to the matrix.

present on the cell surface to cause osteoblast adhesion. Due to the large size of the neurexin and neuroligin genes, and the extensive inter-gene splicing and post-translational modification, over 1000 possible variants of neurexin and numerous neuroligin members could be expressed (Ullrich et al., 1995, Ichtchenko et al., 1996).

It is conceivable that bone-specific partners for AChE exist that may belong to the neurexin family. It has also been hypothesised that neuroligins may act as signalling molecules via neurexins, which may attach to intracellular components signalling systems via PDZ domain proteins, some of which have already been identified in bone and osteoblasts (see figure 6.3) (Nguyen et al., 1997, Littleton et al., 1997, Songyang et al., 1997, Grifman et al., 1998, Spencer et al., 2000).

Thirdly, it may be possible that surface bound AChE via the GPI anchor or the PRiMA subunit could bind to other novel matrix components. Again the neurexin-neuroligin parallel may play a factor, or AChE may bind to other matrix components through it adhesive domain such as lamanin or collagen which are known to bind to AChE (Johnson and Moore, 2003). A graphical representation of these possibilities can be found in Figure 6.3.

The data presented in chapter 4 would suggest that the second possibility discussed might be more favourable, with AChE present as a matrix component for osteoblast attachment. Although the complex regulation and multiple isoforms of AChE suggest that it is likely that many different circumstances with differing needs could be fulfilled. In contrast to a role in mediating cell adhesion, it is also possible AChE could block adhesion. Competition between cell-surface bound, matrix bound and free soluble AChE for receptor molecules such as neurexins may exist. This could be useful both during development and remodelling. Production of secreted AChE may block matrix bound receptor molecules from surface expressed AChE binding allowing cell motility and migration, an important factor in osteogenesis and remodelling (see figure 6.3). In addition, it could block cells from binding to the cell surface and encourage them to form complexes with molecules on other cells to cause cell-cell contact. Again during development this would be essential. Conversely, it could also prevent cell-cell contact and encourage cell adhesion. This is true for a number of bone matrix proteins (e.g. osteonectin, osteopontin) that have been classed along with a number of other proteins as matricellular proteins (Sodek et al., 2002). This new family

of proteins display roles in a variety of tissues in adhesion, migration, and differentiation and often possess these properties secondary to their primary role (Bornstein and Sage, 2002). It has been suggested that such proteins have the ability to maintain an intermediate adhesive state in which no focal adhesions of cells occur; rather cells are associated with the matrix but can freely migrate (Murphy-Ullrich, 2001). This would be of considerable use for osteoblasts, especially during remodelling where bone lining cells and osteoblasts are required to be removed from the surface for osteoclastic bone resorption. It is clear that AChE is capable of self-regulation of function and can provide a number of mechanisms to regulate bone development and remodelling.

AChE as a growth promoting agent

Demonstration of regulated AChE expression in response to growth promoting agents, mechanical loading and regulated expression during osteoblast differentiation, indicated that AChE may play a role in osteoblastogensis. AChE is thought to possess properties that can promote cell proliferation and differentiation. However the specific mechanisms involved have yet to be identified. In cholinergic and other neuronal tissues it has been proposed that AChE can act through a cholinergic mechanism to assert its growth promoting effects. However, increases in AChE expression that parallel cell proliferation and differentiation in other tissues, in which cholinergic signalling is absent, could present us with a novel mechanism. One tissue in which AChE plays a recognised non-cholinergic function is the retina. Characterisation of AChE and BChE expression profiles in retina and retinal spheroid systems has revealed that AChE is specifically expressed in tissues that are differentiating. BChE expression however, is limited to proliferating cells, and is progressively lost in culture as cells become more differentiated (Layer, 1983, Willbold and Layer, 1992, Layer and Willbold, 1994). Indeed, in the developing nervous system AChE/BChE expression profiles are mutually exclusive, with AChE apparent in areas of migration or differentiation, and BChE expressed only in mitotically active areas (Vollmer and Layer, 1986 and 1987, Willbold and Layer 1992). In contrast, AChE expression levels appear to increase in cancerous conditions where dramatically increased cell proliferation is observed (Perry et al., 2003). Although there is enough data to implicate a role for AChE in cell differentiation, in a system that may include BChE, there is no evidence of

a direct involvement. It is possible that cell-cell/cell-matrix interaction could initiate downstream signalling either by allowing interaction with other signalling systems, or by activating its own signalling systems. As discussed above, AChE could be involved in mediating cell-cell matrix interactions in bone, and data from other tissues suggest neurexin-neuroligin like signalling may occur through AChE. In addition, AChE-R was recently found to interact with RACK1 and PKC?II to induce intracellular signalling cascades in response to stress conditions in the brain (Birikh et al., 2003).

It has long been recognised that the expansion of haematopoietic progenitors and the terminal differentiation of certain haematopoietic cells is co-related to increasing levels of AChE expression. Most recently direct evidence both in vitro and in vivo suggests that the AChE-R isoform can control haematopoietic cell fate. Moreover the AChE-R peptide ARP can induce progenitor cell expansion directing cell differentiation toward that of the megakaryoblastic/cytic lineage. The mechanism by which ARP induces such actions has yet to be identified. However it is clear that ARP can exert considerable influence on the differentiation of this cell type. As haematopoietic cells share a common lineage, it is possible that effects on one cell type might well influence differentiation of other cell types as a consequence. Indeed, differences in populations of other cell types have been observed in AChE-R mice, and in mice with AChE expression inhibited by antisense treatments (Soreq et al., 1994, Grisaru et al., 2001, Deutsch et al., 2002). Such influences may also subsequently have down stream effects on osteoprogenitor cell differentiation. Additionally, mesenchymal cells in bone interact with megakaryocytic cells and are thought to influence each other through cross-talk of signalling systems. ARP may represent yet another molecule that can mediate signals between cell types of different tissues which occupy the same biological environment. Cross-talk between the dura mater and the developing calvarium is also an essential mechanism by which ARP may act. The data presented in Chapter 5 already implicate a specific role for the AChE-R isoform in calvarial development.

Conversely, AChE peptides may act directly to affect osteoprogenitor expansion and differentiation. At the suture, this may mean that elevated AChE-R could subsequently give rise to increased levels of ARP, which in turn may cause a prolonged period of osteoprogenitor cell proliferation resulting in greater cell masses within the suture and delayed closure. My data already indicate this could also be true for the

marrow where increased ARP as a result of increased AChE-R expression may cause aberrations in stem cell proliferation with down-steam effects on osteoblastogenesis.

Novel cholinesterases as treatments for bone diseases

From the data presented in this thesis, it is clear that a functional role for AChE exists in bone. The extensive knowledge of AChE in relation to cholinergic signalling and the growing body of data on AChE non-cholinergic activities could yet provide novel approaches in the treatment of bone disorders such as osteoporosis and osteoarthritis. As a relationship between chronic exposure to cholinesterase inhibitors and decreased bone mass has been identified, it is unlikely that already established inhibitors against AChE could be exploited as possible treatments (Compston et al., 1999, Compston et al., 2003). However, because cholinergic signalling pathways have yet to be identified in bone cells, design of novel drugs for AchE, targeted specifically to bone could be developed without any disruption to cholinergic signalling systems. Moreover, since it has become evident that AChE represents only one member of larger family of esterase-like proteins, the possibility of identifying new therapeutic targets is further expanded. Understanding of isoform-specific functions of AChE in bone may also provide an area for targeted treatment development. Peptides such as those found on the C-terminus of AChE, if involved in bone modelling and remodelling, are ideal candidates for the development of new treatments of bone disorders. Both are easy and inexpensive to produce, and these peptides would pose little disturbance to cholinergic signalling.

Future work

As this project has progressed it has moved in directions that were not first envisaged. The analysis of AChE transgenic mice has proved to be essential not only in providing a definitive physiological function for AChE in bone, but in identifying isoform-specific differences in function. Furthermore, significant complexity in the regulation of AChE isoform expression has become evident. However, this leap from characterisation to functionalisation has left behind what I feel are unanswered questions and gaps in the story. Additionally, a number of new avenues of investigation have been identified. Therefore future work could progress in three directions.

1. Further characterisation of osteoblastic AChE regulation
2. Further characterisation of AChE transgenic mice

3. Investigation of isoform-specific functional roles for AChE in osteo/chondrogenesis.

1. Although expression of a number of AChE accessory proteins and cholinesterase-like proteins has been established in this thesis, the exact nature of their role in AChE function is still very unclear. This is an area of its own relevance and interest and should be treated as such.

Functional localisation of AChE, and means of localisation may also prove important. Characterisation of anchoring proteins such as the PRiMA subunit and ColQ in osteoblasts and bone may provide further clarification. Further analysis of the AChE isoforms secreted by osteoblasts may also be important in establishing potential interaction between AChE and the matrix (i.e ColQ or AChE-R).

2. Characterisation of the AChE transgenic mice is still in the very early stages. Thorough histological analysis at varying ages of all skeletal elements is required to determine the extent of reduction in intramembraneous ossification. This should parallel further characterisation of bone mass and volume using DXA, micro CT, or QCT. It would be most interesting to see if the similarities in skeletal morphology observed in AChE-R overexpressing mice, antisense mice and AChE knock out mice are carried over to the effect on bone mass observed in AChE knock out mice. Micro-CT could also be used to analyse developmental delays in suture closure, and to establish differences in suture width in transgenics in comparison to wild type mice. Furthermore, this could be done independantly of any changes in the skull base sychondroses to clarify the nature of the defect. Suture closure could also be investigated by the use of whole calvarial organ cultures systems, to see if specific signalling pathways have been affected by aberrations in AChE expression. Responses of AChE animals to mechanical loading and cytokine administration are also a possibility. Ultimately, transgenic mice with bone targeted AChE disruption using osteoblast and chondrocyte specific promoters would be very beneficial.

3. Transgenic AChE mice are also an invaluable source of tissue for ex vivo experiments. Tissues from AChE transgenic mice could be analysed for alterations in RNA and protein levels of established regulators of bone formation and osteoblastogenesis. Ex vivo culture of bone marrow from mice over expressing AChE have already provided indications of isoform-specific differences in osteoblast

differentiation. To clarify isoform-specific roles for AChE in osteoblast function, differentiation, proliferation, and apoptosis, ex vivo whole marrow cultures and primary cultures of mouse calvaria could be employed. Further to this, transfection of primitive cell lines and primary osteoblast-like cells with dominant-negative and dominant-positive plasmid constructs containing sequences specific to the different AChE isoforms could be also be used. To determine specific functions for AChE, plasmids containing sequences targeted to specific regions of the AChE protein (i.e. adhesive domain, EF calcium binding domain), or mutated sequences could be used to establish effects on osteoblast adhesion and differentiation. This approach could also be used to determine any effects of the C-terminal peptides of AChE, ARP and ASP. As ASP has been linked to translocation and transcriptional activity of a Runx1, the possibility of ASP maintaining a similar influence on Runx2/Cbfa1 in osteoblasts is another potential area for investigation.

I have proposed a functional redundancy and transcriptional feedback response to explain the skeletal phenotype analogies, and in theories of cellular interaction, this is a key point. Therefore it is imperative to establish if a transcriptional feedback response exists within bone and osteoblasts. This could be approached in two ways. Firstly, using cell culture models, and secondly, using cells and tissues from the variety of AChE transgenic mice. Potentially, this could also provide an indication of possible interacting signalling systems for AChE and others established within bone.

Summary and Conclusions : Functional redundancy, or a poor substitue for the real thing?

Multifunctionality and functional redundancy are by no means new concepts. Although it may be easy, it is foolish to assume that a protein may exist for a singular purpose. The basis of this project is rooted in these concepts. The recent discovery of multiple non-cholinergic functions for AChE provided the possibility of numerous roles within bone. The analogy of AChE to other bone matrix proteins, both in expression and structure could also suggest functional redundancy. The data presented in this thesis suggest strongly that AChE has a role as a bone matrix protein. However, functional data are limited, mainly because of the growing number of functional roles of AChE. I have been lucky enough to have access to a number of sources of AChE transgenic mice, from which isoform-specific roles for AChE and a complex regulation of AChE

isoform expression in bone has become evident. Expression of AChE-R, in particular, showed considerable parallels to a number of bone disorders, as well as similarities to the inhibition of AChE expression. This suggests that expression of AChE-R outside of its normal capacity has adverse effects. Again the multifunctional nature of AChE and functional redundancy with other proteins may provide a mechanism by which this is possible. In spite of such potential remedies, AChE-/- mice do display a bone phenotype characterised by reductions in BMD and BMC. Therefore, could it be that a functional redundancy of AChE variants in one of its capacities, could provide for problems in another capacity in which it might be a poor substitute. Functional redundancy may just be a quick fix that presents no real solution to a greater problem. Taken together these data suggest that this intriguing molecule plays a fundamental role in bone of which we have only touched the surface.

List of abbreviations

ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
AChE$^{-/-}$	-	Acetylcholinesterase knock out mice
AChE$^{+/-}$	-	Acetylcholinesterase heterozygous mice
AER	-	Apical ectodermal ridge
ALK	-	Activin receptor-like kinases
AML3	-	Acute myeloid leukaemia factor 3
APP	-	Amyloid precursor protein
ARP	-	C-terminal peptide of AChE-R
ASP	-	C-terminal peptide of AChE-S
ATCI	-	Acetylthiocholine iodide
BCA	-	Bicinchoninic acid
BChE	-	Butrylcholinesterase
BFA	-	Brefeldin A
bFGF	-	Basic fibroblast growth factor
BMPs	-	Bone morphogenic proteins
BODIPY TR ceraminde	-	N-((4-(4,4difuoro-5-(2-thienyl)-4-bora-3a, 4a-diaza-s-indacene-3-yl)phenoxy)acetyl)sphingosine
BSA	-	Bovine serum albumin
BW284C51	-	1,5-bis(4-Allydimethylammoniumphenyl)pentan-5-1 dibromide
Cbfa1	-	Core binding factor alpha 1
CFU-Fs	-	Colony forming unit - fibroblasts
ChAT	-	Choline acetyltransferase

CRE	-	cAMP response elements
CSF	-	Cerebrospinal fluid
CCD	-	Cleidodocranial dysplasia
ColQ	-	Collagen-like tail
dpc	-	Days post coitus
DEPC	-	Diethyl Pyrocarbonate
DFP	-	Diisopropyl Fluorophosphate
DMEM	-	Dulbecco's modified Eagles Medium
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DNTB	-	5'5 dithiobisnitrobenoate
dNTP's	-	2'-deoxynucleoside 5'-triphosphates
DTT	-	Dithiothreitol
EDTA	-	Ethylenediamine tetra-acetic acid disodium salt
ECL	-	Enhanced chemiluminesence
ER	-	Endoplasmic reticulum
FCS	-	Foetal calf serum
FGF	-	Fibroblast growth factor
FGFR	-	Fibroblast growth factor receptor
FITC	-	Fluorescein isothiocyanate
GAPDH	-	Glyceraldehyde-3-Phosphate Dehydrogenase
GDFs	-	Growth differentiation factors
Gla	-	3 gamma-carboxyglutamic acid
GPI	-	Glycophospholipid

HSPGs	-	Heparan sulphate proteoglycans
HRP	-	Horseradish peroxidase
Ihh	-	Indian hedgehog
IGFs	-	Insulin-like growth factors
iso-OMPA	-	Tetraisopropyl pyrophosoramide
kDa	-	Kilodaltons
KOH	-	Potassium hydroxide
MAPK	-	Mitogen activated protein kinase
α-MEM	-	α-Minimum Essential Medium
MDS	-	Myelodysplastic syndromes
ME	-	Myalgic encephalomyelitis
MMPs	-	Matrix metalloproteinases
micro CT	-	Micro computed tomography
MTT	-	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide Thiazolyl blue
NCAMs	-	Neuronal cell adhesion molecules
NMDA	-	N-methyl-D-aspartate
NMJ	-	Neuromuscular junction
NP40	-	Nonylphenoxy polyethoxy ethanol
OSE	-	Osteoblast specific elements
PAS	-	Peripheral anionic site
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase chain reaction
PDGF	-	Platelet derived growth factor
PDZ	-	PSD-95/Dlg1/ZO-1

PEBP2αA	-	Polymavirus enhancer binding protein 2αA
PKC	-	Protein kinase C
PNPP	-	Paranitrophenol phosphate
PRAD	-	Proline rich attachment domain
PTH	-	Parathyroid hormone
PVA	-	Polyvinyl acetate
RGD	-	Arginine-glycine-aspartate
RNA	-	Ribonucleic Acids
Runx2	-	Runt related gene 2
RT-PCR	-	Reverse transcriptase polymerase chain reaction
SARA	-	Smad anchor for receptor activation
SDS	-	Sodium dodecycl sulphate
Shh	-	Sonic hedgehog
SSC	-	saline-sodium citrate
TBS-T	-	Tris buffered solution with Tween 20
TGF	-	Transforming growth factor
TGF-β1	-	Transforming Growth Factor – type β1
TRIS	-	Trisodium citrate
TRIS-HCL	-	Tris solution pH with HCL
UV	-	Ultra violet
VAChT	-	Vesicular acetylcholine transporter
VDREs	-	Vitamin D3 responsive elements
VEGF	-	Vascular-endothelial derived growth factor
WAT	-	trypyophan (W) amphiphilic tetramerization

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