Parallel Analysis of Gene Expression:

Bone Cells as a Model System

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Presented in fulfillment of the requirements for the degree of Doctorate of Philosophy of Medical Research

Griffith University, Gold Coast Campus, Queensland

2003
ABSTRACT

The use of comparative gene expression techniques has expanded considerably in recent years, especially with advances in microarray technology. In this project, a number of these techniques have been used to identify genes worthy of further research as potential mediators of bone cell differentiation and function. Bone is a tissue with many potential as yet unidentified regulatory molecules. The skeleton is constantly undergoing replacement, with old bone being degraded by osteoclasts, bone resorbing cells derived from the haematopoietic lineage, and replaced with new bone by osteoblasts, bone synthesizing cells derived from the mesenchymal stem cell lineage. When the rate of bone resorption exceeds the rate of bone synthesis, osteoporosis can occur. Osteoporosis is the most common form of disease affecting the skeleton, and one of the most common age-related diseases, and is a major social and economic burden. Recent studies have shown that cells of mesenchymal lineage are capable of adopting alternate differentiation fates, suggesting that cell-based therapies may be a useful therapeutic approach for this disease. Therefore, identification of molecular mechanisms involved in regulating the behavior and development of bone forming and bone resorbing cells is essential. The aim of this project, therefore, was to identify genes involved in various stages of bone cell differentiation using comparative gene expression techniques.

The specific objectives of this project became: 1) to identify molecules expressed by osteoblasts which may increase or decrease bone synthesis; these may have potential for exploitation to treat bone loss in osteoporosis, or excess bone deposition in
osteopetrosis; and 2) to identify molecules expressed by osteoclasts which may increase or decrease bone resorption; these may have potential for exploitation to treat excess bone deposition in osteopetrosis, or bone loss in osteoporosis.

The first objective, identification of molecules expressed by osteoblasts involved in bone deposition, was addressed using three techniques: subtractive hybridization, DNA microarray analysis, and DNA macroarray analysis. These techniques were used to identify genes transcribed at different levels between foetal osteoblasts and fibroblasts.

The key difference between DNA arrays, and subtractive hybridization, as techniques is that DNA arrays utilize a cDNA population fixed to a rigid medium as starting material. This means, therefore, that in order to identify a gene as being expressed, the gene must be present on the array, as a member of the cDNA library the original starting array was made from. This inhibits identification of truly novel transcripts, a bias which is removed in techniques such as subtractive hybridization.

The technique of subtractive hybridization is used to identify genes transcribed at higher levels in one DNA sample compared with another. The subtractive hybridization technique described here was modified to enrich a foetal osteoblast phagemid library by removing phagemid which contain transcripts common to foetal osteoblasts and dermal fibroblasts, thus resulting in identification of genes expressed uniquely or at higher levels in osteoblasts. The technique identified 65 genes that were expressed either highly or specifically in osteoblasts when compared with fibroblasts. Some of the genes identified were found in multiple library clones, such as collagen
and fibronectin, both of which are key structural components of bone, abundantly expressed by osteoblasts. Expression of some other identified genes had not previously been detected in osteoblasts, making them interesting targets for further investigation. Interesting genes revealed using this technique included prohibitin, leptin-receptor gene related protein, ornithine decarboxylase antizyme, amyloid precursor peptide and connective tissue growth factor. The usefulness of the technique was verified by performing real-time PCR to confirm the expression of these genes either specifically or abundantly in osteoblast cells.

DNA array analysis was undertaken to identify transcripts previously identified in other tissues, but not investigated in bone cells to date. Micro and macroarray analysis was used to identify known genes that were over or underexpressed.

The microarray comparison of fibroblasts and osteoblasts using cDNA differentially labeled with fluorescent dyes hybridized to glass microarrays, showed that the two cell types were very similar, with just 64 genes found to be regulated 5-fold or more-37 were down-regulated in osteoblasts, and 27 were upregulated in osteoblasts, out of the 19,000 genes represented on the array. Genes shown to be significantly upregulated in osteoblasts by the 19k microarray included several neural proteins and transcription factors, while genes downregulated in osteoblasts included cell signal transducers and other transcriptional activity modifiers.

The set of Atlas cDNA arrays consists of three pairs of arrays, with each pair containing 1176 genes. Two pairs of filters, or 2352 genes, were probed in the
osteoblast/fibroblast comparison, while all three pairs, or 3528 genes, were probed in the osteoclast/macrophage comparison.

The data from the DNA macroarray experiment, in which radioactively-labelled cDNA from osteoblasts and fibroblasts was hybridized to two separate sets of nylon arrays (ATLAS human cDNA arrays) showed that 12 out of the 2352 genes assayed were significantly regulated, with eight upregulated in osteoblasts, and four downregulated in osteoblasts. Genes upregulated in osteoblasts included transcription factor Dp-2, cAMP response element binding protein 1, and transcription factor ATF2. Genes down-regulated in osteoblasts included teratocarinoma derived growth factor, thymosin beta-10, and transcription factor 3.

To address the second objective, and identify molecules expressed by osteoclasts involved in bone resorption, the DNA macroarray analysis approach was repeated. cDNA isolated from osteoclasts was compared with cDNA isolated from macrophages to identify genes differentially transcribed between these two cell types which differentiate from the same developmental lineage in vitro.

DNA macroarray analysis, performed using radioactively labeled cDNA from osteoclasts and macrophages hybridized to ATLAS human cDNA arrays identified 53 genes as upregulated in osteoclasts, including GM-CSF Receptor, signalling molecule calmodulin 1, and transcription regulatory molecule Nuclear Factor of Activated T-cells (NFAT). Seventeen genes were shown to be downregulated in osteoclasts, including transcription factor zpf36, Integrin 5, and X-ray repair complementing protein.
The studies described here suggested that osteoclasts and macrophages have more differentially expressed genes than osteoblasts and fibroblasts, with 1.98% of genes arrayed showing differential expression between osteoclasts and macrophages, but only 0.3% of genes arrayed showing differential expression between fibroblasts and osteoblasts. This suggests that the morphological differences between cell types may be a direct reflection of the molecular differences between them as well, as osteoblasts and fibroblasts are quite similar, while osteoclasts and macrophages are morphologically quite distinct.

From this project, it would appear that comparisons of different populations of cells require the use of different techniques to yield the best results, and that the techniques of array analysis and subtractive hybridization may be best utilized in combination, rather than exclusively of each other. Many of the genes identified as differentially expressed between fibroblasts and osteoblasts using DNA arrays were fairly well-characterised ‘house-keeping’ type genes- metabolic, structural, and not specific or likely to play a significant role in the differentiation of osteoblasts, or the development of bone disease. One possible reason for this is that the arrays are too limited by the number of genes featured, to be able to detect many differences between similar cell types, where there are fewer differences to detect. In contrast, using the same macroarrays to compare the more distinct osteoclasts and macrophages resulted in identification of several interesting candidate genes, showing that some cell type comparisons can be performed adequately using this technology. By using the subtractive hybridization method to enrich a pre-existing phagemid library, any bias related to the genes able to be detected was removed. Although this
technique requires manufacture of a cDNA library of the cell type of interest, it may be worthwhile for the comparison of similar cell types where sensitivity is an issue.

In summary, this project used the techniques of subtractive hybridization and DNA macroarray and microarray analysis to detect genes showing differential expression between osteoblasts and fibroblasts, and used DNA macroarray analysis to detect genes differentially expressed between osteoclasts and macrophages. Of particular note were results for two interesting genes, amyloid precursor protein and ornithine decarboxylase antizyme. Amyloid Precursor Protein was identified as expressed in high levels in osteoblasts by subtractive hybridization, and real-time PCR studies later confirmed that it is expressed specifically in osteoblasts, and not at all in fibroblasts. Differential expression of ornithine decarboxylase antizyme was identified between both osteoclasts and macrophages, and fibroblasts and osteoblasts. Expression of antizyme results in destruction of ornithine decarboxylase, which is required for the production of polyamines. Degrading cells release spermidine, a polyamine, which attracts macrophages. It is possible that differential regulation of the inhibitory antizyme may be an important distinction between the function of macrophages as general tissue and debris scavenging cells, and osteoclasts, which specifically degrade bone. This study has identified some genes which further studies may show to be important regulators of cellular differentiation and behaviour.
CERTIFICATION OF ORIGINALITY

To the best of my knowledge, this thesis does not contain any material previously submitted for a degree or diploma in any university or any material previously written or published by any other person, except where due reference is made in the text.
ACKNOWLEDGEMENTS

I wish to thank the School of Health Science at Griffith University (Gold Coast campus) for enabling me to undertake this project. I wish to thank Dr Nigel Morrison for allowing me to work in his lab, and Professor Lyn Griffiths for supervising submission of this thesis.

I also wish to thank my collaborator, Dr Rebecca Mason, and her research group, at the Department of Physiology at the University of Sydney for supplying osteoblast and fibroblast cell lines, and Dr Geoff Nicholson’s research group at the Geelong Hospital for supplying the earlier batches of osteoclast and macrophage cells used in this project.

I wish to acknowledge Chris Day for his assistance with establishing experiments and proof-reading, and general running of the laboratory, and the other members of the Morrison, Korolik and Beacham groups, both past and present, for their friendship and support.

Finally, I want to acknowledge both my families- my Adelaide family, Mum, Dad, Chris, Gran, and especially Eric, for their unswerving support and love from afar, and my Clagiraba family- Rod, Cathy, Hannah, Elizabeth and Thomas, who made even the most unsuccessful days seem worthwhile.
PUBLICATIONS ARISING FROM THIS WORK

Published Manuscripts:


Manuscripts in preparation:

**WE Simcock**, CJ Day, M Muir, GR Leong, R Mason, NA Morrison and L Griffiths. Comparison of osteoblast and fibroblast gene expression using DNA microarrays and phagemid library subtractive hybridization

Abstracts and Short communications


ABSTRACT ....................................................................................................................... 1
CERTIFICATION OF ORIGINALITY ............................................................................. 7
ACKNOWLEDGEMENTS ................................................................................................. 8
PUBLICATIONS ARISING FROM THIS WORK .................................................................. 9
Manuscripts in preparation: ............................................................................................ 9
Abstracts and Short communications ........................................................................ 9
TABLE OF FIGURES AND TABLES .................................................................................. 13
TABLE OF ABBREVIATIONS ......................................................................................... ERROR! BOOKMARK NOT DEFINED.
CHAPTER 1: INTRODUCTION ...................................................................................... 17
1.1 Overview .................................................................................................................. 17
1.2 The Skeleton- function and importance ................................................................. 17
1.3 Skeletal patterning and formation- chondrogenesis .............................................. 19
1.4 Skeletal metabolism and remodelling- osteoblasts and osteoclasts ...................... 27
1.4.1 Mineralization of the skeleton .......................................................................... 27
1.4.2 Remodelling of the mineralized skeleton .......................................................... 33
1.5 Diseases of the skeleton- relevance and treatment .................................................. 39
1.6 Differentiation of osteoblast and osteoclast precursors ........................................ 44
1.7 Research overview and objectives ....................................................................... 47
CHAPTER 2: MATERIALS AND METHODS ..................................................................... 51
2.1 Overview ................................................................................................................ 51
2.2 Materials ............................................................................................................... 52
2.2.1 Media ............................................................................................................. 52
2.3 Methods .............................................................................................................. 54
2.3.1 Culture of cells and preparation of RNA ......................................................... 54
2.3.2 Preparation of labelled probes, and hybridisation of probes to arrays ............. 56
2.3.3 Enrichment and analysis of phagemid library ................................................. 61
2.3.4 Real-time PCR ............................................................................................... 67
CHAPTER 3: RESULTS PART 1: SUBTRACTIVE HYBRIDISATION STUDIES .................. 72
3.1 Introduction ........................................................................................................... 72
3.2 Results ............................................................................................................... 77
3.3 Discussion ........................................................................................................... 89
3.3.1 Connective Tissue Growth Factor ................................................................... 91
3.3.2 Amyloid Precursor Protein ............................................................................ 94
3.3.3 Other Candidate Genes ................................................................................. 98
3.3.4 Conclusion ..................................................................................................... 104
TABLE OF FIGURES AND TABLES

Figure 1.1: Derivation and destination of mesenchymal stem cells.....21
Figure 1.2: Differentiation of cells with osteoblastic differentiation potential.....23
Figure 1.3: Development of the osteoclast.....35
Table 2.1: Genotypes of all bacterial strains used in this research.....53
Table 2.2: Sequences of primers used for sequencing and real-time PCR amplification of DNA.....68
Figure 3.1: Traditional method of creating a subtracted cDNA library.....75
Figure 3.2: Diagram of modified method of enriching an osteoblast cDNA library using a phagemid library as starting material.....76
Figure 3.3: Screening digest of plasmids isolated from enriched foetal osteoblast cDNA library...78
Table 3.1: Genes revealed as expressed by osteoblasts as a result of enriching a foetal osteoblast library...81
Figure 3.4: Real-time PCR amplification of APP and CTGF relative to 18S housekeeping gene primers. ...88
Figure 3.5: Processing of Amyloid Precursor Peptide protein, resulting in altered functions and cellular effects.....96
Figure 4.1: Atlas arrays probed with radioactively-labelled cDNA.....110
Table 4.1: Genes significantly upregulated in osteoblasts compared with fibroblasts, as identified by the ATLAS macroarray system, using arrays 3.1 and 3.2....111
Figure 4.2: Microarray analysis of differences in gene expression between osteoblasts and fibroblasts.....114
Table 4.2: Genes significantly upregulated in osteoblasts compared with fibroblasts, as identified by the 19k microarray system....115
Figure 4.3: Log plot of the expression levels obtained from the osteoblast vs fibroblast 19k microarray comparison experiment…121

Table 4.3: Genes revealed as being significantly upregulated in osteoclasts compared with macrophages, as identified by the ATLAS human macroarray system………123

Figure 4.4: Expression of transcription factors identified by ATLAS macroarray as being significantly regulated between osteoclasts and macrophages at three time-points of osteoclast differentiation…137

Figure 4.5: Inhibition of osteoclast formation by Cyclosporin A…138

Figure 4.6: Effects of treatment with Cyclosporin A on expression of Cathepsin K, TRAP, and calcitonin receptor…140

Figure 4.7: Inhibition of Cathepsin K and TRAP expression by GM-CSF…141
TABLES OF ABBREVIATIONS

APP- Amyloid Precursor Protein
ATP- Adenosine Triphosphate
BAP37- B-cell associated Protein 37
bHLH- Basic helix-loop-helix (Transcription factors)
BLAST- Basic Local Alignment Search Tool
BLAT- Basic Local Alignment Tool
BMP- Bone Morphogenic Protein
bp- Base Pair
CAM- Cellular Adhesion Molecule
cAMP- Cyclic Adenosine Monophosphate
CBFA1- Core Binding Factor Alpha 1
cDNA- Copy/Complementary Deoxyribonucleic Acid
cpm- Counts per minute
CTGF- Connective Tissue Growth Factor
dCTP- Deoxycytosine Triphosphate
DMEM- Dulbecco’s Minimum Essential Media
dNTP- deoxynucleotide triphosphate
DNA- Deoxyribonucleic Acid
DTT- Dithiothreitol
EDTA- Ethylenediaminetetraacetic acid
FGF- Fibroblast Growth Factor
FGFR- Fibroblast Growth Factor Receptor
GDF- Growth Differentiation Factor
GMCSF/GCSF- Granulocyte/macrophage Colony Stimulating Factor
Hox- Homeobox gene
IGFBP- Insulin Growth Factor Binding Protein
IHH- Indian Hedgehog
IL- Interleukin
kb- kilobase
kDa- Kilodalton
L#- Lumbar vertebrae #
LB- Luria Bertani media
MAPK- Mitogen Activated Protein Kinase
MCSF- Macrophage Colony Stimulating Factor
MEM- Minimal Essential Media
MMP- Matrix Metalloproteases
mRNA- Messenger Ribonucleic Acid
NFATc- Nuclear Factor Activator of Transcription
NFκB- Nuclear Factor κ B
OD- Optical Density
osf-2- periostin
PBS- Phosphate-buffered Saline
PCR- Polymerase Chain Reaction
PEG- Polyethylene Glycol
PFU- plaque-forming units
PTHrP- Parathyroid-related hormone related peptide
RANK- Receptor Activator of NFκB
RANKL- Receptor Activator of NFκB Ligand
RNA- Ribonucleic Acid
Rpm- revolutions per minute
RT-PCR- Real-time Polymerase Chain Reaction
S#- Sacral vertebrae #
SSC- Sodium Sulfonyl Citrate buffer
SDS- Sodium Dodecyl Sulfate
SHH- Sonic Hedgehog
T#- Thoracic vertebrae #
TAE-tris sodium acetate EDTA
Taq- Thermophilus aquaticus (polymerase)
TGF-β- Transforming Growth Factor beta
TIGR- The Institute for Genome Research
TNF-α- Tumour Necrosis Factor alpha
TRIP7- Thyroid Hormone Receptor Interactor 7
TRAP- Tartrate-resistant Acid Phosphatase
UTR- Untranslated Region
VEGF- Vascular Endothelial Growth Factor
CHAPTER 1: INTRODUCTION

1.1 Overview

The research reported in this document was undertaken to investigate the molecular changes that occur during differentiation of precursor cells into bone synthesising or bone resorbing cells. Bone is an important physiological system, because deformities or diseases of the skeleton result in severe consequences for patients, ranging from infant death, for some of the skeletal patterning disorders, through to prolonged hospital stays and an increased risk of death from complications as a result of fractures caused by osteoporosis in the elderly. Identification of the genes involved in bone cell differentiation will lead to an understanding of the genetic abnormalities which lead to these diseases, as well as providing information about how the processes of cell differentiation may be manipulated to treat these diseases using cell-based therapies.

In this introductory section, I will summarise the key research relevant to this project, including a brief discussion of how the skeleton is patterned and formed, how the two cell types primarily responsible for maintaining its integrity are derived, and the disease states resulting when this balance is not adequately maintained, before putting the research undertaken here into context, and outlining the research objectives.

1.2 The Skeleton- function and importance

The skeleton fulfils a number of vitally important functions within all vertebrates. Skeletal elements provide a measure of protection to many of the vital organs,
including the brain, heart and lungs. The skeleton also provides adherence points for
muscle, allowing flexibility of limbs around joints, and giving limbs rigidity between
joints. The skeleton is also the body’s major store of minerals essential for
homeostasis. About 90% of the body’s total calcium, 80% of its total phosphorus, and
significant amounts of magnesium, sodium, citrate and bicarbonate, are stored in the
skeleton at any given point in time (Dempster, 1992). As biological processes
demand, these minerals are released into the bloodstream, a process often mediated by
the endocrine system.

The dry mass of an entire adult skeleton is approximately 1 kilogram. It consists of
two different types of bone: flat bone, such as the skull and pelvis, and tubular bone,
which forms the centre of the limbs and the vertebrae (Woolf, 1998).

Bone, the constitutive material of the skeleton, is a composite material consisting of
two parts, an organic phase and an inorganic phase (Currey, 1984). The inorganic,
mineralized phase is mostly calcium and phosphorus, and other inorganic salts, stored
mainly as hydroxyapatite crystals (Glimcher, 1981). The organic phase of bone
consists of the cell types that make up the skeleton: osteoblasts, osteoclasts,
chondrocytes, and osteocytes, and the proteins secreted by these cells that form the
extracellular bone matrix.

Bones of the skeleton fall into two distinct classes. Most of the long bones are part of
the appendicular skeleton, which includes all the bones of the four limbs. The other
bones form the axial skeleton, the central structure to which the appendicular portions
of the skeleton are attached. The axial skeleton includes the vertebral column, the pelvis, the ribs, and the bones of the skull.

Each individual bone is made up of two distinct types of bony tissue: cortical bone and cancellous bone, also referred to as spongy or trabecular bone. Cortical bone forms the outer surface of bones. It is hard, densely packed tissue, which provides most of the strength of the skeleton, and accounts for about 80% of the total skeletal mass. Cancellous bone is usually found in the centre of the bone surrounding the bone marrow, mostly in the vertebrae and at the ends of the long bones. It has a more open, lattice-like structure consisting of plates and rods of osteoid, called trabeculae. This structure provides maximal strength for minimal mass, acting to protect the bone marrow within the cavities without adding excessive weight to the total skeletal mass. The large surface area of cancellous bone, and its relative fragility compared with cortical bone, means that any disturbances to skeletal integrity are frequently reflected in cancellous bone first. This explains why bones rich in cancellous tissue, such as the vertebrae, are often the first part of the body to manifest symptoms of skeletal stress, such as osteoporosis (Dempster, 1998; Lane, 1999; Woolf, 1998).

1.3 Skeletal patterning and formation- chondrogenesis

From fertilisation onwards, cells of the developing embryo proliferate and adopt progressively more restricted differentiation fates, as cells in different regions of the embryo become more similar to the cell types found in that region of the mature organism. Skeletal patterning commences early in the development of the embryo in much the same way as other organs and body parts are determined. Cells in the region
of the embryo where skeletal elements will be required in the adult differentiate into mesenchymal stem cells, in the first step of restriction of their differentiation potential. Mesenchymal stem cells have the potential to develop into myoblasts, fibroblasts, adipocytes, and more importantly with regard to skeletogenesis, osteoblasts and chondrocytes (Figure 1.1: Derivation and destination of mesenchymal stem cells) (Olson et al, 1999). Mesenchymal stem cells with skeletal developmental potential migrate to the area of the embryo where a skeletal element is to develop in response to signals from molecules including Homeobox genes, members of the TGF-β superfamily, and members of the basic helix-loop-helix family of transcription factors. The mesenchymal stem cells then establish cell-cell interactions with surrounding epithelial cells, and other mesenchymal stem cells, and proceed to proliferate and differentiate, giving rise to a dense population of mesenchymal stem cells with the overall shape of the bone which will eventually develop at that site (Hall and Miyake, 2000).

There are two methods by which bone is produced in humans. The first of these, endochondral ossification, is responsible for the production of the tubular bones, such as the limbs and vertebrae, and relies on the presence of a complete cartilaginous template prior to ossification (Karsenty, 1998). The second method, intramembranous ossification, occurs in the formation of flat bones, such as the clavicles and certain bones of the skull (Thorogood, 1993). This process does not rely on development of a cartilaginous anlagen. Instead, mesenchymal cells differentiate directly into
Figure 1.1: Derivation and destination of mesenchymal stem cells. Chondrogenic precursors can develop from both mesoderm and ectoderm, while the bones of the cranium and axial and appendicular skeleton are all derived from mesoderm.
osteoblasts, and proliferate outwards to form plates or sheets of bone which join together with the edges of other bone sheets when they make contact (Figure 1.2: Differentiation of cells with osteoblastic differentiation potential)

The framework of mesenchymal cells at this stage of development is called the anlagen, and acts as a scaffold for the eventual deposition of bone matrix (Hall and Miyake, 1992; Horton, 1993). At this stage of development, cells of the anlagen express type I collagen, a mesenchymal cell marker, and type IIa collagen, a protein not specific to chondrocytes (Lui et al., 1995). Expression of Sox9, an SRY homologue, has also been observed at this point prior to chondrogenesis (Ng et al., 1997). It is at this stage of skeletal development that the fork between the two pathways of ossification, endochondral and intramembranous, occurs.

In intramembranous ossification, vascularization of the anlagen is followed directly by osteoblast differentiation, as vascularization allows osteoblastic precursors to enter the anlagen, attach to the collagen expressed by it, differentiate into osteoblasts, and begin secreting bone matrix (Thorogood, 1993). It is possible that the invading osteoblastic precursors also secrete signalling molecules capable of recruiting cells of the mesenchymal condensation to the osteoblastic differentiation pathway. Vascularization of the anlagen probably also facilitates the entry of osteoclast precursors into the condensation body, as there may be a requirement for cells of this type to degrade the secreted collagen surrounding the differentiating osteoblasts prior to deposition of osteoid matrix.
Figure 1.2: Differentiation of cells with osteoblastic differentiation potential. Pathways indicated by broken lines have been observed in vitro, and are postulated to occur in vivo, making manipulation of these pathways targets for therapeutic intervention to treat diseases of the skeleton such as osteoporosis. (Adapted from Aubin, 1998).
In endochondral ossification, the anlagen is replaced by a cartilage template, which in turn is replaced by bone, and forms the long bones of the axial and appendicular skeleton (Karsenty, 1998). There are four subpopulations of chondrocytes found during skeletal development: proliferating, hypertrophic, apoptotic, and, in the growth plate, a senescent form, or resting subpopulation.

Proliferating chondrocytes are identifiable from their mesenchymal stem cell precursors by their expression of type II collagen, a chondrocyte-specific marker. Expression of type II collagen is regulated, at least in part, by the transcription factor Sox 9. In the absence of Sox9 activity, mice fail to develop chondrocytes, and suffer a complete lack of endochondral ossification (Lefebvre et al., 2001). Two related transcription factors, L-Sox5 and Sox 6, are also required at later stages of chondrogenesis- the absence of either of these transcription factors in a mouse model results in mild skeletal defects, but the absence of both factors is embryonic lethal, with a complete failure of endochondral skeletal formation. Cells at the centre of condensations express pre-hypertrophic, or proliferating, chondrocyte markers, while cells at the periphery express hypertrophic chondrocyte markers, resulting in disrupted growth plates and bone organisation (Smits et al., 2001).

As the developing foetus grows, so do the skeletal element templates. And after birth, through childhood, and during puberty, the bones of the skeleton undergo longitudinal growth, mediated by a population of undifferentiated cells located just beyond the mineralized front at the end of the bone, called the growth plate. The size of the bone is determined by the number of proliferating chondrocytes, which is tightly regulated
by balancing levels of parathyroid hormone-related peptide (PTHrP) and Indian Hedgehog (IHH).

Pre-hypertrophic, or proliferating, chondrocytes express IHH, which acts through the PTHrP receptor to repress the progression of pre-hypertrophic chondrocytes to hypertrophic chondrocytes (Bitgood and McMahon, 1995; Lee et al, 1996; Vortkamp et al, 1996; Tiet et al, 2003; Pateder et al, 2000). Overexpression of IHH results in decreased hypertrophic chondrocyte numbers, and leads to an increase in the levels of PTHrP secreted by the cells. Over expression of PTHrP also prolongs the prehypertrophic phase of chondrocyte development, via a positive feedback mechanism. Deficiency of PTHrP or its receptor results in Dwarfism, a phenotype of shortened limbs, due to insufficient time spent in the proliferative phase of chondrogenesis, reflected most dramatically in growth plate chondrocytes, causing the long bones to fail to extend longitudinally in the expected manner. In mice, inactivation of both copies of the PTHrP gene results in death shortly after birth, caused by asphyxiation, probably due to the lack of skeletal support for the lungs and cartilaginous support for the trachea. In addition, mice homozygous for the inactivated PTHrP gene display short limbs, domed skulls, short snouts and mandibles, and protruding tongues, which are characteristic of the skeletal dysplasia caused by PTHrP deficiency. Staining for mineralization and cartilage of these mice reveals that premature mineralization of the cartilaginous template occurs in many bones formed by endochondral ossification, while bones formed by intramembranous ossification appear to develop normally (Karaplis et al, 1994).
PTHrP is also involved in regulating the apoptosis of hypertrophic chondrocytes. In late proliferative chondrocytes, high levels of Bcl-2, an apoptotic inhibitor gene, are observed (Amlington et al., 1997). Bcl-2 is regulated by PTHrP, and deficiencies of PTHrP result in decreased levels of Bcl-2 (Lanske et al., 1996). Bcl-2 works by sequestering Bax, a pro-apoptotic gene. As the chondrocyte progresses towards hypertrophy, the levels of Bax increase, and the levels of Bcl-2 decrease, until Bax outcompetes Bcl-2, and the chondrocyte undergoes apoptosis (Oltvai et al., 1993; Miyashita et al., 1994). It is the downregulation of Bcl-2 in PTHrP deficient mice that causes the premature hypertrophy and apoptosis of chondrocytes, and the Dwarfism phenotype (Lanske et al., 1996), which is also observed when Bcl-2 is subjected to targeted inactivation in mice (Amling et al., 1997).

Proliferating chondrocytes express type II, IX, and XI collagens, and matrix GLA protein (Ryan and Sandell, 1990; Sandell et al., 1991; Lui et al., 1995; Luo et al., 1995), components of the extracellular cartilaginous matrix. Furthermore, expression of type I collagen stops at this stage of chondrogenesis (Luo et al., 1995). Hypertrophic chondrocytes primarily express type X collagen, which is assembled to form a hexagonal lattice-like structure around the chondrocyte (Schmid and Linsenmayer, 1990). Collagens secreted earlier during chondrogenesis, such as type II, are only transient molecules. Small in diameter, subsequent to the secretion of type X collagen by hypertrophic chondrocytes, earlier collagens are degraded by collagenases 1, 2, and 3, (MMPs 1, 8, and 13) secreted by cells surrounding the anlagen, and resorbed (Alini et al., 1992), leaving a more open matrix structure suitable for invasion during vascularisation of the cartilaginous template, which is initiated during hypertrophy. MMP9 is also expressed at this stage, but is restricted to
the ends of the long bones, and acts primarily to allow vascularization of the matrix surrounding the chondrocytes without triggering apoptosis, thus preserving the growth plate. These populations of chondrocytes have not entered the hypertrophic phase of chondrogenesis. Instead, these cells, located just beneath the epiphysis (ends) of the bone, remain in the proliferative phase, and become growth plate chondrocytes, which undergo further cycles of proliferation, hypertrophy, and apoptosis in a longitudinal manner to push the ends of the long bones forward during growth in childhood and adolescence (Vu et al., 1998). These cells eventually undergo hypertrophy and apoptosis, and are replaced by osteoid matrix, once the bones have reached their final length (Ehrlebacher et al., 1995). The degradation of the collagenous matrix by MMPs triggers expression of Vascular Endothelial Growth Factor (VEGF), which acts as a directional attractant of nearby blood vessels to facilitate invasion during angiogenesis (Gerber et al., 1999; Vu et al., 1998).

1.4 Skeletal metabolism and remodelling- osteoblasts and osteoclasts

1.4.1 Mineralization of the skeleton

Osteoblastic precursor cells are recruited into the anlagen through the newly-grown blood vessels, and adhere to the anlagen (Bianco et al. 1995). Differentiation is then completed, and the osteoblasts commence secreting osteoid matrix (Bianco and Robey, 1999; Bianco et al., 1991; Bianco et al., 1998; Galotto et al., 1994). Alterations in the extracellular matrix surrounding chondrocytes once the osteoblasts begin secreting osteoid have been shown to impede diffusion of PTHrP between chondrocytes, and result in a decrease in Bcl-2, thereby promoting the apoptosis of
chondrocytes (Yang et al, 1997). Once apoptosis has occurred, osteoblasts invade the cavity left by the chondrocyte, and secrete more osteoid.

The function of the osteoblast is two-fold: firstly, it is responsible for the deposition of bone matrix, and, secondly, it regulates the progress of the bone resorbing cells, the osteoclasts, which are important in bone repair and remodelling. Osteoblasts synthesize bone by secreting a number of proteins which contain motifs capable of binding the mineral phase of bone, hydroxyapatite.

The effects of a complete failure of osteoblasts activity have been demonstrated in a mouse model deficient for CBFA1. CBFA1 is one of only two transcriptional activators of osteoblastogenesis discovered thus far. It is an absolute requirement for the development of osteoblasts in the developing embryo. Otto et al (1997) and Komori et al (1997) generated CBFA1 knockout mice, the most striking phenotypical characteristic of which was the complete lack of osteoblast development, and hence absence of skeletal mineralization. Those skeletal elements normally formed by intramembranous ossification were found to consist of mesenchymal cells in the CBFA1 knockout mouse, and sites of endochondral ossification remained as cartilaginous templates. Although these skeletal elements appeared normal at the gross level, further analysis revealed that the chondrocytes failed to mature fully, revealing an additional role for CBFA1 in chondrocyte development.

The complete lack of mineralized bone tissue in these animals results in their death shortly after birth, caused by suffocation due to lack of skeletal support for
respiration. A heterozygotic form of this gene inactivation in mice has been shown to correspond to a human phenotype called cleidocranial dysplasia. This disorder is characterized by delayed, or absent, suturing of the fontanelles of the skull, and hypoplastic clavicles, both skeletal elements formed by intramembranous ossification (Mundlos et al., 1997). These phenotypic characteristics suggest that both copies of the gene are required to produce normal intramembranous ossification, while one copy is sufficient for endochondral ossification. This may indicate that chondrocytes have some positive feedback mechanism for increasing the amount of CBFA1 available for transcription activation, which is not available in intramembranously-formed elements due to the lack of these cell types, or that the activity of CBFA1 is concentration-dependent in different types of ossification. Further evidence for the importance of CBFA1 in osteoblastogenesis comes from the identification of downstream regulatory targets. Thirunavukkarasu et al. (2000) identified 12 putative CBFA1 binding sites in the promoter of osteoprotegerin, the osteoclastigenic decoy receptor, deletion of which results in almost complete absence of osteoprotegerin expression, thereby strengthening the role of osteoblastic cells as regulators of osteoclastogenesis, and bone remodeling. CBFA1 binding sites have also been identified in the promoters of all the genes expressed by osteoblasts that form the extracellular matrix which undergoes mineralization to form bone. These genes include the type I collagen genes, osteopontin, collagenase-3, bone sialoprotein, and osteocalcin, the most osteoblast-specific gene identified thus far (Ducy et al., 1997; Ducy and Karsenty, 1995, Jimenez et al., 1999; Porte et al., 1999; Sato et al., 1998). Osterix is the only other transcriptional activator of osteoblastogenesis discovered thus far. Osterix is a zinc-finger transcription factor found in all developing bone, and when it is inactivated in a mouse model, results in invasion of mesenchymal cells into
the anlagen, but no mineralisation of either endochondral or mesenchymal skeletal elements. Nakashima et al (2002) observed that Osterix-null mice express CBFA1, but CBFA1-null mice do not express Osterix, causing them to propose that Osterix may occur downstream from CBFA1. The downstream activation targets of osterix are yet to be identified.

Osteocalcin is the major non-collagenous protein of bone matrix (Price and Williamson, 1985; Price, 1988; Hauschka, 1986) secreted exclusively by mature osteoblast cells (Nishimoto and Price, 1980) in an advanced stage of differentiation during mineralization (Ducy and Karsenty, 1995; Stein et al, 1996). As such, it is often used as a marker of both osteoblast maturation and mineralization. Osteocalcin is a 5 kDa calcium-binding protein containing three $\gamma$-carboxyglutamic acid residues that bind divalent cations (Price and Williamson, 1985). The ability of the $\gamma$-carboxyglutamic acid residues to interact with calcium explains the high affinity of this protein for hydroxyapatite, the major mineral component of bone. The role of hydroxyapatite in bone is to provide rigidity and support, allowing a balance between the elasticity afforded by the organic phase of bone, and the sturdiness required by the skeleton. An interesting feature of the hydroxyapatite in bone is that it tends to be organized so that CaCO$_3$ groups are displayed on the surface of the molecule, thereby making them readily accessible when required to act in a buffering role to maintain homeostasis (Neuman and Mulryan, 1967; Bushinsky, 1986). This in turn suggests a role for osteocalcin in the mineralization of bone, through the attraction and binding of mineral salts to the extracellular matrix secreted by the osteoblasts. Experiments examining the effects of osteocalcin deficiency on bone mineralization in osteocalcin knockout mice suggest that bone deposition and mineralization are normal at birth.
(Price, 1988; Desbois, 1995). By several months after birth, increased bone density is observed, and analysis of the mineral phase of bone shows a defect in mineral crystal maturation in the absence of osteocalcin (Boskey et al, 1998). This observation suggests that osteocalcin may act as a regulator of bone formation, ensuring correct assembly of mineral crystals during mineralization, but it is also possible that osteocalcin is involved in bone resorption, by recruiting and mediating the activity of osteoclasts (Lian et al, 1984; Glowacki et al, 1991).

Another protein secreted by osteoblasts into bone matrix shown to interact with osteoclasts is osteopontin. Like osteocalcin, osteopontin has been demonstrated to bind tightly to hydroxyapatite, and it has also been shown that it anchors osteoclasts to hydroxyapatite during the resorption phase of bone remodelling (Reinholt et al, 1990). Another similarity between osteopontin and osteocalcin is that expression of osteopontin is CBFA1 dependent, demonstrated by the lower levels of osteopontin in CBFA1 deficient mice compared with wild-type littermates. In addition, Tsuiji et al (1998) showed that overexpression of CBFA1 results in increased expression of osteopontin in osteoblast-like cell lines.

Osteopontin is a highly phosphorylated and glycosylated protein found in all body fluids and mineralized tissue. Post-translational modification of Osteopontin has also been shown to be necessary for bone resorption. Razzouk et al (2002) found that phosphorylation of serine or threonine residues, or some other post-translational modification of osteopontin was necessary for osteoclastic resorption in vitro, but attachment of osteoclasts required only the RGD-motif of osteopontin, in order to
facilitate integrin-mediated attachment, and not post-translational modification. The role of osteopontin in bone resorption was also investigated by Ihara *et al* (2001). These authors found that while treatment with parathyroid hormone induced bone resorption in wild-type bones, it had no such effect on bones cultured from osteopontin-deficient mice. This suggests a link between osteopontin-induced bone resorption and the bone loss seen in hyperparathyroidism.

Another interesting feature of osteopontin is that it can bind to osteocalcin, which in turn contains binding sites for a number of other proteins expressed by osteoblasts, including osteonectin, fibronectin, and the most abundant protein found in bone, collagen type I. The main role of collagen expressed by osteoblasts into the extracellular matrix appears to be in arranging the other proteins expressed into the extracellular matrix in such a way as to facilitate mineralization. As discussed earlier, hypertrophic chondrocytes express collagen type X. This is structured to form a distinct hexagonal array around the chondrocytes prior to apoptosis. During the phase of bone synthesis directly following angiogenesis, osteoblasts secrete type I collagen into the cavity left by the apoptotic chondrocyte. Type I collagen makes up more than 90% of the total organic phase of bone (Tracey, 1998). It is a trimeric protein consisting of two $\alpha_1$ and one $\alpha_2$ polypeptide chains, trimers of which require the type X collagen array of the chondrocytes as a template for assembly and association (Paschalis *et al*, 1996). Once the type I collagen has been assembled, and the type X collagen has been degraded, collagen type I begins binding osteopontin, which in turn is capable of binding osteocalcin (Denhardt and Noda, 1998). Expression of all these proteins is regulated by CBFA1, while the mature proteins contain calcium-binding sites. Calcium is one of the main components of hydroxyapatite, the mineral phase of
bone. Thus, it is easy to get a general idea of how bone mineralization occurs. The hypertrophic chondrocyte is surrounded by Type X collagen extracellular matrix. This matrix is rearranged by osteoclasts secreting matrix metalloproteases, which rearrange the cartilage and allow VEGF-mediated angiogenesis. Osteoblast precursors enter the anlagen through these newly-grown blood vessels, and, under the control of CBFA1, begin expressing collagen type I, and other matrix proteins. The type I collagen assembles on the type X collagen, which is then degraded, allowing association of type I collagen with osteopontin and osteocalcin. The calcium binding sites contained within the proteins enable the proteins to act as nucleators for mineralization- divalent cations, such as calcium, are attracted and anchored to the protein mesh, which in turn attracts the other ions required for hydroxyapatite formation. These ions attach to form a crystal of hydroxyapatite, and the process of crystal growing continues until the bone is mineralized (Boskey, 1998). The osteoblast, now embedded in bone matrix, either undergoes apoptosis, or becomes an osteocyte, an osteoblast cell embedded in bone thought to measure the stability of the surrounding bone by responding to changes in intracellular hydrostatic pressure, and trigger subsequent repair signalling cascades.

### 1.4.2 Remodelling of the mineralized skeleton

Even once the biomineralization of the extracellular matrix surrounding the osteoblasts is complete, the skeleton is far from being a static system. Rather, it is constantly undergoing remodelling and repair, with old and damaged tissue being resorbed by osteoclasts, and then replaced with new bone, synthesized by osteoblasts. Remodeling of bone can occur in response to a number of triggers, the most dramatic of which is fracture resulting from mechanical insult, but also microdamage caused by
strain being placed on the skeleton by gravitational forces, and changes in the calcium and phosphorus requirements of the body for homeostasis (Turner et al, 1998).

Bowler et al (2001) suggest that recognition of the need for microdamage repair may be triggered by a signalling cascade involving the secretion of extracellular nucleotides. This model of microdamage recognition proposes that damage to bone cells results in an enhanced release of ATP into the microenvironment. The secreted ATP is then thought to bind to P2 receptors, located on the cell surface of both osteoclast and osteoblast cells, resulting in an intracellular signalling cascade leading to increased osteoclastogenesis, possibly via activation of c-fos.

Mature osteoclasts are large, multinucleated cells with anywhere between two and fifty nuclei, although most commonly observed are between ten and twenty nuclei. Osteoclasts become multinucleate as a result of cell fusion, rather then nuclear mitosis. Osteoclasts adhere to the surface of the bone being resorbed by means of a ruffled border, which forms a tight junction between the extracellular matrix of bone, and the plasma membrane of the osteoclast (Suda et al, 1996). It is the secretion of protons, cathepsins, and MMPs across this border that is primarily responsible for the degradation of old bone. (Figure 1.3: Development of the osteoclast). Osteoclasts and osteoclast precursors express high levels of integrins, for which osteopontin, expressed by osteoblasts, is a ligand. Through integrin αvβ3, osteoclasts adhere to the bone matrix through the RGD motif in osteopontin. Other proteins in the bone matrix with similar RGD motifs have also been demonstrated to have a similar role in adhesion, and are likewise capable of mediating interactions through the integrin
Figure 1.3: Development of the osteoclast. The osteoclast develops from a haematopoietic precursor into a myeloid progenitor which, under the influence of M-CSF alone in vitro will develop into a macrophage. When the myeloid progenitor is cultured in the presence of both M-CSF and RANK-L, it develops into an osteoclast.
signalling pathway. Collagen and bone sialoprotein have both been shown to have αvβ3 integrin binding capabilities, and collagen is also able to bind to another integrin, α2β1, also via an RGD motif (Flores et al., 1996; Helfrich et al., 1996). A gene commonly used as a marker of osteoclast differentiation, tartrate resistant acid phosphatase (TRAP) is a phosphatase enzyme capable of removing phosphate groups from osteopontin. It has been shown that dephosphorylated osteopontin is incapable of supporting osteoclast adhesion, suggesting that osteoclast motility and detachment may be regulated by interactions between osteopontin and TRAP. Treatment of cells with bisphosphonates, a group of drugs commonly used to treat osteoporosis, results in a decrease in both TRAP activity, and bone resorption (Moonga et al., 1990). Gene inactivation studies in mice provide further support for a role for TRAP in bone remodelling. Mice with a heterozygous inactivation of TRAP show mild osteopetrosis while homozygous TRAP-deficient mice are defective in endochondral ossification as well, indicating that TRAP is necessary for primary ossification during development (Hayman et al., 1996).

Once osteoclasts have attached to the bone matrix, the plasma membranes of the cells develop an interesting osteoclast-specific phenotype. A ruffled border, a series of invaginations in the plasma membrane, develops on the surface of the osteoclast facing the area of bone to be degraded. This is flanked by the clear zone, or sealing zone, an area of the membrane that forms a tight junction between the bone matrix and the cell (Teti et al., 1991). The cytoplasm near the clear zone tends to be devoid of organelles, but does contain a number of actin filaments, which act as orientation points for a number of adhesion-related molecules, including vinculin and talin. High levels of integrin αvβ3 are expressed on the clear zone portion of the membrane,
probably mediating the adherence of the osteoclast to the underlying bone. The formation of a tight junction between osteoclast and bone matrix is important for maintaining differences in pH between the area of bone to be degraded and the surrounding tissue. One of the main mechanisms used to degrade bone is by generating a proton gradient across the plasma membrane of the osteoclast, resulting in higher pH within the cell than in the underlying bone cavity. This gradient is maintained by a membrane-bound H+/ATPase, known as vacuolar (V-type) electrogenic ATPase. It is homologous to the ATPase used to generate ATP in mitochondria, and generates ATP in the process of pumping protons across the membrane. The exclusion of protons from the cell upsets the balance of charges in the cell, which causes chloride ions to diffuse across the cell membrane in the same direction as the protons via chloride channels through passive diffusion. The supply of chloride ions for diffusion is maintained by a chloride-bicarbonate exchange across the membrane of the cell on the other side of the cell from the ruffled border, while the protons involved in the process are obtained from carbonic acid, formed by the oxidative phosphorylation of CO$_2$, mediated by the osteoclastic enzyme carbonic anhydrase type II.

The net result of these processes is the dumping of a large amount of hydrochloric acid onto the strongly basic hydroxyapatite component of bone. This causes the mineral ions to dissociate, liberating the calcium and phosphate normally contained within bone (Blair et al, 1989; Blair, 1998).
Once the mineral phase has been dissolved, the organic phase must also be degraded. This is done using proteases, in particular Cathepsin K, a lysosomal cysteine protease with optimum function at low pH (Bossard et al., 1996). A Cathepsin K knockout mouse, generated by Gowen et al. (1999) has a phenotype which corresponds to the human disorder Pycnodysostosis, which causes osteosclerosis and short stature (Gelb et al., 1996). In the mouse model, osteoclasts are present in normal numbers, and the morphology of the cell is normal, but only the mineral component of bone is dissolved, leaving the organic phase intact. Another protease enzyme identified as degrading bone is matrix metalloprotease 9 (MMP9). It is expressed by osteoclasts, and cleaves collagens I, III, IV, and V, as well as gelatins.

Once the mineral and organic phases of bone have been degraded, they do not remain in the cavity under the osteoclast. Instead, waste products of bone degradation are engulfed in invaginations of the ruffled border, which pinch off from the rest of the cell, isolating the highly acidic contents from the cytosol. The vesicles are then transported to the other side of the osteoclast, where fusion of the membrane surrounding the transport vesicle results in the release of these products into the extracellular space (Nesbitt and Horton, 1997).

Once the products of bone degradation have been removed from under the osteoclast, the osteoclast either undergoes apoptosis, leaving a cavity to be filled by osteoblasts, or it releases the adherence molecules anchoring the osteoclast to the bone, and moves to a new site, after depositing osteopontin onto the resorption surface of the degraded site (Dodds et al., 1995).
1.5 Diseases of the skeleton - relevance and treatment

When the balance between bone deposition and bone replacement is disrupted, and the rate of bone resorption exceeds the rate of bone replacement, osteoporosis can be the end result.

Osteoporosis is defined as bone mineral density more than 2.5 standard deviations below the mean bone mineral density of young women, while osteopenia, or low bone mass, is bone mineral density between 1 and 2.5 standard deviations below the mean bone mineral density of young women (World Health Organisation, 1994).

Osteoporosis represents a major medical problem worldwide, but particularly in Western countries where an aging population means an increased incidence of this condition, of which age is a key risk factor. Other risk factors include being female, early menopause, family history of osteoporosis, and behavioural risk factors, such as low calcium intake, Vitamin D deficiency, and low body mass (Reviewed in Woolf and Pfleger, 2003). Osteoporosis is often first diagnosed when a patient presents with a fracture resulting from a low-energy impact, such as a fall. The three most common sites for these fractures to occur in are the vertebrae, the hip, and the wrist. Of these three, hip fractures are thought to be the most debilitating, with 20% mortality, and 50% permanent loss of function. Recent studies have also estimated that 54% of postmenopausal women in the USA have osteopenia, and half of all women over 85 in the UK are osteoporotic (Kanis et al, 2000). While osteoporosis is less of a problem in men, it is still significant, affecting 20% of men 85 years or over.
Osteoporosis and osteopenia are most commonly diagnosed by dual emission X-ray absorptiometry (DXA), although diagnostic techniques utilising ultrasound have also been used (reviewed in Blake and Fogelman, 2003). This testing procedure is most commonly performed in post-menopausal women, and is used to identify at-risk patients. Often it is followed by treatment to increase their bone mineral density, to reduce their risk of osteoporotic fracture.

Many osteoporosis patients suffer multiple fractures, and the site of fracture often fails to heal well. Treatment is, therefore, aimed at prevention of fracture by increasing skeletal strength, and prevention of falls, as well as repair of existing fracture sites.

The relationship between osteoporosis and menopause made hormone replacement therapy a popular therapy for many years. Bone resorption increases significantly with the onset of menopause in middle aged women, a phenomenon that is explained by the effects of estrogen on differentiating osteoclasts. At the molecular level, Shevde et al (2000) demonstrated that treating osteoclasts with estrogen or estrogen analogues results in down-regulation of genes which are essential for osteoclastogenesis. In addition, estrogen has been shown to protect against bone loss by promoting apoptosis of osteoclasts, effectively decreasing the amount of bone degraded by decreasing the life-span of the cell (Hughes et al, 1996), while deprivation of estrogen has been shown to cause increased osteoclast development (Jilka et al, 1992). The Women’s Health Initiative study hormone replacement therapy reduced the incidence of osteoporotic fractures in women aged 50-79 by 24% (Rossouw et al, 2002). The benefits of hormone replacement therapy using non-specific estrogen have, however,
been coupled with an increased risk of breast cancer in several studies, making it a less popular course of treatment than it was previously (Fournier et al, 2003).

Other drugs, designed to interfere with the estrogen signalling pathway by binding to the estrogen receptor and blocking conformational change, appear to cause fewer side effects than HRT. A study on the effects of Raloxifene, an example of such a drug, showed that it reduces the risk of vertebral fracture in post-menopausal women with osteoporosis, but failed to show a reduction of risk of fracture at other sites, although it was also found to decrease the risk of breast cancer (Ettinger et al, 1999).

Bisphosphonates are another drug used to treat osteoporosis. These drugs have the effect of binding to hydroxyapatite, and preventing osteoclast activation. Studies have shown that these drugs can greatly reduce both vertebral and non-vertebral fractures, in patients with both diagnosed osteoporosis, and patients who are merely at risk of osteoporosis (Cummings et al, 1998; Black et al, 1996; Harris et al, 1999; Reginster et al, 2000). However, bisphosphonates are poorly absorbed in the gut, require fasting pre- and post-administration, and have been associated with oesophageal erosions, making them a fairly unpalatable option for many patients.

Salmon calcitonin has also been studied as a treatment for osteoporosis. Calcitonin acts by inhibiting osteoclast formation. This treatment was shown recently to reduce vertebral fractures by 33%, but its effects on bone mineral density were minimal, prompting the authors to propose that it may alter the quality of bone deposited, rather than the quantity (Chesnut et al, 2000). An alternative theory about the protective
effects of both calcitonin and bisphosphonates was proposed by Plotkin et al (1999), who observed that bisphosphonates and calcitonin both suppress apoptosis of osteocytes and osteoblasts induced by glucocorticoids.

Parathyroid hormone treatment has also been used to treat osteoporosis. Both osteoclasts and osteoblasts are responsive to parathyroid hormone, with increased bone absorption occurring during long periods of exposure, such as occurs in hyperparathyroidism, but a greatly decreased fracture risk and increased bone deposition occurring in response to intermittent exposure (Neer et al, 2001), as a result of increased osteoblast numbers. A possible mechanism for this phenomenon has been proposed by Bellido et al (2003), who found that parathyroid hormone increases expression of CBFA1, which, as well as promoting osteoblastogenesis, prevents apoptosis of osteoblasts by increasing levels of anti-apoptotic genes. In addition, parathyroid hormone also increases the rate of CBFA1 proteasomal degradation. Thus, intermittent increases in the levels of parathyroid hormone may serve to increase bone deposition by activating anti-apoptotic genes repeatedly, rather than just once as occurs with a sustained increase in parathyroid hormone levels. A recent review on teriparatide, an recombinant form of human parathyroid hormone, indicated that side effects include disrupted calcium levels, nausea, headache, dizziness, and arthralgias (Cappuzzo et al, 2004) while some studies showed that it leads to an increase in osteosarcoma in rats. Thus far, however, not enough is known about its long term side effects to determine its usefulness as a treatment.

Many of these treatments are prescribed in combination with calcium and vitamin D, and supplementation with these two alone is regarded as minimal treatment for low
bone mineral density. Calcium is required for mineralisation of the skeleton: it is a key component of hydroxyapatite, while vitamin D is used to increase the rate of bone turnover. Calcium is required by many systems of the body—thus, calcium homeostasis is tightly regulated. In situations of low calcium, vitamin D is upregulated—it in turn acts on osteoblasts to raise production of RANKL and promote osteoclastogenesis, thus releasing more calcium from the skeleton (Kitazawa et al., 2003). If calcium is administered simultaneously with vitamin D, the rate of bone turnover is still raised, but there is a greater amount of calcium available to be incorporated into the new bone, thus improving the quality of the new bone. However, a study by Cooper et al. (2003) has demonstrated that treatment of women with osteoporosis with vitamin D and calcium provides no greater benefit than treatment with calcium alone if vitamin D serum levels are within the normal range, suggesting that in some cases, the baseline combination therapy of Vitamin D and calcium may not be more beneficial than calcium supplementation alone.

As can be seen from the outline of treatments presently available, there is not really an optimal treatment available for osteoporosis. Many of the treatments currently available have unpleasant side effects, or only indirectly affect bone synthesis, while parathyroid hormone treatment, which actively promotes bone synthesis, can affect other elements of calcium homeostasis. This, combined with the high degree of cellular involvement in osteoporosis, makes it a good candidate disease for cell based therapies, because the disease is not so much caused by a problem with the function of the cells, as an imbalance in the activity of two competing cells types. Furthermore, the cell types involved can be differentiated from and into other abundant cell types, as will be discussed in the next section.
1.6 Differentiation of osteoblast and osteoclast precursors

Osteoblasts arise from a lineage of multipotential mesenchymal cells that also gives rise to adipocytes, chondroblasts, fibroblasts, and myoblasts (Figure 1.1; Figure 1.2)(Aubin, 1998). In vitro, these multipotential mesenchymal cells present as osteoprogenitors with two phenotypes. The first of these types of progenitor cells can be induced to form active bone-forming osteoblasts by culture in the presence of dexamethasone, progesterone, or bone morphogenetic proteins (Ishida and Heersche, 1997; Hughes et al, 1995). The other population of osteoprogenitor cells capable of developing an osteoblastic phenotype do so under standard culture conditions as a default developmental pathway (Turksen and Aubin, 1991), suggesting that these progenitors are either more committed to osteogenic differentiation, or that the other osteoprogenitors may be partially committed to following an alternative developmental pathway, which is reversed by the presence of steroid hormones or BMPs. BMPs are thought to play an important role in mediating the transition of mesenchymal osteoprogenitor cells into osteoblasts. Ectopic application of BMPs into soft tissue leads to bony nodule formation, which can only occur in the presence of fully differentiated osteoblasts. BMP-2 has been demonstrated to have an inhibitory effect on pre-adipocyte maturation (Gimble et al, 1995) while having a stimulatory effect on osteoblasts (Fromigue et al, 1998). Exposure to BMP-2 causes an increase in the expression levels of CBFA1 and osteopontin (Gori et al, 1999).

Interchangeability of developmental fate is not an uncommon phenomenon in mesenchymal cells. Gimble et al (1996) found that it is likely that a bipotential cell phase, with the ability to differentiate into either adipocytes or osteoblasts exists, and
that external agents may alter the commitment pathway of the cells, or even reverse commitment altogether. Similarly, highly differentiated adipocytes have been demonstrated to dedifferentiate to a proliferative fibroblastic state, before adopting an osteoblastic phenotype in certain culturing conditions (Owen, 1998), while osteoblasts expressing marker genes indicative of late osteoblast differentiation have been demonstrated to revert to adipocytes (Nuttall et al, 1998). There are three key molecules which have been implicated as regulating the plasticity of fate between osteoblasts and adipocytes—CBFA1, PPAR, and CEPB.

Wu et al (1996) demonstrated that expression and activation of peroxisome proliferator activated repressor \( \gamma \)1 (PPAR\( \gamma \)1) caused fibroblasts to differentiate into adipocytes, while Tontonoz et al (1994) demonstrated a similar phenomenon with PPAR\( \gamma \)2. Further studies have shown that PPAR\( \gamma \)2 expression can cause osteoblast cells expressing CBFA1 and a number of other osteoblast marker genes to differentiate into adipocytes, by repressing CBFA1 activity, and suppressing the osteoblastic phenotype (Lecka-Czernik et al, 1998).

As well as depositing bone, osteoblasts are responsible for regulating osteoclastogenesis. Osteoblasts secrete a soluble form of a tumour necrosis family receptor called osteoprotegerin, expression of which is controlled by CBFA1 (Thirunavukkarasu et al, 2000) and BMPs (Wan et al, 2001). Osteoprotegerin acts as a decoy receptor to inhibit osteoclastogenesis by binding to Receptor Activator NFκB Ligand (RANKL) (Simonet et al, 1997; Yasuda et al, 1998a; Yasuda et al, 1998b). Osteoblasts also express RANKL on the cell surface, and, for osteoclastogenesis to
occur in vivo, RANKL must bind to RANK on the surface of an osteoclast precursor before the osteoclast will become functional.

Osteoclasts develop from the same haematopoietic lineage as macrophages. In culture, osteoclasts can be derived from monocytes by treatment with Receptor Activated Nuclear factor κ B Ligand (RANK-L) and Macrophage Colony Stimulating Factor (M-CSF). Treatment with M-CSF alone results in differentiation into macrophages (Quinn et al., 1998; Suda et al., 1996; Roodman, 1996).

Gene inactivation studies in mice have implicated a number of genes in the regulation of the progression of osteoclastogenesis. Inactivation of PU1, a transcription factor specific for myeloid and B-cell lineages, causes severe osteopetrosis, with a complete lack of both osteoclasts and macrophages, suggesting that it acts very early along the differentiation pathway of the myeloid lineage (Tondravi et al., 1997). Another gene deletion model which causes an osteopetrotic phenotype, but which appears to operate later in the differentiation process of osteoclasts, is that of the c-src null mutant. These mice are osteopetrotic, due to poor bone resorption and enhanced bone formation (Marzia et al., 2000). C-src null mice have increased numbers of osteoclasts at the surface of bone, but these osteoclasts fail to develop ruffled borders and remain inactive (Boyce et al., 1992; Lowe et al., 1993).

TRAP expression is commonly used as a marker of osteoclast differentiation. Heterozygous inactivation of TRAP in a mouse model causes mild osteopetrosis while homozygous TRAP-deficient mice are defective in endochondral ossification as
well, indicating that TRAP is necessary for primary ossification during development, as well as osteoclast activity during skeletal remodelling (Hayman et al, 1996).

The ability to alter the developmental fate of cell involved in skeletal remodelling makes them a good candidate for cell-based therapies. These observations raise the possibility of pharmacological agents which treat osteoporosis by altering the cells involved. This strategy could operate to increase bone deposition, by increasing osteoblast numbers by repressing PPAR, thus diverting adipocytes into osteoblast development, or other mechanisms designed to transform fibroblasts into osteoblasts. An alternative strategy would be to reduce osteoclast activity, either by decreasing the number of osteoclasts, by diverting undifferentiated cells down an alternative pathway, or by interfering with the activity of those cells, and preventing them from becoming fully activated. The first possibility could present side-effects, as it may result in an increased number of immune cells, which could present other medical problems; the second would require interfering with the differentiation of osteoclasts after differentiation fate has been determined, but before differentiation is completed.

1.7 Research overview and objectives

In order to develop therapies to treat diseases such as osteoporosis by altering cellular differentiation or behaviour, it is necessary to know how the cell types concerned differ from one another. For example- if you are going to treat a disease by converting one cell type, such as a fibroblast, into another cell type, such as an osteoblast, it is important to know a great deal about the molecular differences between the two cell
types. The reasons for performing studies like the one described here are two-fold—firstly, comparisons of the genetic profiles of two cell types may identify genetic pathways better suited to therapeutic intervention than those identified to date; secondly, by identifying other pathways and activities occurring within the cell prior to therapeutic intervention, it may be possible side-effects of candidate treatments may be identified and addressed in the early stages of therapeutic development.

The recent development and refinement of several techniques has made possible the comparative analysis of gene expression between RNA samples isolated from different cell types and tissues, and cells at different stages of differentiation. These techniques, including DNA arrays and subtractive hybridisation facilitate the discovery of genes regulated by different influences, which in turn increases the level of understanding of how cell behaviour and development is regulated. In addition, high-throughput techniques such as these which allow the simultaneous analysis of many different genes produce data which can be used in the design of therapeutic agents, as well as identifying indicator genes which may prove useful as markers of disease when used in genotyping studies.

The current project has been performed to investigate genes important in the differentiation of bone and related cell types. This is a useful model system in many respects, as there is a wide range of relevant starting materials that can be investigated. This includes cells cultured both in vitro and isolated from whole animals, and cDNA stored in phage libraries made from rare cell types. This diversity of starting material allows us to examine the versatility of three techniques to
investigate the molecular basis of bone cell differentiation: DNA microarrays, macroarrays, and subtractive hybridization.

In order to identify key genes expressed by osteoblasts, gene expression in osteoblast cells has been examined by direct comparison with fibroblasts, cells derived from the same developmental lineage. For this comparison, three separate techniques have been used. The first, microarray analysis, relies on differential fluorescent labelling of cDNA isolated from cells of each type cultured *in vitro* and hybridising the labelled cDNAs to a glass chip. The second technique, macroarray analysis, is fundamentally similar to the first, in that cDNA from in vitro cultured osteoblasts and fibroblasts was radioactively labelled and hybridised to two separate identical nylon arrays.

In the third technique, the principle of subtractive hybridisation was modified to isolate osteoblast abundant or specific genes from within a phagemid library. This was done by attaching an excess amount of fibroblast mRNA to magnetic beads using biotinylated oligo-dT, allowing phagemid isolated from the library to hybridise to the fibroblast mRNA, and liberating those phagemid that do not hybridise to the fibroblast mRNA. Non-hybridising, or osteoblast-specific, phagemid were then characterised and sequenced, to identify genes expressed specifically by osteoblasts as compared with fibroblasts.

In order to identify genes expressed predominantly by osteoclasts, as opposed to macrophages, I have repeated the process of macroarray analysis to compare gene expression.
Hence, the specific objectives of this project were:

i) to identify molecules expressed by osteoblasts which may increase or decrease bone synthesis; and

ii) to identify molecules expressed by osteoclasts which may increase or decrease bone resorption.

Overall the aim of this project was to identify genes involved in various stages of bone cell differentiation. By undertaking this project, I have identified a large number of novel genes that have not previously been implicated in bone development. The project was essentially one of gene discovery, and not gene characterization, and for that reason, many different approaches were used to identify novel bone genes. Further characterisation of the genes identified in this project, in particular growth factors and receptors, and transcription factors, is warranted as these may be considered potential therapeutic targets for specific bone disorders.
CHAPTER 2: MATERIALS AND METHODS

2.1 Overview

This project can be split into two separate parts- comparative study of osteoblasts and fibroblasts, and comparative study of osteoclasts and macrophages.

For the comparative study of osteoblasts and fibroblasts using DNA arrays, cells were isolated and cultured by collaborators at the University of Sydney. The cells were then lysed with guanidium thiocyanate, and the RNA was isolated and used to synthesise cDNA. The two populations of cDNA were then labelled with fluorescent and radioactive labels, and hybridized to glass and nylon DNA array chips containing identified cDNA samples in known locations, allowing positional analysis of genes expressed by each cell type.

In addition to doing microarray analysis on osteoblasts, we developed a technique to enrich a phagemid library containing cDNA isolated from foetal osteoblast cells for osteoblast specific transcripts. We hypothesised that this technique would allow us to detect transcripts which were expressed at levels beyond the sensitivity range of the arrays, or transcripts simply not represented on the arrays. We already possessed a library of cDNA expressed by foetal osteoblast cells contained within a phagemid vector, which preliminary characterization suggested contained many housekeeping gene clones. Therefore, we wished to enrich it for osteoblast specific or abundant
gene transcripts. This was done by expressing a portion of the library as single-stranded circular DNA, and passing it through a matrix of oligo-dT labelled beads attached to polyA mRNA from fibroblasts. Non-hybridising phagemid were then eluted from the matrix and transformed into bacteria, where they were converted to plasmid. After plasmid isolation, the clones were sequenced, and their identity determined by comparison against the NCBI database.

A comparison of differential gene expression between osteoclasts and macrophages was done by culturing cells from peripheral blood mononuclear cells by the addition of RANK-L and M-CSF, or M-CSF alone, respectively. Once cultured, RNA was isolated from the cells, and cDNA synthesized from the two cell populations. The two cDNA populations were then radioactively labelled separately, and hybridized to nylon DNA arrays. Positional analysis was performed to determine the identity of hybridizing genes.

2.2 Materials

A list of materials used, and their suppliers, can be found in Appendix 1.

2.2.1 Media

All bacterial growth media were prepared using Milli-Q® water, and prepared media were sterilized by autoclaving. When required for selection of transformants, ampicillin (100µg/ml) or tetracycline (50µg/ml) was added after the sterilized media
had cooled to 50°C. DH5α cells were cultured using LB media and plates, while NZY media and plates were required for XL1-Blue MRF’ and XLOLR strains. Plates were made by making the media according to the manufacturers instructions, and then adding 2% w/v bacteriological grade agar prior to autoclaving. Where required, the media were supplemented with maltose and magnesium sulfate. SM buffer, used to culture the phagemids, was made as follows: 5.8g NaCl, 2g MgSO₄·7H₂O, 50 ml 1M Tris-HCl (pH7.5), 5ml 2% (w/v) gelatin, to final volume 1L with Milli-Q® H₂O, prior to autoclaving. The genotypes of all bacterial strains used in this research are shown in Table 2.1.

Minimal Essential Medium, supplemented with Earle’s salts, L-glutamine, and non-essential amino acids, without sodium bicarbonate, supplied in powdered form by GiboBRL was used to culture osteoclasts and fibroblasts. Dulbecco’s Minimum Essential Medium, with high glucose, 25mM HEPES buffer, pyridoxine.HCl, without L-glutamine or sodium pyruvate, also supplied by GibcoBRL, was used to culture fibroblasts and osteoblasts. Powdered media were diluted in Milli-Q® water and then filter sterilized, according to the manufacturers’ instructions.

Table 2.1: Genotypes of all bacterial strains used in this research.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>sup E44, Δlac U169, (●80 lacZΔM15) hsdR17, recA1 endA1, gyrA96, thi –1, relA1</td>
</tr>
<tr>
<td>XL1-BlueMRF’</td>
<td>Δ(mcrA) 183 ΔmcrC hsdSMR-mrr 173 endA1 supE44 hi-1 rec A</td>
</tr>
<tr>
<td></td>
<td>gyr 96 relA1 lac [ F’ proAB lac lº ZΔM15 Tn 10 (Tet’)]</td>
</tr>
</tbody>
</table>
2.3 Methods

2.3.1 Culture of cells and preparation of RNA

Osteoblasts and fibroblasts used for the DNA array experiments and real-time PCR were prepared by the Bone and Skin Research Group, Department of Physiology, University of Sydney, Australia.

2.3.1.1 Culture of fibroblast cells in vitro

Fibroblasts were prepared from foreskin samples collected in 10mls of serum-free media, before being washed with 70% ethanol. The samples were then washed in D-PBS, first with penicillin-streptomycin, then without penicillin-streptomycin. The samples were then trimmed of subcutaneous tissue, and dissected into 3mm fragments, which were then washed with Betadine, and added to 0.1% dispase in D-PBS for overnight incubation at 4°C. The following day, the epidermal layers were removed from the tissue sample, placed in trypsin/EDTA, and incubated at 37°C for 15 minutes, after fractionation of tissue sample using a pipette. The trypsinised cell reaction was then transferred to a centrifuge tube containing an equal volume of calcium-supplemented media, and centrifuged at 2000 rpm for 8 minutes at room temperature. The pelleted cells were then plated in fibroblast growth media, and
incubated at 37°C until confluent (Method adapted from Dissanayake et al, 1993). The cells were then separated and cultured in DMEM media supplemented with penicillin/streptomycin for a week, until four flasks (25cm²) of high confluency (approx. 80-90%) were obtained. RNA was then prepared from the cells as outlined in 2.3.1.4.

2.3.1.2 Culture of osteoblast cells in vitro

Fetal osteoblasts were prepared by isolating trabecular bone from whole bone samples, and dissecting the trabecular bone into 3mm fragments. The fragments were then added to DMEM, and vortexed for 30 seconds. The media was then discarded, and the fragments washed and vortexed in fresh media a further two times, before the fragments were added to a tissue culture flask containing fresh DMEM. The cells were then incubated at 37°C until confluent (Adapted from Slater et al, 1994).

2.3.1.3 Culture of osteoclasts and macrophages in vitro

Peripheral blood mononuclear cells were isolated from 50mls of whole blood taken in EDTA-coated vacuette tubes. Blood was then divided between four 50ml tubes, and made up to final volume of 30mls using PBS. Ficoll-Paque (15mls) was then layered underneath the blood/PBD mixture in each of the tubes, and the tubes were then centrifuged at 2300rpm for 30 minutes. The serum was then discarded, and the white cell layer divided between four fresh 50ml tubes, and each tube made up to a final volume of 50mls with PBS. The tubes were then centrifuged at 1000rpm for 10 minutes. The supernatant was discarded, and the pellets resuspended in 10mls PBS. The resuspended cell fractions were then pooled, and distributed between six 25cm²
tissue culture flasks. After being left to adhere for 2 hours at 37°C, the cells were washed with 10mls of supplemented MEM. After draining, the cells were cultured in 10mls of supplemented MEM, with 25ng/ml M-CSF, for macrophages, or 25ng/ml M-CSF and 20ng/ml RANK-L for osteoclasts. The cells were incubated at 37°C for three weeks, with replacement of media supplemented with M-CSF, or M-CSF/RANK-L occurring every 3-7 days. At the end of three weeks, the cells were lysed, and the RNA harvested.

2.3.1.4 Lysis of cells and recovery of RNA from cultured cell populations
The cells were resuspended in 2.5ml 4M Guanidium Thiocyanate 0.1M Tris-Cl (pH 7.5) 1%β-mercaptoethanol. The suspension was then sheared using a 23-g needle, and diluted with sodium lauryl sarcosine to a final concentration of 0.5%. The lysate was then layered onto a 5.7M CsCl, 0.01M EDTA (pH7.5) gradient, before centrifugation at 40,000 rpm overnight at 20°C. The RNA pellet was then resuspended in TE (pH7.6) 0.1% SDS, and precipitated using 3M sodium acetate (pH5.2) and 100% ethanol. The RNA was incubated at 0°C for thirty minutes, and then centrifuged at 27000xg for ten minutes at 4°C. The remaining pellet was then drained and resuspended in H2O.

2.3.2 Preparation of labelled probes, and hybridisation of probes to arrays

2.3.2.1 cDNA synthesis from isolated RNA
The RNA was then converted into cDNA by adding 3.2pmoles final concentration of 16mer dT cDNA synthesis primer to 2µg RNA, and incubating it with 200 units of
Superscript version 2 reverse transcriptase, 5μM dNTPs, and 5x First-strand synthesis buffer (50mM Tris-HCl, pH8.3, 375mM KCl, 15mM MgCl2, 50mM DTT) in a final volume of 20μl at 42°C for 1 hour.

2.3.2.2 Preparation of probe for ATLAS macroarrays

Probe was prepared by adding 5μg of cDNA to a reaction mix containing 4μl of 5xRT reaction buffer (minus DTT), 1μl 100mM DTT, 2μl of each CDS primer, 0.5μl 13-mer primer (AAGCTTTTACCGC), 1μl 100μM dNTP’s (no C), 0.1 μl 100μM dCTP, and 6.7μl MQ H2O.

This reaction mix was denatured at 94°C for 3 minutes, and then cooled to 42°C prior to the addition of 1μl reverse transcriptase. The reaction was incubated at 42°C for a further 15 minutes, before 3.5 μl of dCTP-α32P (10,000Ci/mMol) and 1 unit of Taq polymerase was added. The reaction was again denatured at 94°C for 3 minutes, primed at 42°C for two minutes, and extended at 65°C for 1 minute. Thermocycling was then performed for twenty cycles, each consisting of a 30 second denaturation step at 94°C, followed by a 42°C priming step for 1 minute and a 65°C extension step for 1 minute. A final 5 minute extension step at 72°C concluded labelling of the probe.

Unincorporated radioactive nucleotide was removed from the probe by separation through a G-50 Sephadex Nick Translation column (Amersham Pharmacia). The probe was eluted in TE buffer, and the second 400μl fraction eluted from the column
was collected. One µl of each probe was taken for scintillation counting to assess the specific activity of the probe. A further microlitre of the probe was also run on a 6% acrylamide gel to ensure that the size range of cDNA’s labelled was suitable for hybridisation (> 200 bps).

The probe was then boiled for 5 minutes, and added to 10 mLs of hybridisation solution (0.75M NaCl, 0.145M Na_2HPO_4, 25mM NaH_2PO_4, 2mM Na_4P_2O_7.10H_2O, 2%SDS, 5× Denhardt’s solution, 0.15M Tris-Cl pH 7.5, 4µg sheared herring sperm DNA) in which the ATLAS arrays had been pre-hybridised overnight at 42°C.

The probe was then hybridised to the arrays in a rolling drum hybridisation oven at 42°C overnight. The arrays were then washed in 0.2L of 2× SSC (0.3M NaCl, 3mM C_6H_5Na_3O_7.2H_2O) for 5 minutes. The filter was removed from the hybridisation cylinder and placed in a plastic tub with 0.2L of wash buffer 1 (2× SSC, 1% SDS) pre-warmed to 68°C and washed for 10-30 minutes. This was repeated until 1L of solution was used, disposing of used buffer between washes. The filter was then washed with 0.2L of wash buffer 2 (0.6× SSC, 1% SDS) pre-warmed to 68°C for 10-30 minutes. This was repeated until 1L of solution was used, disposing of used buffer between washes.

The washed filter was wrapped in plastic or placed between two screen protecting films and placed in light excluding cassette with a phosphor screen overnight. The phosphor screen was developed using a personal molecular imager and the results were viewed and quantified using Quantity One software, and analysed using Microsoft Excel.
2.3.2.3 Preparation of probe for 19k microarrays

RNA recovered as per 2.3.1 was quantitated using a spectrophotometer, and equal amounts of RNA from each population being compared set aside, approximately 80µg. The RNA was diluted into a final volume of 100µl, and an equal volume of chloroform was added, before mixing and centrifugation at 12,000 rpm for 2 minutes. The upper aqueous layer was then extracted, and placed in a fresh tube with 10% volume 3M Na acetate pH 5.2, and 2.5× volume ice-cold 100% ethanol. After mixing, centrifugation was repeated for 20 minutes at 4°C at 27000×g. After draining the ethanol, the RNA pellet was allowed to air-dry on ice, before being resuspended in 19µl of water. To the resuspended pellet 3µl of 2µg/µl oligo dT (anchored) primer was added. This mixture was then heated at 70°C for ten minutes, before being placed immediately on ice. To this mix was added 2µl 20×dNTPs low C, 3µl of either Cy5 or Cy3, depending on which population of RNA was being labelled with each probe, 10 µl 5x reaction buffer, 1µl 0.1M DTT, and1.5µl superscript. This reaction was then incubated in the dark at 42°C for 30 minutes, before the addition of a further 1µl of superscript, and further incubation, again in the dark, for an hour.

The labelling reaction was then stopped by the addition of 4µl 0.5M EDTA (pH 8.0), and 2µl 10M NaOH, followed by incubation at 65°C for 5 minutes. The reaction was then neutralized by adding 4µl 5M Acetic acid and 51µl 100% isopropanol, and incubated on ice for 10 minutes.
The labelled probe was then precipitated by centrifugation at 14000rpm for 10 minutes at 4°C. After washing the pellet with 70% ethanol, the probes were resuspended in 10µl human Cot-1 DNA (1µg/µl) 2µl Poly dA (10µg/µl) and 40µl hybridization buffer. The probes were then incubated at 45°C for 10 minutes, before being pooled and spotted onto the microarray coverslip, which was then placed on the microarray, ensuring the removal of any visible bubbles. The microarray was then placed in the hybridization chamber, along with 20µl of water in the humidity reservoirs, and the chambers sealed, before being submerged in a 65°C waterbath in the dark overnight.

The following day, the chamber was retrieved from the water bath, and the microarrays washed by gentle shaking in filter-sterilised 1xSSC heated to 65°C for 10 minutes in the dark. The array was then transferred to a wash of filter-sterilised 1xSSC 1%SDS heated to 65°C, and shaken in the dark for a further 10 minutes. The step was repeated three times, before another 10 minute wash in filter-sterilised 1x SSC heated to 65°C. The array was then dipped in MQ water, and placed in a clean dry tube, and centrifuged at 1000xg for 5 minutes. The microarray was then transferred to another clean tube for transportation to the array scanner.

The arrays were scanned, and the images were analysed using Imagene software. All target genes contained within the array were duplicated. Data from Imagene was then exported into Genespring v6.1, where it was normalised using Lowess normalization, and filtered to remove data which was unreliable due to low expression levels. A 5-fold or greater limit was set to identify potentially interesting genes.
2.3.3 Enrichment and analysis of phagemid library

2.3.3.1 Titration of the primary phagemid libraries

An overnight culture of XL1BlueMRF’ cells in 50mls of LB supplemented with 0.2% maltose and 10 mM MgSO$_4$ was established and grown at 30°C with shaking. A 1/100 dilution of the overnight culture in another 50ml of supplemented media was established the following day, and incubated at 37°C with shaking until an OD600 of 1 was obtained. The cells were then diluted to an OD600 of 0.5 using fresh LB. Serial dilutions of the original phage library in SM buffer between $10^{-2}$ and $10^{-6}$ were made. To 1 µl of each serial dilution of phage, 200µl of cells were added, and the resulting cultures incubated at 37°C for 15 minutes. To each culture, 3 ml of top agar were added, and the cultures plated on NZY plates, which were incubated overnight. The number of plaques formed was counted, and used to determine the concentration of the library based on the serial dilutions.

2.3.3.2 Titration of the helper phage

Helper phage was diluted in TE buffer at concentrations between $10^{-4}$ and $10^{-7}$. A microlitre from each dilution was added to 200 µl of XL1BlueMRF’ cells cultured to concentration at OD$_{600}$ of 1. The cells and phage were incubated at 37°C for 15 minutes, and 3 ml of top agar added and poured onto NZY plates. The plates were
incubated overnight at 37°C, and the number of plaques produced used to calculate the titre of the helper phage.

2.3.3.3 Excision of single-stranded phagemid DNA

Overnight cultures of XL1Blue MRF’ cells in NZY media supplemented with 0.2% maltose and 10mM MgSO₄ were grown at 37°C with vigorous shaking. These cells were pelleted, and resuspended in MgSO₄ to a final concentration at OD₆₀₀ of 1, or 8×10⁸ cells per mL. In order to excise a total of 5×10⁵ pfu from the primary library, phagemid from the library were added to the XLBlue MRF’ cells at a multiplicity of infection of 1:10 phage to cells. ExAssist helper phage were added at a ration of 1:1 helper phage to cells. The phagemid titre of the primary library used was 1.42×10⁸ pfu/mL, therefore this experiment required adding 37.5 μl of the primary library to 1.64 μl of ExAssist helper phage (titre 6.1×10¹⁰ pfu/ml) and 10 ml of XL1Blue MRF’ cells at 8×10⁸ cells/ml. The cells and phagemids were then incubated at 37°C for 15 minutes without shaking to allow the phage to adhere to the cells. NZY broth (20mls) was added to the culture, and incubated for a further three hours with shaking at 37°C. The cultures were then heated at 70°C for 20 minutes, and the cellular debris pelleted by centrifugation at 1000×g for ten minutes. The supernatant was removed and retained, and 200 μl of 10%PEG in 4MNaCl per 1.2 ml of supernatant was added. The supernatant was then vortexed and centrifuged at 12,000 rpm for 2 minutes. The supernatant was then drained off, and the remaining pellet resuspended in 150 μl of TE buffer per 1.2 ml of initial supernatant.
2.3.3.4 Subtraction of fibroblast-common cDNA clones from library

The fibroblast RNA was heated at 65°C for 10 minutes prior to the addition of 3µl of Biotinylated oligo dT probe, and 13µl of 20×SSC. The mixture was cooled slowly to room temperature. The streptavidin magnetic beads were washed three times in 0.3ml 0.5×SSC by flicking the tube, capturing the beads using the magnetic stand, pipetting off the old supernatant, and resuspending the beads in replacement supernatant, before final resuspension in 100µl of 0.5×SSC. The cooled primer/RNA mix was then added to the beads, and incubated at room temperature for 10 minutes, with mixing by inversion every two minutes. The beads were then captured using the magnetic stand, and the supernatant removed. The beads were then washed four times in 300µl of 0.1×SSC. The single-stranded osteoblast phagemid DNA (2.3.6) was then added to the beads, along with 300µl of 0.1×SSC, and incubated at 42°C for one hour with gentle shaking. The beads were then captured, and the supernatant reserved. The column was washed with 300µl of 0.1×SSC, and the supernatant pooled with that eluted immediately after incubation, to constitute the osteoblast-specific portion of the original library.

2.3.3.5 Production of chemically-competent *E.coli* cells for transformation

A single colony of *E. coli* DH5α cells was used to inoculate 2ml of LB and was grown overnight at 37°C with shaking. The following day, 330µl of the overnight culture was subcultured into 10 ml of LB, and grown until it reached OD₆₀₀ of 0.6. A
A total of 5µl of the subtracted library was transformed into 500µl of chemically competent cells. The library was added to the cells, which were placed on ice for 30 minutes, heat-shocked at 42°C for 2 minutes, and then incubated on ice for a further minute. The cells were then added to 4ml of LB, and incubated for one hour at 37°C. The cells were then centrifuged at 12000 rpm for 2 minutes, and the pellet resuspended in 500 µl of LB. Aliquots of 100µl were then plated out on 20cm diameter plates containing LBAmpl agar, and incubated overnight at 37°C.
Colonies were picked individually from the plates, and used to inoculate 2 ml of LBAmp media. The cultures were incubated overnight at 37°C with shaking. The following morning, 100µl of the cultures were added to an equal volume of 30% glycerol and frozen at –80°C to make stocks. The remaining cultures were centrifuged at 12000rpm for 2 minutes to pellet the cells, and drained. The cells were then resuspended in 100µl of TE buffer. After vortexing, 200µl of solution 2 (0.2 M NaOH, 1% SDS) was added, and mixed by inversion. A further 150µl of solution 3 (60 ml 5M potassium acetate, 11.5 ml glacial acetic acid, 28.5ml H2O) was then added, and mixed prior to centrifugation at 12000rpm for 5 minutes. The supernatant was transferred to a fresh tube, and two volumes of ethanol were added. The plasmid DNA was then precipitated out of solution by centrifuging at 27000×g for 5 minutes. The DNA pellet was then washed with 70% ethanol, and allowed to dry, before being resuspended in TE buffer.

2.3.3.8 Restriction digestion of plasmid DNA

To identify plasmids containing insert, 5µl of the resuspended DNA was digested by restriction enzyme. In addition to the 5µl of DNA, 2µl of restriction buffer, 1 unit of Rsa1, and 12.5µl of H2O were incubated at 37°C for 2 hours. Of the resulting digestion, 10 µl were added to 2µl of 6×loading dye, and the 12µl run on a 1% agarose gel run at 80Volts for 3 hours.

2.3.3.9 Preparation of plasmid DNA for sequencing insert-containing clones
Plasmids from clones identified as containing insert were prepared using the Eppendorf Direct bind plasmid preparation kit in a 96-well format. In this protocol, 1.25 mls of culture were grown in ampicillin-supplemented LB in a 2ml 96-well tray for 36 hours. The tray was then centrifuged in a plate centrifuge at 1900xg for 5 minutes. The supernatant was drained off, and the cells resuspended in 300µl of buffer 1. After the addition of 400µl of buffer 2, the lysates were mixed, before the addition of 400µl of buffer 3. The plate was then sealed with a plate sealer, and inverted five times. The lysate from each cell of the tray was then transferred to the corresponding cell of tray DB, positioned over tray A in the vacuum manifold used with the kit. A vacuum was applied for five minutes. The effect of this particular step is to draw all the lysate through to the bottom column, leaving the protein debris precipitated by the addition of buffer 3 in the upper tray. Tray DB was then removed from the manifold, and tray A was placed on top of the original 2ml culture tray, and both were replaced in the manifold. A further 400µl volume of binding buffer was added to the cells of tray A, and drawn through under vacuum for 2 minutes. Following the addition of 400µl of wash buffer, the vacuum was reapplied for five minutes. The culture tray was then removed from the manifold, and replaced by a collection plate. The underside of plate A was blotted on paper, and replaced in the manifold. The DNA was eluted by adding 70µl of molecular biology grade water to each cell, incubating for two minutes, and then applying the vacuum for a further 5 minutes. Sequencing was performed on these plasmids by using 11µl of plasmid prep, following the procedure outlined in 2.3.3. Chromatograms obtained from the sequencing gel were then used to identify the DNA contained in the plasmid using the NCBI and TIGR databases.
2.3.3.10 Sequencing

The volume of DNA used in a sequencing reaction varied greatly, depending on the concentration of the DNA in solution, but within the range of 5-11µl of purified DNA. Sequencing reactions also contained 1µl of sequencing primer, and 8µl of terminator ready reaction mix, in a final volume of 20µl. This reaction was cycled as follows:

Cycle 1: 94°C for 1 minute (1×)
Cycle 2: 94°C for 10 seconds, 50°C for 10 seconds, 60°C for 4 minutes (26×).

At the end of the sequencing cycle, the product of the reaction was precipitated by adding 2µl of 3M sodium acetate pH5.2, and 50µl of 100% ethanol, and centrifuged at 27000×g for 20 minutes. The pellet was then washed with 70% ethanol, and left to air-dry overnight, before being sent off-campus for fluorescent sequencing.

2.3.4 Real-time PCR

2.3.4.1 Design of primers for real-time PCR

The sequence information obtained was then used to design primers suitable for real-time PCR analysis. The DNA sequence of the entire gene, including intron/exon boundaries (where known) was downloaded from the BLAT (genome.ucsc.edu) database, and primers to amplify products of between 70-150 basepairs of cDNA product, with an annealing temperature of 57°C, were designed by eye (Table 2.2).
Where possible, these primers were designed to be intron-spanning, to identify and genomic DNA contamination within the cDNA stocks.

Table 2.2: Sequences of primers used for sequencing and real-time PCR amplification of DNA.

**T7 sequencing primer (forward) (osteoblast library): ATTAACCCTCACTAAAG**

**APP forward real-time primer: ACATGCAGTGAGAAGAGTACC**

**APP reverse real-time primer: TGCTCCGCCACCACCAGACAT**

**CTGF forward real-time primer: CAGGCGTTGCGAAGCTGAC**

**CTGF reverse real-time primer: TCTTCATGCTGGTGAGCCCA**

**18S forward real-time primer: CTTAGAGGGACAAGTGCG**

**18S reverse real-time primer: ACGCTGAGCCAGTCAGTGGA**

**c-jun forward real-time primer: GCGTTAGCATGAGTTGGCAC**

**c-jun reverse real-time primer: CGCATGAGGAACCGCATCGC**

**Calmodulin forward real-time primer: GGCATTCCGAGTCTTTGACAA**

**Calmodulin reverse real-time primer: CCGTCTCCATCAATATCTGCT**

**CathepsinK forward real-time primer: TGAGGCTTCTCTTGGTGATG**

**CathepsinK reverse real-time primer: CATACTGTGTCTTCTTGGGA**

**CD44 forward real-time primer: TGGCACCGCTATGGCG**

**CD44 reverse real-time primer: GTAGCAGGGATTCTGCTG**

**CDK7 forward real-time primer: ATGGCTCTGGACGTGAAGTC**
CDK7 reverse real-time primer: CTTAATGGCGACAATTTGGTTG
CSF2Rα forward real-time primer: GGCACGAGGCGAGAGAAGA
CSF2Rα reverse real-time primer: ACGCAAACATCGCCGCTTCT
CTR forward real-time primer: TGGTGCCAACCACTATCCATGC
CTR reverse real-time primer: CACAAGTGCCGCCATGACAG
FBP forward real-time primer: CATGGCGATGGACCCGGGA
FBP reverse real-time primer: AGGTTCGTCAGCACCGATGT
GABPα forward real-time primer: AAAGAGCGGCGAGGATTTCAG
GABPα reverse real-time primer: CCAAGAAATGCAGTCTCGAG
GABPβ forward real-time primer: CCCAGAGAGTCTGACACT
GABPβ reverse real-time primer: TCTGAAGAATTGGACAATGG
NF90 forward real-time primer: AGGCCTACGCTGCTTTCTCTGC
NF90 reverse real-time primer: GCCGAAGCCAGGGTTATGTG
NFATc forward real-time primer: GCATCACAGGAAGACCGTGTC
NFATc reverse real-time primer: GAAGTTCAATGTCGGAGTTTCTGAG
TRAP forward real-time primer: GACCACCTTGCAATGTCTCTCTG
TRAP reverse real-time primer: TGGCTGAGGAAAGTCAATCGGAGTTCTTTG
ZFP 33a forward real-time primer: AAGATGTGACTGTGGGCTTCA
ZFP 33a reverse real-time primer: GATCACCTCTGGTTTGTGAAC

2.3.4.2 Optimisation of primers for real-time PCR
Primers were optimized by establishing Magnesium chloride gradients for each primer pair. PCRs were set up containing 2µl of Magnesium chloride between 15mmol and 35mmol, 2µl of 10x PCR buffer, 1. 2µl 2.5µmol dNTPs, 4µl of 10 µg/µl genomic DNA, 1µl of each 5µM primer, 0.5µl SYBR green, and 0.1µl Taq polymerase, in a final volume of 20µl. The reactions were then cycled as follows: Cycle 1: 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute(5×); cycle 2: 94°C for 20 seconds, 55°C for 30 seconds, 72°C for 30 seconds(35×); cycle 3: 72°C for 5 minutes, 4°C.

Following cycling, 10µl of the product was run on a 12% polyacrylamide gel to identify the magnesium chloride level producing optimum amounts of product with minimal amounts of primer dimer. Once identified, subsequent real-time PCR experiments on cDNA were performed at that magnesium chloride concentration. Primer concentrations at the optimum MgCl₂ concentration were then decreased by 0.5mM increments until primer dimer was abolished.

2.3.4.3 Real-time PCR

Real-time PCR was then performed at the optimal MgCl₂ and primer concentrations, substituting 2µl cDNA for the 2µl gDNA used in the optimization step, and adding 0.2µl of F-cal to each reaction. A dilution series of cDNAs was used to test for linearity of response using standard curves between 10 and 10⁹ copies of cDNA. Each cDNA reaction was set up in triplicate, along with a genomic DNA control, and a DNA-free water control. PCR products were analyzed by polyacrylamide gel
electrophoresis (PAGE) to verify the specificity of amplification and exclude the possibility of genomic DNA contamination. Similarly, melt curve analysis was performed to ensure that the products detected by the real-time PCR machine were PCR product, and not primer dimer. Results from the housekeeping gene were used to correct for the amount of cDNA present in each reaction.

The fold difference in gene expression between samples was calculated using the cycle threshold (Ct) obtained from the amplification curves of the real-time PCR. The formula used to calculate this is: $\text{Fold difference} = 2^{\Delta \Delta Ct}$ (Livak and Schmittgen, 2001). Correlation of Q-PCR results to array results was performed using SPSS statistical analysis software.
CHAPTER 3: RESULTS PART 1: SUBTRACTIVE
HYBRIDISATION STUDIES

3.1 Introduction

While DNA arrays are useful for identifying known genes or ESTs differentially expressed between cell types, a limitation of these experimental systems is that they are limited to identifying differentially regulated transcripts that are contained within the cDNA library printed onto the array. In many cases, the success or failure of an array experiment designed to identify a single key regulatory molecule may depend on something as arbitrary as array selection. For this reason, techniques such as subtractive hybridization have been developed.

Subtractive hybridization is the process used to enrich a library of cDNA transcripts for transcripts specific to the cell type the library was made of, by removing transcripts common to both that particular cell type, and the other cell type used as the subtractor. Usually this results in identification of genes that are expressed at significantly higher levels in one specific cell type during differentiation.

The earliest reported techniques of subtractive hybridization were described in the 1980’s (Hedrick et al, 1984; Sargent and Dawid, 1983; Davis et al, 1984). After further refinement, the technique was commercialized by Clontech as the PCR-select cDNA Subtraction kit. According to the protocol supplied with the kit, 0.5-2 µg
polyA+RNA from two different cell populations are converted to cDNA, and then separately digested with Rsa I. The digested fragments from the population specific transcripts are sought from are then divided into two subgroups, each of which is ligated to a different adaptor molecule. Each subpopulation is then hybridized to the cDNA fragments derived from the RNA of the alternate cell population, or subtractor population. After this initial hybridization, the two hybridization mixes are pooled, and further hybridized. A PCR performed on the hybridized cDNAs, using primers specific for the two adapter molecules is supposed to amplify only cell-type specific transcripts, as these should, in theory, be the only transcripts with both the required adaptor molecules (common transcripts are expected to hybridise with the subtractor population cDNAs) (Figure 4.1). These PCR products are then cloned into a vector, and transformed into *E. coli* for plasmid isolation and sequencing.

A limitation of the commercial kit is that it requires RNA as the starting material, and at the time we commenced this experiment, we had no access to osteoblast cells to make cDNA from, only to a phagemid library containing cDNA isolated from foetal osteoblast cells. The original foetal osteoblast library was made by cloning cDNA fragment derived from foetal osteoblasts into the multiple cloning region of the pAD-GAL4 phagemid vector, which can be used in bacteria, yeast, or eukaryotic cells, and can produce single-stranded anti-sense phagemid DNA. Therefore, in light of these considerations, a novel method for enriching our pre-existing library for osteoblast-specific or prolific transcripts was developed.
Under the method developed here, a pre-existing library contained in a phagemid-competent host was used to create a single-stranded antisense phagemid library. The antisense library was then hybridised to an excess amount of subtractor mRNA, attached to magnetic beads, which were then captured, leaving phagemid containing inserts specific to the library species in the supernatant. These free phagemid were transformed into *E. coli*, and used as an enriched library (Figure 3.2).
Figure 3.1: Traditional method of creating a subtracted cDNA library. The DNA to be subtracted is divided into two subpopulations, each of which is restricted with a blunt-cutter enzyme, and ligated to a different adaptor molecule. Each population of adaptor-ligated DNA is then hybridized to subtractor DNA. The hybridized pools are then pooled, and allowed to hybridise to each other. The resulting mix is used as a template for a PCR containing primers directed against each of the adaptor molecules which results in the amplification of only hybridization products with both of the two adaptor molecules, specific to the DNA subpopulation of interest.
Phagemid containing insert common to fibroblasts and osteoblasts is also trapped by the magnet through hybridization to the fibroblast magnetic bead complex.

Phagemid containing insert specific to osteoblasts is not trapped, and so is eluted from the column, ready for transformation into E. coli.

**Figure 3.2: Diagram of modified method of enriching an osteoblast cDNA library using a phagemid library as starting material.**
Fibroblasts were selected as the population of cells against which the osteoblast would be subtracted for two reasons: firstly, fibroblasts are relatively easy to grow in culture; and secondly, fibroblasts are of the same cellular lineage as osteoblasts—indeed, fibroblastic development is the default pathway of osteoblastic precursor cells grown in vitro without the appropriate supplements.

### 3.2 Results

The initial titre of the phage library was $1.422 \times 10^8$ plaque forming units per ml (pfu/ml), while the initial titre of the helper phage was $6.1 \times 10^{10}$ pfu/ml. 1 mg of total RNA was isolated from fibroblasts to use as the subtractor, against $5 \times 10^5$ pfu of single-stranded phagemid.

Transformation of 5 µl of the subtracted phagemid into 1 ml of chemically competent DH5α cells (at $10^8$ competency) resulted in 1800 clones. Rsa I digestion showed that approximately 90% of all clones from the subtracted library contained one of several contaminating plasmids of unknown origin. By screening the original library, it was discovered that approximately 10% of the original library, which was made by a company based overseas, contained the contaminating plasmids. The enrichment of the contaminating plasmids in the process of the subtraction had a hidden benefit, because while approximately 90% of all colonies of the subtracted library screened were the wrong vector, the fact that the plasmid contaminants accounted for only 10% of clones in the original unsubtracted library verified that the process really had enriched the library for non-fibroblast-expressing transcripts (Figure 3.3).
Figure 3.3: Screening digest of plasmids isolated from enriched foetal osteoblast cDNA library. Plasmids were isolated from colonies grown on selective media, and restricted with Rsal. The resulting digest was run on a 1% agarose gel with 1kb+ markers (lane M). Plasmids from lanes with restriction patterns corresponding to the predicted restriction pattern of the pAD-GAL4 plasmid with insert (marked with a *) were sequenced. Plasmids with unfamiliar restriction patterns (marked with a #) were discarded. (Attempts to sequence these contaminating plasmids with universal sequencing primers proved unsuccessful).
After elimination of colonies containing the foreign plasmids, 186 colonies containing pAD-GAL4 plasmid with insert remained. The inserts from these colonies were sequenced, and the sequences compared with the DNA sequences lodged in the NCBI and TIGR databases to identify the inserts. These results appear in Table 3.1, along with a brief description of the functions of these genes, where known.

From this list, two genes represented by multiple non-overlapping clones were selected for verification by real-time PCR. Connective Tissue Growth Factor was selected because the literature revealed surprisingly little about any role it may play in skeletal metabolism, while Amyloid Precursor Protein was selected as it is known to be involved in another age-related disease, Alzheimer’s Disease. Primers were designed to amplify small regions of the 18S gene, the CTGF gene, and the APP gene (less than 200bp, intron-spanning where possible) from cDNA, and larger products (400bp or more) from genomic DNA (Appendix 2). Conventional PCRs were performed on genomic DNA and cDNA to optimise primer concentration, and the products run on a polyacrylamide gel to confirm no primer dimer formation. When the only primer dimer formation observed occurred in the DNA free water control lane, and products of the correct size were observed in both the genomic DNA and cDNA lanes, the primers were deemed to be optimised. Real-time PCR was then performed in the presence of fluorophores under the optimised conditions, across a dilution series of cDNA, to yield the real-time PCR data used in analysis. Again, products were run on a polyacrylamide gel to confirm no primer dimer formation (data not shown). The two genes, Connective Tissue Growth Factor and Amyloid Precursor Protein, were both shown to be differentially regulated between osteoblasts and fibroblasts. Figure 3.4 D shows the melt curve trace for the CTGF primers. As
can be seen from the trace, both osteoblast and fibroblast derived cDNA amplified a product with a higher melting temperature than the product amplified by the water control (primer dimer). This indicates that both cell types transcribe the CTGF gene. The differences in threshold cycle (Figure 3.4E) were then used to calculate the difference in fold transcription. The threshold cycles were corrected for differences in starting cDNA concentration relative to 18S, which is supposed to be expressed equally in both cell types. 18S primers amplified a product from fibroblast cDNA at cycle 22, and from osteoblast cDNA at cycle 26, indicating that 4 cycles must be subtracted from the osteoblast threshold cycle for CTGF to correct for differences in starting material concentration. The raw threshold cycle for amplification of CTGF in osteoblasts was 29 cycles- corrected, it showed amplification at cycle 25. This resulted in a ∆ct value between osteoblasts and fibroblasts of 2. Calculation of the formula to determine fold regulation showed that CTGF is transcribed at levels 4-fold higher in osteoblasts than fibroblasts (Livak and Schmittgen, 2001).

The melt curve trace shown in figure 3.4B indicates that the 18S primers amplified a product from the osteoblast, fibroblast, and genomic DNA templates, while only primer dimer was formed in the reaction containing no template DNA, as shown by the lower melting temperature of the product from the DNA free control (primer dimer product is shorter than amplified target DNA, so melts at a lower temperature). This result was confirmed by analysis of the product by polyacrylamide gel electrophoresis (data not shown). The melt-curve data for the APP primers (figure 3.4 C) shows that the two samples containing cDNA made from osteoblasts melted at a much higher temperature than did the fibroblast and genomic DNA template samples,
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Number of non-overlapping clones</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A kinase anchor protein 9 (AKAP; Yotiao)</td>
<td>1</td>
<td>Anchors synaptic modulators to neurotransmitter receptors (Westphal et al., 1999)</td>
</tr>
<tr>
<td>Actin, beta</td>
<td>1</td>
<td>Cytoskeletal molecule-facilitates organelle transport and cytokinesis (Liesel et al., 1999)</td>
</tr>
<tr>
<td>Actin, gamma</td>
<td>1</td>
<td>Cytoskeletal molecule-facilitates organelle transport and cytokinesis (Liesel et al., 1999)</td>
</tr>
<tr>
<td>Adenosine monophosphate deaminase E</td>
<td>1</td>
<td>Purine nucleotide interconverting enzyme (Yamada et al., 1992).</td>
</tr>
<tr>
<td>Adhesion regulating molecule 1</td>
<td>1</td>
<td>Transmembrane protein which, when over-expressed, greatly increases cellular adhesion to different cell types (Simins et al., 1999)</td>
</tr>
<tr>
<td>ADP-ribosylation factor 1</td>
<td>1</td>
<td>Guanine nucleotide exchange factor which recruits the coatamer complex to the membrane to facilitate transport from the endoplasmic reticulum to the Golgi apparatus (Presley et al., 2002)</td>
</tr>
<tr>
<td>Amyloid beta precursor protein</td>
<td>3</td>
<td>Peptide implicated in neuronal growth, and Alzheimer’s plaque formation. See discussion section of this chapter.</td>
</tr>
<tr>
<td>Annexin A2</td>
<td>2</td>
<td>Calcium-dependent membrane and phospholipid binding protein. Increases osteoclast formation and resorption (Takahashi et al., 1994). Acts as a calcium channel to promote chondrocyte maturation and apoptosis, and mineralisation</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Quantity</td>
<td>Function</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>----------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Antizyme (ornithine decarboxylase)</td>
<td>1</td>
<td>Regulates polyamine levels by inhibiting biosynthesis and cellular uptake (Matsufuji et al, 1996)</td>
</tr>
<tr>
<td>Calpain, small subunit</td>
<td>1</td>
<td>Calcium-activated thiol protease which regulates protein activity through limited proteolysis-known to regulate BMP activity, and bone matrix mineralization (Yajima and Kawashima, 2002)</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>1</td>
<td>Calcium storage in endoplasmic reticulum (McCauliff et al, 1990) transcription regulator (Burns et al, 1994; Dedhar et al, 1994)</td>
</tr>
<tr>
<td>Cargo selection protein (mannose 6 phosphate receptor)</td>
<td>1</td>
<td>Lysosomal transport (Diaz and Pfeffer, 1998)</td>
</tr>
<tr>
<td>Chaperonin containing T Complex Polypeptide 1, subunit 2 (beta)</td>
<td>1</td>
<td>Protein folding, substrates include actin, tubulin, alpha-transducin and cyclin E (Won et al, 1998)</td>
</tr>
<tr>
<td>CLL-associated antigen KW-12</td>
<td>1</td>
<td>Function unknown</td>
</tr>
<tr>
<td>Collagen type 1 alpha 1</td>
<td>6</td>
<td>Primary structural protein of bone (Tracey, 1998)</td>
</tr>
<tr>
<td>Collagen type 6 alpha 1</td>
<td>2</td>
<td>Involved in cell-extracellular matrix adhesion (Lamande et al, 1998)</td>
</tr>
<tr>
<td>Collagenase inhibitor</td>
<td>1</td>
<td>Growth factor for erythryocytes, also inhibits collagenase degradation by binding tightly to collagen molecules (Docherty et al, 1985)</td>
</tr>
<tr>
<td>Complement component 1 r subcomponent</td>
<td>1</td>
<td>Component of the complement cascade</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Score</td>
<td>Pathway of immune response</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Connective tissue growth factor</td>
<td>3</td>
<td>Growth factor involved in osteoblast development. See discussion section, this chapter.</td>
</tr>
<tr>
<td>Cysteine sulfinic acid decarboxylase-related protein 2</td>
<td>1</td>
<td>Associated with the metabolic pathway converting cysteine sulfinate to taurine</td>
</tr>
<tr>
<td>Cytoskeleton-associated protein 4</td>
<td>1</td>
<td>Cytoskelon-associated molecule- facilitates organelle transport and cytokinesis</td>
</tr>
<tr>
<td>Damage-specific DNA binding protein 2 (p48)</td>
<td>1</td>
<td>Required for repair of UV induced DNA damage (Hwang et al., 1999)</td>
</tr>
<tr>
<td>Dynein, cytoplasmic, heavy polypeptide 1</td>
<td>1</td>
<td>Microtubule-dependent ATP-ase used to transport organelles and chromosomes throughout the cell (Vaisberg et al., 1993)</td>
</tr>
<tr>
<td>EGF-containing fibulin-like extracellular matrix protein 2</td>
<td>1</td>
<td>Putative calcium-binding extracellular matrix protein - nb- authors observed low expression in fibroblasts(Katsanis et al, 1999)</td>
</tr>
<tr>
<td>Enolase 1 alpha</td>
<td>1</td>
<td>Metabolic enzyme involved in glycolysis</td>
</tr>
<tr>
<td>Enoyl coenzyme A hydratase short chain</td>
<td>1</td>
<td>Metabolic enzyme involved in mitochondrial beta oxidation of fatty acids</td>
</tr>
<tr>
<td>Epithelial membrane protein</td>
<td>2</td>
<td>Putative N-glycosylated transmembrane protein involved in cell-cell contact (Ben-Porath and Benvenisty, 1996)</td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1</td>
<td>3</td>
<td>Binds aminoacyl tRNAs to 80s ribosomes using GTP hydrolysis as an energy source</td>
</tr>
<tr>
<td>Ferritin, light polypeptide</td>
<td>1</td>
<td>Structural subunit of the major intracellular iron storage protein</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Reference</td>
<td>Description</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-----------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Fibronectin 1</td>
<td></td>
<td>Surface glycoprotein involved in cell-cell and cell-extracellular matrix contact, containing collagen, heparin and hyaluronic acid binding domains. Loss of expression results in loss of contact inhibition in transformed cells.</td>
</tr>
<tr>
<td>Glycyl tRNA synthetase (GARS)</td>
<td>1</td>
<td>Catalyses aminoacylation of tRNA (Williams et al, 1995)</td>
</tr>
<tr>
<td>Laminin receptor 1</td>
<td>2</td>
<td>Permits cell adhesion via the basement membrane protein laminin (Gehlsson et al, 1988)</td>
</tr>
<tr>
<td>Latent transforming growth factor beta binding protein 1</td>
<td>1</td>
<td>Required for assembly of TGF-B molecules (Kanzaki et al, 1990)</td>
</tr>
<tr>
<td>Leptin receptor gene related protein</td>
<td>1</td>
<td>Recently discovered peptide of unknown function. See discussion section, this chapter.</td>
</tr>
<tr>
<td>Leucine-rich neuronal protein</td>
<td>1</td>
<td>Member of the AMIGO family of cell adhesion molecules required for formation and myelination of fiber tracts in neuronal tissue (Kuja-Panula et al, 2003)</td>
</tr>
<tr>
<td>LIM and SH3 protein 1 (LASP1)</td>
<td>1</td>
<td>Function unknown- contains both LIM and SH3 domains (Tomasetto et al, 1995)</td>
</tr>
<tr>
<td>LIM domain only 7, transcript variant 3</td>
<td>2</td>
<td>Cysteine-rich zinc finger protein binding domain (Putilina et al, 1998)</td>
</tr>
<tr>
<td>Mitogen activated protein kinase kinase 2</td>
<td>1</td>
<td>Secondary signalling kinase involved in MAP kinase signal transduction (Zheng et al, 1993)</td>
</tr>
<tr>
<td>Myosin regulatory light chain</td>
<td>1</td>
<td>Regulates myosin ATPase activity, required for contraction and relaxation of muscle by stabilising the structure of the myosin head</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone complex)</td>
<td>1</td>
<td>First complex of the electron transport chain in the mitochondria</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Gene ID</td>
<td>Functions/Properties</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Netrin 4</td>
<td>2</td>
<td>Axon guidance molecule- other family members implicated in neural patterning in bone (Togari et al, 2000)</td>
</tr>
<tr>
<td>Nuclear domain 10 protein</td>
<td>2</td>
<td>Function unknown- normally colocalises with nuclear matrix, but is redistributed following viral infection (Korioth et al, 1995)</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>1</td>
<td>Regulates transcription of ribosomal RNA by RNA polymerase I, required for ribosome maturation and assembly and nucleocytoplasmic transportation of ribosomal components</td>
</tr>
<tr>
<td>Ornithine decarboxylase antizyme</td>
<td>1</td>
<td>Involved in degradation of polyamines. See discussion section, this chapter.</td>
</tr>
<tr>
<td>Osteoblast-specific factor 2 (OSF-2)</td>
<td>4</td>
<td>Protein involved in bone and dental ligament development. See discussion section, this chapter.</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>4</td>
<td>Major protein component of bone- contains Ca++-binding domains (Stenner et al, 1986)</td>
</tr>
<tr>
<td>p35srj</td>
<td>1</td>
<td>Hypoxia/deferoxamine induced transcriptional regulatory molecule (Bhattacharya et al 1999)</td>
</tr>
<tr>
<td>Phosphoglycerate kinase 1</td>
<td>1</td>
<td>Catalyses conversion of 1,3-diphosphoglycerate to phosphoglycerate to produce ATP</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor</td>
<td>1</td>
<td>Member of the Serpin family of serine proteinase inhibitors, induced by stress and implicated in thrombosis (Ny et al, 1986; Yamamoto et al, 2002)</td>
</tr>
<tr>
<td><strong>Proteasome activator 26S subunit (prosome, macropain)</strong></td>
<td>2</td>
<td>Involved in degradation of short-lived and misfolded proteins. Required for efficient antigen processing in immune cells (Preckel et al, 1999)</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>---</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Protein phosphatase 1</strong></td>
<td>2</td>
<td>Serine/threonine phosphatase required for signalling (catalytic subunit)</td>
</tr>
<tr>
<td><strong>Protein phosphatase 2</strong></td>
<td>1</td>
<td>Serine/threonine phosphatase required for signalling (regulatory subunit)</td>
</tr>
<tr>
<td><strong>Protein tyrosine phosphatase IVA</strong></td>
<td>1</td>
<td>Tyrosine phosphatase required for signalling</td>
</tr>
<tr>
<td><strong>Repressor of estrogen activity/D-prohibitin</strong></td>
<td>1</td>
<td>Tumour supressor gene which prevents proliferation of cells, frequently mutated in breast cancer (Sato et al, 1992)</td>
</tr>
<tr>
<td><strong>Ribosomal protein L4</strong></td>
<td>1</td>
<td>Ribosomal subunit- function unknown</td>
</tr>
<tr>
<td><strong>RNA binding motif protein 5</strong></td>
<td>1</td>
<td>Specifically binds poly G mRNA (Drebkin et al, 1999)</td>
</tr>
<tr>
<td><strong>Signal sequence receptor gamma</strong></td>
<td>2</td>
<td>Transmembrane protein involved in protein trafficking across the endoplasmic reticulum (Hartmann et al, 1993)</td>
</tr>
<tr>
<td><strong>Slit homolog 3</strong></td>
<td>1</td>
<td>Involved in neural patterning, and development of endocrine system (Itoh et al, 1998)</td>
</tr>
<tr>
<td><strong>Thyroid hormone receptor interactor 7 (TRIP 7)</strong></td>
<td>1</td>
<td>Interacts with thyroid hormone receptors to facilitate hormone-dependent transcription (Lee et al, 1995)</td>
</tr>
<tr>
<td><strong>Tissue inhibitor of metalloproteinase 3</strong></td>
<td>1</td>
<td>Regulates deradation of extracellular matrix by inhibition of matrix metalloproteases (Langton et al 1998)</td>
</tr>
<tr>
<td><strong>Transforming growth factor, beta-induced, 1</strong></td>
<td>1</td>
<td>Putative bone adhesion molecule, similar to OSF-2 (Munier et al, 1997)</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Count</td>
<td>Description</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>UDP-glucose ceramide glucosyltransferase</td>
<td>1</td>
<td>Produces glucosylceramide, required for synthesis of glycosphingolipids (Ichikawa et al., 1996)</td>
</tr>
<tr>
<td>Vacuolar sorting protein 29</td>
<td>1</td>
<td>Forms part of a multimeric complex responsible for intracellular protein sorting (Haft et al., 2000)</td>
</tr>
<tr>
<td>v-Ha-ras Harvey rat sarcoma viral oncogene homolog</td>
<td>1</td>
<td>Initiates intracellular signal transduction via the MAP kinase signalling pathway (Seger and Krebs, 1995)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>4</td>
<td>Mesenchymal cell specific intermediate cytoskeletal filament</td>
</tr>
<tr>
<td>Zinc finger protein 22 (KOX 15)</td>
<td>1</td>
<td>Putative transcription factor (Bray et al., 1991)</td>
</tr>
</tbody>
</table>
Figure 3.4: Real-time PCR amplification of APP and CTGF relative to 18S housekeeping gene primers.

A) shows the PCR trace for 18S and APP, along with threshold cycles.

B) The melt curve data for the 18S primers.

C) The melt-curve data for the APP primers.

D) The melt curve data for the CTGF primers.

### Amplification data for 18S and APP primers

<table>
<thead>
<tr>
<th>Template</th>
<th>Primer set</th>
<th>Colour</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblast</td>
<td>18S</td>
<td>blue</td>
<td>13</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>18S</td>
<td>purple</td>
<td>14</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>18S</td>
<td>maroon</td>
<td>14</td>
</tr>
<tr>
<td>DNA-free control</td>
<td>18S</td>
<td>magenta</td>
<td>26</td>
</tr>
<tr>
<td>Osteoblast</td>
<td>APP</td>
<td>green</td>
<td>21</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>APP</td>
<td>green</td>
<td>20</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>APP</td>
<td>blue</td>
<td>24</td>
</tr>
<tr>
<td>DNA-free control</td>
<td>APP</td>
<td>aqua</td>
<td>31</td>
</tr>
</tbody>
</table>

### Melt curve data for 18S primers

<table>
<thead>
<tr>
<th>Template</th>
<th>Colour</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblast</td>
<td>magenta</td>
<td>89°C</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>yellow, green</td>
<td>90°C</td>
</tr>
<tr>
<td>Water control</td>
<td>aqua</td>
<td>82°C</td>
</tr>
</tbody>
</table>

### Melt curve data for APP primers

<table>
<thead>
<tr>
<th>Template</th>
<th>Colour</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblast</td>
<td>blue, dark blue</td>
<td>91°C</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>red, aqua</td>
<td>84°C</td>
</tr>
<tr>
<td>Water control</td>
<td>yellow, green</td>
<td>84°C</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>magenta, brown</td>
<td>84°C</td>
</tr>
</tbody>
</table>

### Melt curve data for CTGF primers

<table>
<thead>
<tr>
<th>Template</th>
<th>Colour</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblast</td>
<td>red, green</td>
<td>89°C</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>yellow, purple</td>
<td>89°C</td>
</tr>
<tr>
<td>Water control</td>
<td>dark green</td>
<td>86°C</td>
</tr>
</tbody>
</table>
which had the same melting temperature as the water control, indicating that they contained only primer dimer. Because the APP primers were designed to be intron-spanning, the lack of amplified product in the genomic DNA sample was expected. Because APP failed to amplify from fibroblast cDNA, while 18S amplified from the same batches of cDNA and PCR reagent in the control reaction, no fold difference could be determined for APP expression between fibroblasts and osteoblasts. The experiment was repeated using three different batches of fibroblast cDNA, from three different primary sources, and the 18S PCR primers successfully produced the correct fragment for all three batches of cDNA, demonstrating that the cDNA was of sufficient quality. Furthermore, osteoblast cDNA and genomic control DNA successfully amplified APP product under the same reaction conditions using the same master mix for the PCR, indicating that the PCR reagents and conditions were all functioning adequately for amplification. These three factors confirm the probable absence of any APP gene product in the fibroblast cDNA, thereby verifying the initial isolation of APP from the subtracted library as an osteoblast-specific gene (relative to fibroblasts).

3.3 Discussion

As can be seen from the table of clones sequenced from the osteoblast-enriched library, this process has revealed a large number of different targets for further research. While many of the sequences have been assigned functions, and isolated in other tissues previously, a number of clones contained genes previously identified only by clone numbers from other cDNA libraries. Obviously these genes are the ones
most likely to yield novel information about intra- and inter-cellular processes in
general, but the very fact that so little is known about them makes it difficult to select
targets for further research. Further support for the effectiveness of the subtraction
comes from the absence of the more abundant ubiquitously-expressed genes in the
list of sequences, such as 18S and tRNA genes.

Many of the previously identified genes sequenced in the osteoblast-enriched library
have previously been assigned functions in other cell types, as mentioned previously,
however these genes have either not been tested for in bone, or the function of these
genes in bone is yet to be determined. CTGF falls into the latter category, APP into
the former. I selected APP and CTGF as candidate genes for verification using real-
time PCR for several reasons: firstly, both genes were isolated from the library a
number of times, suggesting that these gene transcripts really were present in
osteoblasts at a higher level that in fibroblasts. Secondly, each gene is relevant to bone
disease in view of the research related to each of them. Based on what was previously
known about CTGF, and the function and expression pattern of the gene, it seems
likely that it should be involved in skeletogenesis at some level. APP seemed a good
candidate gene for verification for precisely the opposite reason: no previous
published studies identified APP in bone or suggested a role for APP in bone. Finding
it several times suggested it was either a novel observation, or the technique used to
enrich the library was flawed in some way, and the presence of APP was an artefact.

That real-time PCR verified the presence of both genes at higher levels in osteoblasts
than fibroblasts suggests that the subtraction did work, and that other genes isolated
from the library are also likely to be expressed at higher levels in osteoblasts than fibroblasts.

3.3.1 Connective Tissue Growth Factor

Connective tissue growth factor (CTGF) was initially isolated from a cDNA library made from human umbilical vein endothelial cells by Bradham et al (1991). CTGF has been since been identified in a number of cell types, including fibroblasts, osteoblasts, osteocytes, hypertrophic chondrocytes, and other tissues including liver, kidney, brain and testis.

CTGF is a member of the CCN protein family, and possesses the characteristics of that family including an amino-terminal secretory signal, followed by an IGFBP domain, a von Willebrand factor type C repeat, a thrombospondin type 1 repeat, and a carboxy-terminal cysteine knot domain, consisting of a cysteine-rich region of 38 cysteine residues (Oemar and Luscher, 1997).

CTGF has been shown to be a downstream signalling molecule in the TGF-β signalling pathway. Cortisol has also been shown to upregulate CTGF expression in osteoblasts (Pereira et al, 2000), while Yamashiro et al (2001) showed that mechanical stress of bone causes upregulation of CTGF in osteocytes. This observation increases in significance when the earlier observations of Nishida et al are considered (2000). These authors examined the effects of CTGF on osteoblasts, and established that CTGF can promote the differentiation of osteoblasts from precursor...
cells. This data combined suggests that mechanical stress in bone may result in an increase in CTGF expression by osteocytes, which is then secreted extracellularly to act as a paracrine signal to recruit any nearby osteoblast precursors to differentiate ready to replace the stress-damaged bone surrounding the osteocytes once it has been excised. In this way, a role for osteocytes in priming osteoblast precursors is suggested, which may provide part of the answer to the elusive function of osteocyte cells.

Another interesting observation relating to the function of CTGF comes from the same study by Nishida et al (2000). Previous work by the same group showed that CTGF is expressed extensively by hypertrophic chondrocytes (Nakanishi et al, 1997) and that CTGF promotes the maturation and hypertrophy of chondrocytes (Nakanishi et al, 2000). Furthermore, work by Shimo et al (1999) demonstrated that CTGF induces angiogenesis in vitro and in vivo. Combined with the work by Nishida et al (2000) demonstrating that CTGF promotes osteoblast differentiation, it seems likely that CTGF is mediating at least three components of the process of endochondral ossification.

Briefly, endochondral ossification is the process by which most of the bones of the body are formed including the limbic bones and the vertebrae. During endochondral ossification, mesenchymal cells condense, and differentiate into chondrocytes, which in turn undergo proliferation, differentiation, and hypertrophy, while secreting the cartilaginous skeletal template. This template is then invaded by blood vessels, which introduce osteoblast cells to the template, which in turn secrete osteoid bone matrix.
From the work by Nishida’s group, therefore, it seems likely that CTGF is acting firstly to promote hypertrophy in chondrocytes, secondly to trigger angiogenesis by acting on nearby endothelial cells and promoting vascularisation, and thirdly by acting on osteoblast precursors brought into the cartilaginous template by the newly-formed blood vessels to promote osteoblast differentiation.

This model is supported by another study by Nakanishi et al. (2001) which found that overexpression of CTGF under the control of a collagen promoter caused dwarfism and decreased bone density in mice. Dwarfism is the phenotype usually observed when chondrocytes exit one of the stages of chondrocyte development prematurely. This early exit means that insufficient proliferation of cells within the growth plate occurs, preventing the longitudinal extension of the ends of the bone, with the end result of shorter bones, or a dwarf phenotype. As it has been shown that CTGF promotes hypertrophy in chondrocytes, overexpression of CTGF probably forces chondrocytes into hypertrophy too early for normal bone development. The lower bone density observed in these mice suggests a similar thing may be happening with osteoblasts- the excess CTGF may be forcing osteoblast precursors to differentiate before adequate proliferation has occurred, resulting in a lower number of bone-forming cells overall, and hence decreased bone density.

That we isolated CTGF from the osteoblast-enriched library is not surprising when the current research is considered. It would be interesting to see the effects of a CTGF knockout mouse, and determine whether the resulting phenotype matches that of the CBFA1 mouse exactly, or whether the tiny areas which stain for mineralized bone in
the CBFA1 knockout mouse, co-localising with sites of intramembranous ossification, are affected by CTGF inactivation, as mesenchymal cells have already been shown to express CTGF.

Some other researchers have found that osteoblasts express CTGF at low levels, or not at all (Nakanishi et al., 1997; Shimo et al., 1998). Our observation that it is expressed at levels 3.7-fold higher in osteoblasts than fibroblasts is not necessarily a contradiction of the literature, but may simply be explained by the stage of development the osteoblasts were at when isolated. The osteoblasts used in the present study were isolated from fetuses, while the osteoblasts used in the studies showing little or no expression of CTGF were all obtained from adult rats or mice. This may indicate that CTGF is involved in primary ossification, or could be a reflection of the rarity of active osteoblast cells in adult bone.

3.3.2 Amyloid Precursor Protein

Much of the research performed to date on Amyloid precursor protein and its proteolytic products has focused on the brain. This is largely because a proteolytic processing product of the amyloid precursor product, known as Aβ has been demonstrated to be the main component of the extracellular protein plaques located within the brain associated with Alzheimer's disease, as well as the dementia often found in older patients with Down’s syndrome, and various forms of neuropathies with Lewy bodies, including Parkinsons’ disease (Masters et al., 1985).
However there are a number of alternative amyloid precursor protein proteolytic products, several of which have postulated functions suggesting a role for these variants in healthy cells and tissues, which are often overlooked.

The gene for amyloid precursor protein is located on chromosome 21, indicative of the reason amyloid plaques are often observed in the brains of Down’s syndrome patients (Robakis et al, 1987). The gene is arranged into 19 exons, which are alternatively spliced into a number of isoforms, all of which result in a multidomain transmembrane protein, which spans the lipid bilayer once (Yoshikai et al, 1990). In addition to the transmembrane domain, APP has a number of features suggesting a variety of roles for the functional proteolytic product.

The transmembrane domain contains three secretase cleavage sites for processing of the protein. Under what are believed to be ‘normal’ processing conditions, the peptide is cleaved at the alpha secretase site to generate a soluble extracellular peptide, known as the alpha fragment, and another fragment, which contains the cleavage site for the gamma secretase (Esch et al 1990; Sisodia et al, 1990; Weidemann et al, 1989). Cleavage of this remaining fragment at the gamma site produces two very small peptide fragments, neither of which display obvious aggregation properties. In the proteolytic pathway that leads to the deposition of amyloid plaques, however, the amyloid precursor peptide is cleaved at the beta secretase site, slightly upstream of the alpha site. This results in release of the soluble alpha fragment, but leaves the remaining c-terminus fragment of the peptide intact. This c-terminal fragment is then cleaved by gamma secretase, generating either a 40 or 43 amino acid fragment which
Figure 3.5: Processing of Amyloid Precursor Peptide protein, resulting in altered functions and cellular effects. Cleavage by the $\alpha$-secretase is proposed to produce a transcriptional activator. Cleavage by the $\beta$– and $\gamma$-secretase results in production of the 40/43 aa amyloidogenic fragment, which aggregates to form senile plaques in dementia. It is thought that a similar pathway of misprocessing of $\alpha$-synuclein (also known as Non-A-beta component) results in the amyloidogenic plaques of Parkinson’s disease involving Lewy bodies.
is amyloidogenic, or capable of aggregating to form plaques (Haass et al, 1993; Shoji et al, 1992) (Figure 3.5). The 43-amino acid fragment demonstrates a greater capacity for aggregation than does the 40-amino acid fragment, however both forms have been isolated from plaques. These plaques have been demonstrated to trigger a cascade of inflammatory responses which result in the death of the surrounding neurons, thus causing the memory loss and other symptoms seen with Alzheimer’s dementia. Excessive production of APP, or an imbalance in processing resulting in greater production of either of the beta fragments, leads to an increase in plaque formation (Pike et al, 1995; Citron et al, 1996).

Aside from the interest in APP because of the causative relationship its misprocessing has on Alzheimer’s disease, APP has a number of other properties. Interestingly, in vitro studies have demonstrated a role for APP in neuroprotectivity- adding soluble APP to neuronal cultures has been shown to protect the cells from the effects of hypoglycaemia and oxidative stress, as well as toxicity caused by deposition of amyloidogenic plaques (Goodman et al, 1994; Mattson et al, 1993). Various groups have identified metal binding domains within the molecule, and copper and zinc binding has been demonstrated (Bush et al, 1996; Daigle and Li, 1993; Hess et al, 1994). This binding activity to reactive metals suggests that APP may perform a role in sequestering generators of free radicals within the cell, thus acting in a protective role. A serine protease inhibition domain has also been identified within APP, leading to speculation that it may be involved in regulation of blood coagulation (Oltersdorf et al, 1989). APP has also been demonstrated to contain both collagen and chondroitin sulfate binding sites (Beher et al, 1996), which suggests that as a molecule it may be capable of attaching to both bone matrix and cartilaginous matrix found during
primary osteogenesis. Indeed, when these binding sites are considered along with another observation, that APP can trigger neurite outgrowth (Williamson et al., 1996), it becomes possible to see that APP may be necessary for development of the neural network required to support the skeleton. The ability of the molecule to bind to molecules located within both the types of extracellular matrix located at the primary ossification front, combined with an ability to trigger neurogenesis, suggest that the two properties may be linked in such a way as to explain why we isolated the cDNA for APP in a foetal-osteoblast-enriched library.

Also worthy of consideration is the fate of the cytoplasmic domain of the cleavage product of gamma secretase cleavage. Cao and Sudhof (2001) showed that the cytoplasmic fragment is capable of associating with a nuclear adaptor protein and a histone acetyltransferase, with resulting activation of a reporter gene, suggesting that the remaining intracellular cleavage fragment may act as a regulator of transcription, although the target sequence, and hence target genes of this complex are yet to be identified.

### 3.3.3 Other Candidate Genes
Candidate genes with obvious potential include ornithine decarboxylase antizyme, osf-2, or periostin, B-cell associated repressor of estrogen activity, thyroid hormone receptor interactor 7 and the leptin receptor gene related protein.
3.3.3.1 Ornithine decarboxylase antizyme

While most proteins within the cell undergo ubiquitination prior to proteasomal degradation, the polyamine-synthesizing molecule ornithine decarboxylase does not (Bercovich et al., 1989; Rosenberg-Hasson et al., 1989). Instead, a molecule called ornithine carboxylase antizyme binds to ornithine decarboxylase and targets it to the proteasome for destruction (Hayashi et al., 1996).

The polyamine synthesis pathway is a metabolic pathway required by the cell to produce the three polyamines, spermine \( \text{H}_2\text{N}\left(\text{CH}_2\right)_3\text{NH}\left(\text{CH}_2\right)_4\text{NH}\left(\text{CH}_2\right)_3\text{NH}_2 \) spermidine \( \text{H}_2\text{N}\left(\text{CH}_2\right)_3\text{NH}\left(\text{CH}_2\right)_4\text{NH}_2 \), and putrescine \( \text{H}_2\text{N}\left(\text{CH}_2\right)_4\text{NH}_2 \). The three polyamines are positively charged, hydrophobic cations which are required for DNA replication transcription and protein synthesis through their ability to interact with RNA and DNA (Watanabe et al., 1991). Mitogens have been shown to increase polyamine synthesis, while mutations and drugs which serve to reduce polyamine synthesis result in arrested cell growth and proliferation, largely because spermine is required for post-translational hypusination of translation initiation factor 5A (reviewed in Coffino, 2001a). Mouse strains engineered to alter polyamine levels revealed that tight regulation of polyamine levels is essential to prevent carcinogenic transformation (Pegg et al., 2003). Overexpression of ornithine decarboxylase under the control of skin or stomach- specific promoters resulted in increased sensitivity to UV and chemical induced cancer, while overexpression of ornithine decarboxylase antizyme leads to decreased cancer susceptibility. Unpublished data reviewed in Coffino (2001b) suggests that ornithine decarboxylase is not essential for development in a gene inactivation mouse model, but that mice homozygously
deficient for the gene have higher rates of perinatal death than wild-type litter-mates. Unfortunately no later data about the rate of carcinogenesis in older mice is available.

Antizyme utilizes translational frameshifting to control levels of polyamines within the cell. The mRNA for antizyme contains two overlapping open reading frames, the first of which contains the initiation codon. After translation commences, the translation apparatus either terminates translation at the end of the first open reading-frame, or a frame-shift occurs, which results in translation of the remainder of the antizyme protein. As polyamine levels within the cell increase, so does the efficiency of the frameshift increase. In this way, it is only when polyamine levels are high that active antizyme, which inhibits polyamine synthesis by targeting ornithine decarboxylase for destruction, is produced (Matsufuji et al, 1995).

3.3.3.2 Periostin

Periostin is a protein preferentially expressed in bone cells about which relatively little is known. First cloned by Takeshita et al in 1993 from a mouse osteoblastic cell line, periostin is an 811 amino acid protein with a high level of homology to an insect protein called fasciclin, an adhesion molecule. Horiuchi et al (1999) subsequently demonstrated that periostin is expressed primarily in the periosteum and periodontal ligament, and that periostin promotes spreading and adhesion of osteoblasts, which was inhibited during treatment with anti-periostin antisera. The same authors also demonstrated that TGF-beta induces periostin expression in vitro, which reflects in vivo observations that TGF-beta acts to induce osteoblastogenesis in osteoblastic precursor cells. The transcriptional regulatory mechanisms of periostin were partially
revealed by Oshima et al (2002), who demonstrated that there is a Twist binding site in the promoter of periostin, and that Twist, a basic helix-loop-helix transcription factor involved in cell-type determination, activates transcription of periostin both in vitro and in cotransfection cell assays.

Periostin expression has also been observed in cancerous ovarian tissue, but not in healthy ovarian tissue. Periostin has recently been shown to support adhesion and migration of malignant ovarian cancer cells via interactions with the α(V)β(3) and α(V)β(5) integrins (Gillan et al, 2002). This observation is particularly interesting because it demonstrates that periostin is a ligand for the same integrin (α(V)β(3)) that osteoclasts utilize to adhere to proteins in the extracellular matrix, suggesting that it may be one way osteoblasts and osteoclasts can directly adhere to one another.

3.3.3.3 B-cell associated protein 37/ repressor of estrogen receptor activity/ D-prohibitin/B-cell receptor associated protein

Montano et al (1999) identified a protein which they termed repressor of estrogen activity through its ability to bind to estrogen receptor and repress transcription of gene targets. This protein was later identified as D-prohibitin, or BAP37, and was characterized as an inner mitochondrial membrane protein (Nijtmans et al, 2000). Deletion of homologous genes in yeast result in premature aging of the cells (Coates et al, 1997), a finding confirmed in mammalian cells by the observation of Coates et al (2001) that levels of BAP37 and its binding partner Phb1p decrease heterogeneously during cellular aging and onset of senescence. The same authors
went on to show a role for prohibitin in the maintenance of mitochondrial respiration, particularly under circumstances of mitochondrial stress. It has been widely documented that estrogen deficiency can accelerate osteoporosis. A protein such as D-prohibitin, which has been shown to repress estrogen-dependent transcription, and which has been demonstrated to be involve in cellular aging and mitochondrial stress could well prove interesting to examine further, to gain a clearer understanding of the relationship between estrogen and osteoblast metabolism in healthy cells.

3.3.3.4 Thyroid hormone receptor interactor 7 (TRIP 7)

Thyroid hormone receptor interactor 7 was originally identified from a yeast two-hybrid screen using the Thyroid hormone receptor β1 as bait (Lee et al, 1995), where it was shown to interact with thyroid receptor and retinoic acid receptor in a ligand-dependent manner, to regulate transcription. TRIP7 belongs to a family of nuclear proteins which bind specifically to nucleosomes to loosen the structure of chromatin to facilitate access to promoter sites by transcription complexes (West et al, 2001). At present, little is known about the precise function of this protein, however given that it can potentially regulate gene transcription in a thyroid-dependent manner, and that the thyroid/parathyroid axis is heavily involved in calcium homeostasis, which affects skeletal calcium storage, it may be a potential therapeutic target for intervention to modify osteoblast activity.
3.3.3.5 Leptin receptor gene-related protein

A role for leptin in the regulation of skeletal integrity has been postulated since the observation that obesity partially protects people from developing osteoporosis (Tremollieres et al., 1993). Leptin is a molecule secreted primarily by adipocytes, which circulates through the blood before binding to receptors, which are distributed in a variety of tissues, but most prominently in the hypothalamus. It is the activity of the hypothalamus which potentiates the ability of leptin to signal satiety. Deficiency of leptin (ob/ob mouse model) and leptin receptor (db/db mouse model) results in increased bone mass and body mass (Halaas et al., 1995), while intracerebroventricular injection of leptin into ob/ob mice causes a decrease in bone density (Ducy et al., 2000). The increased bone density found in ob/ob mice is due to increased synthesis of bone matrix by a normal number of osteoblasts, rather than an increase in the number of osteoblasts themselves. A direct role for leptin in bone remodeling is yet to be confirmed, as there is some debate as to whether osteoblasts express leptin receptors (Lee et al., 2002) or not (Ducy et al., 2000).

The transcript identified by this project, leptin receptor gene-related protein, is expressed from the same promoter as the leptin receptor, and shares the first two 5’UTR exons, but is then alternatively spliced to produce a protein with a completely distinct amino acid sequence (Bailleul et al., 1997). Northern blot analysis of the leptin receptor and leptin receptor gene related protein shows that the transcription of leptin receptor gene related protein is as widespread as leptin receptor (Cioffi et al., 1996). The sequence of leptin receptor gene related protein in man is found to be highly similar to the murine counterpart, differing by only six out of 131 amino acids.
Similarly, the organization of the gene within the genome is conserved between the two species (Bailleul et al., 1997). This level of conservation of gene structure and protein sequence, and similarity of tissue expression pattern lead the authors to propose that there may be some as yet unidentified function for this gene which requires its expression to be closely coordinated with that of leptin. Whether the function of leptin receptor gene related protein is involved in bone remodeling at all remains to be determined, along with all other aspects of its functions.

3.3.4 Conclusion

From this experiment, 186 colonies showed the presence of the correct vector when digested with *Rsa I*. Of these colonies, 99 clones containing non-overlapping regions of DNA from 66 known genes were identified, some of which contained different regions of the same gene. A further 39 plasmids contained inserts identified only by sequencing database identifiers. Further bioinformatics studies failed to shed any further light on these clones. The remaining 48 clones either failed to sequence (5) or did not match any of the sequences within the TIGR or NCBI databases when these databases were probed with the DNA sequence isolated from these clones.

Of those genes identified, the seven discussed above are worthy of further investigation, as the literature suggests these genes play important roles in the regulation of other physiological systems. Of particular interest are those genes implicated in other disease states, particularly age-related diseases, as examining the effect of the same aberrant gene across two separate disease systems can provide great
insight into the function of the gene. Many of the other genes identified are structural or involved in basic cellular metabolic processes such as translation or transcription. While these processes are undoubtedly essential, the fact that these genes are so fundamentally vital as to be found in many different cell types suggests that their effects may not be specific enough to make them targets for therapeutic intervention to treat bone disease, as any treatment targeting these processes would be likely to have extensive side effects.

The ‘unknown’ genes found during this study- the ones which do not produce any matches when compared with the sequencing database- seem likely to yield the most novel regulatory molecules of bone cell differentiation, however until more information becomes available, it is difficult to postulate roles for molecules expressed from these sequences.
CHAPTER 4: RESULTS PART 2: DNA ARRAY STUDIES
COMPARING OSTEOBLASTS WITH FIBROBLASTS
AND OSTEOCLASTS WITH MACROPHAGES

4.1 Introduction

Very few osteoblast-specific gene products and regulatory molecules have been identified to date. Thus far, only Osterix and CBFA1 have been identified as regulatory molecules primarily involved in osteoblastogenesis, and relatively little is known about how genes identified from studies on other cell types may regulate bone synthesis and remodelling. This chapter describes the use of microarrays and macroarrays containing known gene targets, some of which are yet to be assigned a function, to identify new candidate genes involved in the regulation of osteoblast activity and development.

Similarly, there is much that remains unclear about the mechanisms regulating the maturation and function of osteoclasts. In vitro culture of osteoclasts is still a relatively new technique, facilitated by the identification a few years ago of RANK-L as the key osteoclastogenic differentiation factor. Prior to this discovery, osteoclasts could only be cultured as co-cultures with osteoblasts, as RANK-L is produced by osteoblasts. Figure 1.6 shows the differentiation events regulating the development of bone cells in light of the relationship between osteoblasts and osteoclasts. Since the
identification of RANK-L as the key osteoclastogenic factor, and the commercialization of a soluble form, osteoclasts have been readily cultured from blood cell preparations in vitro. Macrophages are the comparison cell type used in the research performed in this laboratory, because treatment of monocytes with M-CSF alone results in macrophage development, while the addition of RANK-L and M-CSF combined triggers osteoclastogenesis. Effectively this model examines the differences caused by exposure to RANK-L.

In this chapter, the results of three experiments comparing gene expression profiles are presented. Firstly, gene expression in osteoblasts was compared with gene expression in fibroblasts utilising DNA microarray analysis. The same comparison was then repeated using DNA macroarrays. Finally, the same set of DNA macroarrays were used to compare gene expression between osteoclasts and macrophages.

In this section, two different types of DNA array were used to identify genes with differential expression between cell types. The Clontech cDNA Expression Array kit (macroarray) uses specific primers in a PCR reaction with radioactive nucleotides to amplify gene targets located on the array. In this way, the only labelled DNA present in the hybridisation is specific for the target genes on the array. The set of Atlas cDNA arrays consists of three pairs of arrays, with each pair containing 1176 genes. Two pairs of filters, or 2352 genes, were probed in the osteoblast/fibroblast comparison, while all three pairs, or 3528 genes, were probed in the osteoclast/macrophage comparison.
The microarrays used in this experiment require incorporation of fluorescently-labelled nucleotides into the probe using a direct incorporation step mediated by reverse transcriptase, rather than PCR amplification. Many of the genes on this array do not have identified names or functions, and were simply isolated as cDNAs from libraries, which differs from the ATLAS array, where all genes are known and have some attributed functions. The library of genes represented on the 19k microarray was distributed across two glass slides.

RNA was isolated from osteoblasts, fibroblasts, osteoclasts and macrophages as outlined in section 2.3.1. The RNA was labelled with either radioactive nucleotides or Cy3 and Cy5. Cy3 was used to label fibroblast RNA, and Cy5 was used to label osteoblast RNA to probe the 19k microarray, while RNA from all four cell types was labelled with radioactive nucleotide to probe the ATLAS arrays.

After hybridisation of the Cy3 and Cy5 labelled probe to the microarray, fluorescent images of the array were captured using Imagene software, and the relative intensity of Cy3 emission and Cy5 emission for each target cDNA spot was then measured. This data was then imported into Genespring version 6.1(Silicon Genetics), where it was analysed. Analysis consisted of performing Lowess normalization on the data, which serves to correct for any intensity-dependent effects within the data. Then, the data was filtered to remove any genes with potentially unreliable readings as a result of low expression levels. This is a standard approach used to analyse microarray data (Reviewed by Quackenbush, 2002). Genes with greater than 2-fold expression were included in the list of regulated genes derived from this experiment.
Analysis of the radioactively labelled macroarray was performed by subtracting the background emission from the total emission of each spot, correcting for differences between filters using the control spots on each filter, and then calculating the ratio of intensities for the replicate spot on each filter. After calculating the ratio, a standard acceptance cut-off of three-fold regulation was used to identify significantly regulated targets. This is a widely-accepted method of analyzing data from Southern and Northern blots, and has previously been accepted for use on radioactively-labelled macroarrays.

4.2 Results

Figure 4.1 shows ATLAS human Arrays 1.2.I and 1.2.III probed with radioactively-labelled probes made from cDNA from fibroblasts and pre-mineralizing osteoblasts cultured in vitro. The lists of regulated genes yielded by this experiment are shown in table 3.1, along with a brief description of its known function. Overall, the data from the ATLAS macroarray experiments showed that 11 out of the 2352 genes assayed were significantly regulated, with eight upregulated in osteoblasts, and four downregulated in osteoblasts (Table 4.1).
Figure 4.1: *Atlas arrays probed with radioactively-labelled cDNA*. A) shows two entire filters arrayed with identical targets, but hybridised to probes made from different cell types. B) shows a close view of the same region from each of the two arrays. The red box indicates a gene expressed by the cell type the top panel was probed with, but not by the cell type used to probe the bottom panel, while the blue box shows a target transcript expressed by both cell types, but at different levels.
Table 4.1a: Genes significantly upregulated in osteoblasts compared with fibroblasts, as identified by the ATLAS macroarray system, using arrays 3.1 and 3.2.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold regulation</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription factor Dp-2 (E2F dimerization partner 2)</td>
<td>5</td>
<td>Transcription factor which heterodimerises with E2F for activation (Zhang and Chellappan, 1995)</td>
</tr>
<tr>
<td>cAMP response element binding protein (CRE-BP1)</td>
<td>4</td>
<td>Phosphorylated form binds to the cAMP response element in promoters of cAMP regulated genes to activate transcription (Montminy and Bilezikjian, 1997; Parker et al, 1996)</td>
</tr>
<tr>
<td>Transcription factor ATF2</td>
<td>4</td>
<td>Forms either homodimers, or heterodimers with jun to activate transcription of cAMP response element containing genes, through histone acetyltransferase activity (Kawasaki et al, 2000)</td>
</tr>
<tr>
<td>Diazepam binding inhibitor (GABA receptor modulator, Acyl-Coenzyme A binding protein)</td>
<td>4</td>
<td>Endogenous ligand for GABA receptors which inhibits chloride influx mediated by the chloride channel portion of the GABA receptor, activated by binding of GABA to the GABA receptor, and prolonged by benzodiazepines (Gray et al, 1996)</td>
</tr>
<tr>
<td>Catechol-O-methyltransferase</td>
<td>4</td>
<td>Methylates catecholamine neurotransmitters, including epinephrin, norepinephrin, and dopamine as part of the destruction pathway. Required to maintain a steady-state pool of catecholamine neurotransmitters within the brain, important for emotional and sexual behaviour in mice (Gogos et al, 1998)</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 2B, subunit 5</td>
<td>3</td>
<td>Guanine nucleotide exchange factor required for recycling of translation initiation factor eIF2 (Asuru et al, 1996)</td>
</tr>
<tr>
<td>Dihydrolipoamide acetyl transferase</td>
<td>3</td>
<td>Component of the pyruvate decarboxylase complex. Required to prevent lactic acidemia (Robinson et al, 1990)</td>
</tr>
<tr>
<td>Neurotrophin 3</td>
<td>3</td>
<td>Promotes survival and growth of neural crest derived neurons, required for development of peripheral sensory and sympathetic neurons (Kalcheim et al, 1992; Ernfors et al, 1994)</td>
</tr>
</tbody>
</table>
Table 4.1b: Genes significantly upregulated in fibroblasts compared with osteoblasts, as identified by the ATLAS macroarray system, using arrays 3.1 and 3.2.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold regulation</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teratocarcinoma-derived growth factor 3</td>
<td>5</td>
<td>Required for midline and forebrain patterning in developing organisms, interacts with NODAL patterning signals in mouse (de la Cruz et al., 2002)</td>
</tr>
<tr>
<td>Thymosin, beta 10</td>
<td>4</td>
<td>Actin-sequestering protein (Lin and Morrison-Bogorad, 1991)</td>
</tr>
<tr>
<td>Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)</td>
<td>3</td>
<td>Transcription enhancer required for Immunoglobulin expression- deficiency leads to absence of B-cell development, Zhuang et al. (1994) Bain et al. (1994)</td>
</tr>
<tr>
<td>Myosin, light polypeptide 6</td>
<td>3</td>
<td>Motor-protein which travels along actin cytoskeletal filaments in the opposite direction to all other myosins. Required for cell migration (Rock et al. 2001; Geisbrecht and Montell, 2002)</td>
</tr>
</tbody>
</table>
Figure 4.2 shows one of the 19k microarray chips probed with fluorescently-labelled cDNA synthesized from osteoblasts and fibroblasts. As can be seen from the figure, spots containing cDNAs that are expressed in only one population of cells appear either red or green, while spots containing cDNAs expressed in both osteoblasts and fibroblasts are varying shades of yellow, with a greater intensity of red or green to reflect differential expression.

Overall, 64 genes were found to be regulated 5-fold or more- 37 were down-regulated in osteoblasts, and 27 were upregulated in osteoblasts, as shown in Table 4.2. Figure 4.3 shows a log plot of the data obtained from the microarray after Lowess normalisation was performed, and the data had been filtered to remove unreliable replicates, and genes with unreliable low levels of expression. A cut-off of 5-fold difference in expression levels was used to identify significantly regulated genes, as is marked by the two outer green diagonal lines, while the middle line indicates equivalent gene expression between the two RNA populations.
Figure 4.2: Microarray analysis of differences in gene expression between osteoblasts and fibroblasts. A) is an image of a microarray chip scanned in two channels. B) is a close up of an area of the same chip. The red spots represent genes expressed specifically in osteoblasts, the green spots represent genes expressed in fibroblasts, and the yellow spots represent genes expressed in both osteoblasts and fibroblasts.
Table 4.2a: Genes significantly upregulated in osteoblasts compared with fibroblasts, as identified by the 19k microarray system.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Description</th>
<th>Genbank number</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100 CALCIUM-BINDING PROTEIN, BETA (NEURAL)</td>
<td>71</td>
<td>Calcium binding protein</td>
<td>NM_005980</td>
</tr>
<tr>
<td>MATRIX METALLOPROTEINASE 19</td>
<td>32</td>
<td>Extracellular matrix remodelling</td>
<td>NM_022792</td>
</tr>
<tr>
<td>SERINE (OR CYSTEINE) PROTEINASE INHIBITOR, CLADE A (ALPHA-1 ANTIPROTEINASE, ANTITRYPSIN), MEMBER 6</td>
<td>31</td>
<td>Serine protease inhibitor</td>
<td>NM_001756</td>
</tr>
<tr>
<td>GROWTH ARREST-SPECIFIC 11</td>
<td>19</td>
<td>Cell-cycle arrest induced protein</td>
<td>AF050079</td>
</tr>
<tr>
<td>VAMP (VESICLE-ASSOCIATED MEMBRANE PROTEIN)-ASSOCIATED PROTEIN B AND C</td>
<td>19</td>
<td>Intracellular membrane protein, possibly involved in vesicle trafficking.</td>
<td>NM_004738</td>
</tr>
<tr>
<td>POLYMERASE (DNA DIRECTED), EPSILON</td>
<td>17</td>
<td>DNA polymerase subunit</td>
<td>NM_019896</td>
</tr>
<tr>
<td>FREQUENIN (DROSOPHILA) HOMOLOG</td>
<td>17</td>
<td>Calcium-dependent regulator of G protein-coupled receptor phosphorylation, possibly involved in neurosecretion; may substitute for calmodulin</td>
<td>NM_014286</td>
</tr>
<tr>
<td>DOPACHROME TAUTOMERASE (DOPACHROME DELTA-ISOMERASE, TYROSINE-RELATED PROTEIN 2)</td>
<td>9</td>
<td>Metabolic enzyme required for conversion of dopachrome into 5,6-dihydroxyindole-2-carboxylic acid</td>
<td>NM_001922</td>
</tr>
<tr>
<td>ZINC FINGER PROTEIN 137</td>
<td>9</td>
<td>Putative zinc finger protein</td>
<td>NM_003438</td>
</tr>
<tr>
<td>O-LINKED N-ACETYLGLUCOSAMINE TRANSFERASE (UDP-N-ACETYLGLUCOSAMINE POLYPEPTIDE-N-ACETYLGLUCOSAMINYL TRANSFERASE)</td>
<td>8</td>
<td>Catalyses glycosylation of serine and threonine residues</td>
<td>NM_181672</td>
</tr>
<tr>
<td>ALCOHOL DEHYDROGENASE 4 (CLASS)</td>
<td>8</td>
<td>Involved in metabolism of alcohols and steroids</td>
<td>NM_000670</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Accession</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>ELL-RELATED RNA POLYMERASE II, ELONGATION FACTOR</td>
<td>NM_012081</td>
<td>RNA polymerase subunit Member of a protein family including maternal transcript regulating proliferation of cells during zygotic development</td>
<td>NM_012081</td>
</tr>
<tr>
<td>SPINDLIN-LIKE</td>
<td>NM_019003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PREGNANCY-ASSOCIATED PLASMA PROTEIN A</td>
<td>NM_002581</td>
<td>Secreted metalloproteinase, cleaves IGF-BPs; involved in bone remodelling</td>
<td></td>
</tr>
<tr>
<td>EUKARYOTIC TRANSLATION INITIATION FACTOR 3, SUBUNIT 7 (ZETA, 66/67KD)</td>
<td>NM_003753</td>
<td>Subunit of the translation initiation complex Catalyzes the conversion of GTP to cGMP; main receptor for nitric oxide and nitrovasodilator drugs</td>
<td></td>
</tr>
<tr>
<td>GUANYLATE CYCLASE 1, SOLUBLE, ALPHA 3</td>
<td>NM_000856</td>
<td>Bind long-chain fatty acids; probably required for transport and cellular uptake</td>
<td></td>
</tr>
<tr>
<td>FATTY ACID BINDING PROTEIN 1, LIVER</td>
<td>NM_001443</td>
<td>Apoptosis inhibitor which operates by binding to Bcl family members and caspase regulators</td>
<td></td>
</tr>
<tr>
<td>CELL DEATH REGULATOR AVEN</td>
<td>AF283508</td>
<td>Integral membrane protein associated with presynaptic vesicles in neuronal cells; may be required for synaptic transmission</td>
<td></td>
</tr>
<tr>
<td>SYNAPTOGYRIN 1</td>
<td>BT007135</td>
<td>Binds PDGF-response element in promoter of MMP3; acts as an enhancer of JUN and SP1</td>
<td></td>
</tr>
<tr>
<td>TRANSCRIPTION FACTOR 20 (AR1)</td>
<td>NM_005650</td>
<td>Transmembrane receptor tyrosine kinase protein Integris-binding secreted extracellular matrix</td>
<td></td>
</tr>
<tr>
<td>EPHRIN-B3</td>
<td>NM_001406</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIBULIN 2</td>
<td>BC051690</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene Name</td>
<td>Fold Change</td>
<td>Description</td>
<td>Genbank number</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-------------</td>
<td>----------------</td>
</tr>
<tr>
<td>HEMOGLOBIN, ALPHA 1</td>
<td>5</td>
<td>Subunit of haemoglobin complex</td>
<td>NM_000558</td>
</tr>
<tr>
<td>COAGULATION FACTOR XIII, A1 POLYPEPTIDE</td>
<td>5</td>
<td>Catalytic subunit of coagulation factor, involved in crosslinking of fibrin and fibronectin</td>
<td>NM_000129</td>
</tr>
<tr>
<td>RAN BINDING PROTEIN 9</td>
<td>5</td>
<td>Binds GTPase RAN to permit transport of RNA and proteins through nuclear pore complex</td>
<td>NM_005493</td>
</tr>
<tr>
<td>HOAT4</td>
<td>5</td>
<td>Transporter of organic anions including estrone sulfate and dehydroepiandrosterone sulfate</td>
<td>AB026116</td>
</tr>
<tr>
<td>V-MYC AVIAN MYELOCYTOMATOSIS VIRAL ONCOGENE HOMOLOG</td>
<td>5</td>
<td>Activator of transcription</td>
<td>BC058901</td>
</tr>
</tbody>
</table>

Table 4.2b: Genes significantly upregulated in fibroblasts compared with osteoblasts, as identified by the 19k microarray system.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Description</th>
<th>Genbank number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPATITIS B VIRUS X-INTERACTING PROTEIN (9.6KD)</td>
<td>154</td>
<td>Alters replication of hepatitis B virus by inactivating hepatitis B protein X</td>
<td>NM_006402</td>
</tr>
<tr>
<td>SOLUTE CARRIER FAMILY 1 (NEURONAL/EPITHELIAL HIGH AFFINITY GLUTAMATE TRANSPORTER, SYSTEM XAG), MEMBER 1</td>
<td>127</td>
<td>Transports glutamate across membrane into the brain for neurotransmission</td>
<td>NM_004170</td>
</tr>
<tr>
<td>ZINC FINGER PROTEIN HOMOLOGOUS TO ZFP92 IN MOUSE</td>
<td>40</td>
<td>Zinc finger transcription factor protein</td>
<td>NM_152626</td>
</tr>
<tr>
<td>ARIADNE (DROSOPHILA) HOMOLOG 2</td>
<td>38</td>
<td>Nuclear protein upregulated during retinoic acid induced granulocytosis</td>
<td>BC000422</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Description</td>
<td>accession</td>
<td>Function</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>BROMODOMAIN ADJACENT TO ZINC FINGER DOMAIN, 2B</td>
<td>Putative role in chromatin-dependent transcription regulation</td>
<td>NM_013450</td>
<td></td>
</tr>
<tr>
<td>CD24 ANTIGEN (SMALL CELL LUNG CARCINOMA CLUSTER 4 ANTIGEN)</td>
<td>Cell surface signal transducer, which regulates B-cell behaviour, including apoptosis</td>
<td>NM_013230</td>
<td></td>
</tr>
<tr>
<td>KARYOPHERIN BETA 2B, TRANSPORTIN</td>
<td>Participates in mRNA transport from the nucleus</td>
<td>NM_013433</td>
<td></td>
</tr>
<tr>
<td>FLAVIN CONTAINING MONOOXYGENASE 5</td>
<td>Involved in metabolism of drugs, pesticides, and amino acids</td>
<td>NM_001461</td>
<td></td>
</tr>
<tr>
<td>AMPLIFIED IN OSTEOSARCOMA</td>
<td>Function unknown</td>
<td>NM_006812</td>
<td></td>
</tr>
<tr>
<td>EHM2 GENE</td>
<td>Function unknown</td>
<td>AB032179</td>
<td></td>
</tr>
<tr>
<td>CEREBRAL CELL ADHESION MOLECULE</td>
<td>Function unknown</td>
<td>AF177203</td>
<td></td>
</tr>
<tr>
<td>BROMODOMAIN-CONTAINING 2</td>
<td>Mitogen activated kinase implicated in growth control</td>
<td>NM_005104</td>
<td></td>
</tr>
<tr>
<td>SULFOTRANSFERASE, ESTROGEN-PREFERRING</td>
<td>Transfers sulfo moiety to and from estrone, in metabolism of hormones and drugs</td>
<td>NM_005420</td>
<td></td>
</tr>
<tr>
<td>UDP-N-ACETYL-ALPHA-D-GALACTOSAMINEPOLYPEPTIDE N-ACETYLGLACTOSAMINYLTRANSFERASE 7 (GALNAC-T7)</td>
<td>Required for O-glycosylation of certain proteins</td>
<td>NM_017423</td>
<td></td>
</tr>
<tr>
<td>PHOSPHATIDYLINOSITOL GLYCAN, CLASS H</td>
<td>Required for glycosylphosphatidylinositol-anchor biosynthesis on the cell membrane</td>
<td>NM_004569</td>
<td></td>
</tr>
<tr>
<td>NUCLEOLAR PHOSPHOPROTEIN P130</td>
<td>Binds GTP/ATP to act as GTPase/ATPase during mitosis</td>
<td>Z34289</td>
<td></td>
</tr>
<tr>
<td>SWI/SNF RELATED, MATRIX ASSOCIATED, ACTIN DEPENDENT REGULATOR OF CHROMATIN, SUBFAMILY E, MEMBER 1</td>
<td>Component of SWI/SNF chromatin remodelling complex</td>
<td>NM_003079</td>
<td></td>
</tr>
<tr>
<td>HYPOXIA-INDUCIBLE PROTEIN 2</td>
<td>Transcription factor which mediates hypoxia</td>
<td>NM_013332</td>
<td></td>
</tr>
<tr>
<td>Gene Name</td>
<td>Function</td>
<td>Gene ID</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>STEROL-C4-METHYL OXIDASE-LIKE</td>
<td>induced gene transcription through the hypoxic response element</td>
<td>NM_006745</td>
<td></td>
</tr>
<tr>
<td>HHDC FOR HOMOLOG OF DROSOPHILA HEADCASE</td>
<td>Contains metal-binding domains; putatively implicated in cholesterol biosynthesis</td>
<td>NM_016217</td>
<td></td>
</tr>
<tr>
<td>INHIBIN, BETA B (ACTIVIN AB BETA POLYPEPTIDE)</td>
<td>Human function unknown; highly expressed by some cancer types; required for adult development in Drosophila</td>
<td>NM_002193</td>
<td></td>
</tr>
<tr>
<td>PLEIOMORPHIC ADENOMA GENE 1</td>
<td>Developmentally regulated zinc finger protein</td>
<td>NM_002655</td>
<td></td>
</tr>
<tr>
<td>MESODERM DEVELOPMENT CANDIDATE 2</td>
<td>Required to establish embryonic polarity, and induce mesoderm differentiation</td>
<td>XM_370880</td>
<td></td>
</tr>
<tr>
<td>MAJOR HISTOCOMPATIBILITY COMPLEX, CLASS II, DQ ALPHA 1</td>
<td>Leukocyte antigen determinant</td>
<td>XM_371812</td>
<td></td>
</tr>
<tr>
<td>CYCLIN A2</td>
<td>Activates CDK kinases to promote transition through G1/S and G2/M during cell cycle</td>
<td>NM_001237</td>
<td></td>
</tr>
<tr>
<td>INTERLEUKIN ENHANCER BINDING FACTOR 2, 45KD</td>
<td>Binds to NFAT transcription factor and enhances gene expression</td>
<td>NM_004515</td>
<td></td>
</tr>
<tr>
<td>FIBROUSHEATHIN II</td>
<td>Putative sperm fibrous sheath protein</td>
<td>AF088868</td>
<td></td>
</tr>
<tr>
<td>BUTYROPHILIN, SUBFAMILY 3, MEMBER A2</td>
<td>Basic leucine zipper transcription factor required for cellular detoxification and glutathione synthesis</td>
<td>NM_007047</td>
<td></td>
</tr>
<tr>
<td>NUCLEAR FACTOR (ERYTHROID-DERIVED 2)-LIKE 2</td>
<td>Intracellular thiol protease inhibitor,</td>
<td>NM_000100</td>
<td></td>
</tr>
</tbody>
</table>

119
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD33 ANTIGEN (GP67)</td>
<td>NM_001772</td>
<td>Membrane glycoprotein expressed specifically by cells of haematopoietic lineage</td>
</tr>
<tr>
<td>CASPASE 8, APOPTOSIS-RELATED CYSTEINE PROTEASE</td>
<td>NM_001228</td>
<td>Cysteine aspartic acid protease implicated in apoptosis, especially of neuronal cells</td>
</tr>
<tr>
<td>LYMPHOCYTE ADAPTOR PROTEIN</td>
<td>NM_005475</td>
<td>Links cell-surface receptors to intracellular signalling pathways</td>
</tr>
<tr>
<td>ELLIS VAN CREVELD SYNDROME</td>
<td>NM_014556</td>
<td></td>
</tr>
<tr>
<td>APOLIPOPROTEIN B (INCLUDING AG(X) ANTIGEN)</td>
<td>NM_000384</td>
<td>Main apolipoprotein of chylomicrons and LDL lipoproteins</td>
</tr>
<tr>
<td>HELICASE-MOI</td>
<td>AB028449</td>
<td>RNA helicase required to produce small RNA molecules used to repress gene expression</td>
</tr>
<tr>
<td>ZINC FINGER PROTEIN 36 (KOX 18)</td>
<td>XM_168302</td>
<td>Zinc finger transcription factor protein</td>
</tr>
</tbody>
</table>
Figure 4.3: Log plot of the expression levels obtained from the osteoblast vs fibroblast 19k microarray comparison experiment. The graph shows only those genes found to be significantly regulated between the two cell types, using a significance cut-off of 5-fold (represented by the two outer diagonal green lines). The middle green diagonal line shows where unregulated genes, with equivalent expression levels, would lie. All data shown have undergone Lowess normalisation, and genes with unreliably low levels of expression have been filtered out. The x-axis shows the log of the expression level in the osteoblast RNA population, while the y-axis show the log of the expression level in the fibroblast population. Analysis was performed by, and the graph imported from, Genespring v6 (Silicon Genetics).
To confirm that the results obtained from the array experiments were consistent with previously published information, the gene lists were examined to identify any genes known to be preferentially expressed by osteoblasts compared with fibroblasts. Of those genes checked for (osteocalcin, osteopontin, osteonectin, various collagens, CBFA1, and osterix) none were significantly regulated on the microarray used, if they were even present.

Table 4.3 shows the list of genes found to be differentially regulated between osteoclasts and macrophages in the ATLAS macroarray experiments. Out of the 3528 genes examined, 17 were found to be significantly downregulated in osteoclasts, and 53 were found to be significantly upregulated in osteoclasts.

A number of the regulated genes were demonstrated to be involved in activation or modulation of transcription either as transcription factors or transcription factor subunits. Nuclear Factor of Activated T-cells, Cytoplasmic 1 (NFATc1) was present on both Atlas 1.2 I and II arrays, showing greater than 16 fold up regulation. Early growth response factor 1 (EGR1) was up regulated to a similar level to NFATc1. Zinc Finger Protein 33a was the most differentially expressed transcription factor, down regulated 115 fold in osteoclasts compared with macrophages (Table 4.3b).

Cytokines and cell surface proteins were also commonly regulated between osteoclasts and macrophages (Table 4.3a and b). The GM-CSF receptor (or Colony stimulating factor 2 receptor alpha: CSF2Rα) and HLA-C show the greatest
Table 4.3: Genes revealed as being significantly upregulated in osteoclasts compared with macrophages, as identified by the ATLAS human macroarray system.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold regulation</th>
<th>Gene function</th>
<th>Genbank number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF receptor (CSFR2) a, low-affinity</td>
<td>43.2</td>
<td>Cell surface or cytokine related protein</td>
<td>17648</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 7</td>
<td>30.1</td>
<td>Component of the cyclin-dependent kinase activating kinase complex, required for phosphorylation of CDKs to permit progression through the cell cycle (Fisher and Morgan, 1994)</td>
<td>L20320</td>
</tr>
<tr>
<td>Calmodulin 1 (phosphorylase kinase, delta)</td>
<td>27</td>
<td>Required for signal transduction through the calcium/calmodulin dependent protein kinase 1 signalling pathway (Chin et al, 1997)</td>
<td>M27319</td>
</tr>
<tr>
<td>Nuclear factor of activated T-cells, cytoplasmic 1 (1.2 I)</td>
<td>23.3</td>
<td>Binds to elements in the promoters of cytokines to facilitate gene expression. Activated by dephosphorylation, performed by calcineurin (Park et al, 1996; Arumburu et al, 1998)</td>
<td>U08015</td>
</tr>
<tr>
<td>40S ribosomal protein S20 (RPS20)</td>
<td>19.8</td>
<td>Ribosomal subunit protein</td>
<td>L06498</td>
</tr>
<tr>
<td>Far upstream element (FUSE) binding protein 1 (FBP)</td>
<td>17.3</td>
<td>FUSE binding protein interacts with the far upstream element (FUSE) in the promoter of the MYC oncogene. When activated, FUSE stimulates differentiation of undifferentiated cell populations (Duncan et al, 1994)</td>
<td>U05040</td>
</tr>
<tr>
<td>Nuclear factor of activated T cells, 90kD (ILF-3)</td>
<td>16.4</td>
<td>Binds to elements in the promoters of cytokines to facilitate gene expression. Activated by dephosphorylation, performed by calcineurin (Park et al, 1996; Arumburu et al, 1998)</td>
<td>U10324</td>
</tr>
<tr>
<td><strong>Nuclear factor of activated T-cells, cytoplasmic 1 (1.2 II)</strong></td>
<td><strong>16.2</strong></td>
<td>Binds to elements in the promoters of cytokines to facilitate gene expression. Activated by dephosphorylation, performed by calcineurin (Park et al, 1996; Arumburu et al, 1998)</td>
<td>U08015</td>
</tr>
<tr>
<td><strong>cadherin 5, VE-cadherin (vascular epithelium)</strong></td>
<td><strong>16.1</strong></td>
<td>Calcium-dependent adhesion protein required for endothelial cell-cell contact, and endothelial cell survival via the b-catenin signalling pathway (Huber et al, 1996; Carmeliet et al, 1999)</td>
<td>X79981</td>
</tr>
<tr>
<td><strong>Interleukin enhancer binding factor 3</strong></td>
<td><strong>16</strong></td>
<td>Phosphorylated RNA binding protein, proposed to regulate gene expression at the post-transcriptional stage by binding to double-stranded RNA/DNA hybrids and interfering with transcription elongation (Patel et al, 1999; Saunders et al, 2001)</td>
<td></td>
</tr>
<tr>
<td><strong>GA-BP transcription factor, alpha subunit 60kD</strong></td>
<td><strong>15.5</strong></td>
<td>DNA binding protein involved in gene transcription. Upregulates expression of nuclear-encoded transcription factor TFAM, which triggers transcription of mitochondrial genes, including cytochrome C oxidase (Virbasius and Scarpulla, 1994)</td>
<td>D13318</td>
</tr>
<tr>
<td><strong>adenosine A1 receptor</strong></td>
<td><strong>15.1</strong></td>
<td>G-protein coupled receptor required in the mediation of adenosine signalling, which in turns regulates cardiac rate and contractility, smooth muscle tone, sedation, release of neurotransmitters, platelet function, lipolysis, renal function, and white blood cell function (Libert et al, 1991; Stiles 1992)</td>
<td>S56143</td>
</tr>
<tr>
<td><strong>TNF (ligand) superfamily, member 7 (CD70)</strong></td>
<td><strong>14.6</strong></td>
<td>Receptor involved in TNF signalling, to induce apoptosis and CD4 and CD8 positive T cell expansion during infection (Prasad et al, 1997; Hendricks et al, 2000)</td>
<td>L08096</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Gene ID</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>---------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>A kinase (PRKA) anchor protein 5</td>
<td>14.4</td>
<td>Anchors protein kinase A near the synapse, where it is required for neurotransmission (Carr et al., 1991; Carr et al., 1992)</td>
<td></td>
</tr>
<tr>
<td>CD44 antigen</td>
<td>14.3</td>
<td>Transmembrane glycoprotein receptor which functions as a cellular-Extracellular matrix adhesion molecule. Known ligands include hyaluronate and osteopontin, also implicated in limb bud development (Krainer et al., 1991; Weber et al., 1996; Sherman et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>Tumour necrosis factor (ligand) superfamily,</td>
<td>14</td>
<td>Receptor involved in TNF signalling, to induce apoptosis and CD4 and CD8 positive T cell expansion during infection (Prasad et al., 1997; Hendricks et al., 2000)</td>
<td></td>
</tr>
<tr>
<td>member 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early growth response 1</td>
<td>13.3</td>
<td>Tumour supressor gene which regulates transcription of TGF b and fibronectin, and is required for p53-mediated apoptosis (Liu et al., 1999; Das et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Discs, large (Drosophila) homolog 1</td>
<td>12.8</td>
<td>Interacts with cytoskeletal proteins, p56ck tyrosine kinase, and Shaker type Kv1.3 voltage-gated potassium channel. Proposed role in coupling tyrosine kinase to the ion channel and membrane (Lue et al., 1994; Hanada et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>LIM homeobox protein 1</td>
<td>12.5</td>
<td>Transcription regulatory protein required for patterning of the head regions, and axonal guidance in the limb of developing embryos (Dong et al., 1997; Shawlot and Behringer, 1995; Kania et al., 2000)</td>
<td></td>
</tr>
<tr>
<td>Potassium voltage-gated channel, shaker-related subfamily member 2</td>
<td>12</td>
<td>Component of the complex required for transmission of action potentials across the synapse, and post-transmission recovery, in neuronal signalling (Ramaswami et al., 1990)</td>
<td>L02752</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Score</td>
<td>Description</td>
<td>Accession</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Colony stimulating factor 3 receptor</td>
<td>12</td>
<td>Transmembrane receptor for Granulocyte colony stimulating factor, secreted by macrophages exposed to inflammatory challenge. G-CSF activates granulocyte proliferation through the STAT signalling pathway. A portion of the receptor is required for negative feedback inhibition of STAT mediated proliferation (McLemore et al, 1998; Dong et al, 2001)</td>
<td>M59818</td>
</tr>
<tr>
<td>Potassium voltage-gated channel, shaker-related subfamily, 2</td>
<td>11.9</td>
<td>Component of the complex required for transmission of action potentials across the synapse, and post-transmission recovery, in neuronal signalling (Ramaswami et al, 1990)</td>
<td>L02752</td>
</tr>
<tr>
<td>G-CSF receptor (CSF3R)</td>
<td>11.8</td>
<td>Cell surface or cytokine related protein</td>
<td>M59818</td>
</tr>
<tr>
<td>Tuberous sclerosis 2</td>
<td>11.6</td>
<td>Shown to stimulate GTPase activity of Rab5, which is critical for endosomal docking and sorting, and complexes with tuberous sclerosis 1 to inhibit ribosomal activity and translation elongation factor 4 activity (Xiao et al, 1997; Inoki et al, 2002)</td>
<td>X75621</td>
</tr>
<tr>
<td>small inducible cytokine A5 (RANTES)</td>
<td>11.3</td>
<td>Interacts with receptor CCR5 to regulate cell survival and differentiation in the forebrain of the developing foetus during the first trimester. Also impairs replication of HIV, as it is a natural ligand of the CCR5 receptor, which is required as a co-receptor by HIV during infection (Bakhiet et al, 2001; Liu et al, 1999)</td>
<td>M21121</td>
</tr>
<tr>
<td>Thrombospondin 2</td>
<td>11</td>
<td>Multipurpose protein containing binding sites for thrombin, fibrinogen, heparin, fibronectin, plasminogen, plasminogen activator, collagen, laminin, and other extracellular matrix proteins. Involved in cell migration and adhesion,</td>
<td>L12350</td>
</tr>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Gene ID</strong></td>
<td><strong>Description</strong></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 2</td>
<td>U03882</td>
<td>7-span transmembrane G-protein coupled receptor which activates signalling pathways required for leukocytes activation and migration in response to immune challenge. Also implicated in formation of atherosclerotic plaques (Charo et al, 1994; Combadiere et al, 1995)</td>
<td></td>
</tr>
<tr>
<td>Caspase 10, apoptosis-related cysteine protease</td>
<td>U60519</td>
<td>Involved in programmed cell death. Inactive precursor is cleaved to form subunits which dimerize to form the active complex. Responsible for apoptosis-related degradation of poly(ADP-ribose) polymerase, and binds FADD, CD95 and TNFR1 death receptors (Fernandes-Alnemri et al, 1996; Vincenz and Dixit, 1997)</td>
<td></td>
</tr>
<tr>
<td>PCTAIRE protein kinase 1</td>
<td>X66363</td>
<td>Serine/threonine protein kinase related to cdc2/CDC28 cell cycle regulating protein kinases (Meyyerson et al, 1992)</td>
<td></td>
</tr>
<tr>
<td>Integrin, alpha L (antigen CD11A (p180))</td>
<td>Y00796</td>
<td>Expressed on the surface of lymphocytes and phagocytic cells, and mediates attachment of cytotoxic T cells, and migration and adherence of granulocytes, monocytes, and B- and T-lymphocytes (Marlin et al, 1986; Lu and Cyster, 2002)</td>
<td></td>
</tr>
<tr>
<td>Transformation/transcription domain-associated protein</td>
<td></td>
<td>Required in early embryonic development for blastocyst proliferation. Required for ckd1 expression, to maintain cell cycle checkpoint integrity and mitotic arrest in the case of aberrant cytokinesis or chromosomal missegregation during proliferation (Herceg et al, 2001)</td>
<td></td>
</tr>
<tr>
<td>DNA-directed RNA polymerases 1,2,3, 7kD polypeptide</td>
<td>9</td>
<td>Transcriptional protein required for gene expression (Cramer et al, 2000)</td>
<td></td>
</tr>
<tr>
<td>v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3</td>
<td>9</td>
<td>Heresulin receptor with tyrosine kinase activity capable of autophosphorylation (Carraway et al, 1994)</td>
<td></td>
</tr>
<tr>
<td>Discoidin domain receptor family, member 1</td>
<td>9</td>
<td>Receptor tyrosine kinase which binds nerve growth factor in non-neuronal tissues (DiMarco et al, 1993)</td>
<td></td>
</tr>
<tr>
<td>Integrin, alpha E (antigen CD103)</td>
<td>9</td>
<td>Adhesion molecule required for epithelial and intestinal lamina distribution of T-cells (Schon et al, 1999)</td>
<td></td>
</tr>
<tr>
<td>TNF superfamily, member 1 (TNF-b)</td>
<td>8.7</td>
<td>Cell surface or cytokine related protein</td>
<td></td>
</tr>
<tr>
<td>Neurogranin</td>
<td>8</td>
<td>Neuronal protein phosphorylated by protein kinase C, which binds to calmodulin in the absence of calcium, and is regulated at the transcriptional level by thyroid hormone (Baudier et al, 1991; Martinez de Arrieta et al, 1999)</td>
<td></td>
</tr>
<tr>
<td>Lymphotoxin alpha (TNF superfamily member 1)</td>
<td>8</td>
<td>Mediator of TNF signalling in immune response; also implicated in the formation of atherosclerotic plaques, as a result of its ability to upregulate expression of adhesion molecules including V-CAM (Ozaki et al, 2002)</td>
<td></td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>8</td>
<td>Required for synaptic transmission in nerves and skeletal muscle; exposure to stressors results in rapid and long-lasting changes in the acetylcholinesterase isoform expression profile in the brain and at synapses (Feng et al, 1999; Meshorer et al, 2002)</td>
<td></td>
</tr>
<tr>
<td>Transcription factor AP-2 alpha</td>
<td>8</td>
<td>Activator of transcription regulated developmentally, and by induction with retinoic acid. Required for cranial closure and body patterning of the</td>
<td></td>
</tr>
<tr>
<td>Protein Name</td>
<td>GI Number</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-----------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>abdomen, cranium, and neural tube (Zhang et al, 1996; Schorle et al, 1996)</td>
<td>8</td>
<td>Required for normal spermatogenesis, germ cell apoptosis and male fertility in mice (Dix et al, 1996)</td>
<td></td>
</tr>
<tr>
<td>Interleukin 5 (eosinophil colony stimulating factor)</td>
<td>8</td>
<td>Main regulator of B-cell and eosinophil growth and differentiation, required for protection against parasite infection, and respiratory and digestive infection (Yokota et al, 1987; Rodrigues et al, 1996)</td>
<td></td>
</tr>
<tr>
<td>SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4</td>
<td>8</td>
<td>Part of the human homolog of the yeast swi/SNF transcription complex. Contains a DNA-dependent ATPase which can rescue mitosis in swi-/- yeast cells. Required at the perimplantation stage of murine development (Khavari et al, 1993; Bultman et al, 2000)</td>
<td></td>
</tr>
<tr>
<td>Kruppel-like factor 5 (intestinal)</td>
<td>8</td>
<td>Transcription factor which activates transcription of platelet-derived growth factor A, which is required for formation of the gastrointestinal tract, and also for cardiac remodelling in response to stress (Shindo et al, 2002)</td>
<td></td>
</tr>
<tr>
<td>interleukin 5 (CSF-eosinophil)</td>
<td>7.8</td>
<td>Cell surface or cytokine related protein X04688</td>
<td></td>
</tr>
<tr>
<td>DNA-directed RNA polymerases 2, 14.4kD polypeptide</td>
<td>7</td>
<td>Yeast homolog forms part of a clamp holding the DNA for access for the RNA transcription complex to bind and transcribe (Cramer et al, 2000)</td>
<td></td>
</tr>
<tr>
<td>Discs (large) homolog 2 (chapsyn-110)</td>
<td>7</td>
<td>Guanylate kinase which interacts with PSD95 to form a multisubunit complex which anchors voltage-gated and ligand-dependent channels and receptors to the foci of incoming signals and synapses (Kim et al, 1995; Kim et al, 1996)</td>
<td></td>
</tr>
<tr>
<td>Transcription factor 6-like 1</td>
<td>7</td>
<td>Nuclear-encoded transcription factor required for bi-</td>
<td></td>
</tr>
<tr>
<td>Gene name</td>
<td>Fold regulation</td>
<td>Gene function</td>
<td>Genbank number</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>----------------</td>
<td>---------------------------------------------------------------------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase</td>
<td>7</td>
<td>Protein required for negative feedback inhibition of TNF alpha. Induced by the same stimuli as TNF alpha, this protein binds to the AU-rich element in the mRNA of TNF alpha and destabilises it, thus limiting the amount of translation occurring from any one transcript, and minimising the negative effects of high levels of TNF alpha (Carballo et al, 1998)</td>
<td></td>
</tr>
<tr>
<td>Zinc finger protein homologous to Zip-36 in mouse</td>
<td>7</td>
<td>Contains a metalloenzyme required to cleave NEDD8 from the ubiquitin ligase complex for protein targetting for degradation (Cope et al, 2002)</td>
<td></td>
</tr>
<tr>
<td>COP9 (constitutive photomorphogenic) subunit 5</td>
<td>7</td>
<td>Motor protein involved in cytokinesis and sarcomere contraction (Collins et al, 1992)</td>
<td></td>
</tr>
<tr>
<td>integrin, alpha 3 (antigen CD49C)</td>
<td>6.2</td>
<td>Cell surface or cytokine related protein</td>
<td>M59911</td>
</tr>
<tr>
<td>TNF (TNF superfamily, member 2) (TNF-a)</td>
<td>5.1</td>
<td>Cell surface or cytokine related protein</td>
<td>X01394</td>
</tr>
</tbody>
</table>

Table 4.3b: Genes revealed as being significantly downregulated in osteoclasts compared with macrophages, as identified by the ATLAS human macroarray system.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Entry Number</th>
<th>Description</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine decarboxylase antizyme (ODC-AZ)</td>
<td>147</td>
<td>Antizyme is induced by high levels of polyamine synthesis within the cell, which results in translational frameshifting from the default rate to a more efficient level of codon usage. Ornithine decarboxylase is the key enzyme of polyamine biosynthesis, and binding to antizyme targets the enzyme to the 26S ribosomal subunit for destruction, in addition to inhibiting cellular polyamine uptake (Rom and Kahana, 1994; Matsufuji et al, 1995; Matsufuji et al, 1996)</td>
<td>D78361</td>
</tr>
<tr>
<td>Zinc finger protein 33A (ZNF KOX31)</td>
<td>115</td>
<td>Putative transcription factor</td>
<td>X68687</td>
</tr>
<tr>
<td>60S ribosomal protein L17 (RPL17)</td>
<td>96.1</td>
<td>Ribosomal protein (Mager and Freeman, 1990)</td>
<td>X53777</td>
</tr>
<tr>
<td>X-ray repair complementing defective repair 1</td>
<td>22.6</td>
<td>Interacts with polynucleotide kinase, DNA polymerase-beta and DNA ligase III to repair single-strand DNA breaks caused by reactive oxygen species and ionizing radiation (Whitehouse et al, 2001)</td>
<td>M36089</td>
</tr>
<tr>
<td>Alpha-synuclein</td>
<td>15.9</td>
<td>Pre-synaptic negative regulator of dopamine signalling through the neurological system (Abeliovitch et al, 2000)</td>
<td>L08850</td>
</tr>
<tr>
<td>Deltex</td>
<td>15.4</td>
<td>Negative regulator of the Notch signalling pathway, a highly conserved pathway implicated in differentiation of a wide variety of cell types (Izon et al, 2002)</td>
<td>AF053700</td>
</tr>
<tr>
<td>40S ribosomal protein S20</td>
<td>14</td>
<td>Ribosomal protein (Chu et al, 1993)</td>
<td></td>
</tr>
<tr>
<td>Histocompatibility Complex class I C (HLAC)</td>
<td>11.9</td>
<td>Protein involved in antigenic display-polymorphisms within this gene determine whether it binds to a pro-inflammatory or inhibitory signalling pathway receptor (Kostyu et al, 1997; Yen et al, 2001)</td>
<td>M11886</td>
</tr>
<tr>
<td>REL-B</td>
<td>11.4</td>
<td></td>
<td>M83221</td>
</tr>
<tr>
<td>v-rel avian reticuloendotheliosis viral oncogene homolog B</td>
<td>11</td>
<td>Binds to p50 or p52 to form the NFKB transcriptional activation complex (Bours et al, 1994)</td>
<td>M83221</td>
</tr>
<tr>
<td>Protein arginine N-methyltransferase 3</td>
<td>10.9</td>
<td>Required for post-translational methylation of arginine residues,</td>
<td>AF059531</td>
</tr>
<tr>
<td>Protein/Factor</td>
<td>Score</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------------</td>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>putatively histone ribonucleoproteins (Katsanis et al, 1997)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-hydroxytryptamine (serotonin) receptor 3A</td>
<td>10</td>
<td>Ligand-gated ion channel receptor which opens upon binding of the neurotransmitter serotonin to binding site (Miyake et al, 1995)</td>
<td></td>
</tr>
<tr>
<td>Hepatic leukemia factor</td>
<td>10</td>
<td>Produces a basic heli-loop-helix transcription factor as a result of a chromosomal translocation between chromosomes 17 and 19. Interferes with thymic and splenic development, resulting in T-cells with a high susceptibility to malignant transformation (Inaba et al, 1993; Honda et al, 1999; Smith et al, 1999)</td>
<td></td>
</tr>
<tr>
<td>Purkinje cell protein 4</td>
<td>8</td>
<td>Expressed in foetal brain tissue- over-expression possibly contributes to Downs syndrome phenotype (Cabin et al, 1996; Chen et al, 1996)</td>
<td></td>
</tr>
<tr>
<td>60S ribosomal protein L27A</td>
<td>7</td>
<td>Ribosomal protein (Frigerio et al, 1995)</td>
<td></td>
</tr>
<tr>
<td>Protein kinase C-like 1</td>
<td>7</td>
<td>Putative mediator of rho-GTPase signalling (Amano et al, 1996)</td>
<td></td>
</tr>
</tbody>
</table>
regulation in this group, more than 40 fold up for CSF2Rα and more than 40 fold down for HLA-C. Other CSF related genes (CSF3R and IL-5) were also expressed more highly by osteoclasts, as were three TNF related genes including CD70 which, at 14 fold, was the most strongly regulated cytokine between osteoclasts and macrophages.

Further work was performed by other members of the research group to verify the gene expression difference between the two cell populations as demonstrated by the macroarray experiment (Day et al, 2004). Intro-spanning primers were designed (Appendix 3), and real-time PCR was performed on eight genes from the array, using two different preparations of cells from different donors. Six of the genes that were up regulated, one gene that was not regulated and one gene that was strongly down regulated in osteoclasts were selected for verification by real-time PCR. All eight genes showed comparable levels of differences in fold regulation between osteoclasts and macrophages when Real-time PCR results were compared to the array result (correlation coefficient 0.98). The genes verified were: c-Jun, ZFP33a, CD44, NF90, Calmodulin 1, GABP-α, CSF2Rα and NFATc1 (Table 4.4). The average difference in the estimated ΔΔCt value over the seven genes was 0.3 cycles, confirming that the data was reproducible in different cell preparations. These data, coupled with the similarity in array and Q-PCR data, indicate reproducibility in the differentiation model and a high correlation between array data and Q-PCR data in the eight genes analysed, suggesting that the array data for the other genes are reliable.

A time-course experiment was also performed to map changes in expression levels of six transcription factors over the three week differentiation course of osteoclasts in this model. NFATc1, NFAT90 (ILF3), KOX31, FBP, GABPα and c-Jun all showed
up-regulation at the three week time point by both array analysis and real-time PCR. The time course data showed different kinetics of induction, with NFATc1, GABPα and NF90 demonstrating higher expression levels later in differentiation, while FBP and c-Jun expression increased earlier in the differentiation process, and then decreased (Figure 4.4).

From the results of the macroarray, which suggested that NFATc1 was involved in regulating osteoclastogenesis, a further experiment using cyclosporin to inhibit of NFATc1 was performed to study the effects of NFATc1 on differentiation of osteoclasts. Differentiating cells were treated with cyclosporin A (seven different concentrations between 10-1000 ng/ml) over three week culture, which resulted in atypical cell morphology. Rather than differentiating into TRAP positive multinucleated giant cells, the cells decreased in size across the dose response until at 1000ng/mL the majority of cells were mono-nuclear and TRAP positive (Figure 4.5).

These data reflect the observations of Ishida et al, who performed a similar experiment in the mouse RAW264.7 cell model, and found that cyclosporin repressed the formation of multinucleated cells, resulting in a majority of mononuclear cells that were TRAP positive. This suggested that NFATc1 might be a late transcription factor in the osteoclast differentiation cascade, involved in multi-nucleation. This was tested using real-time PCR to monitor the expression levels of three osteoclast markers, TRAP, cathepsin K, and calcitonin receptor across the dose response course.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Array</th>
<th>Real-time PCR 1</th>
<th>Real-time PCR 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Jun</td>
<td>1.45</td>
<td>1.52</td>
<td>2.3</td>
</tr>
<tr>
<td>Calmodulin 1</td>
<td>27.0</td>
<td>22.1</td>
<td>20.5</td>
</tr>
<tr>
<td>CD44</td>
<td>14.3</td>
<td>9.9</td>
<td>8.9</td>
</tr>
<tr>
<td>CSF2Rα</td>
<td>43.2</td>
<td>21.7</td>
<td>21.2</td>
</tr>
<tr>
<td>GABPα</td>
<td>15.5</td>
<td>24.3</td>
<td>22.3</td>
</tr>
<tr>
<td>NF90</td>
<td>16.4</td>
<td>12.7</td>
<td>11.0</td>
</tr>
<tr>
<td>NFATc1</td>
<td>23.3, 16.2</td>
<td>27.8</td>
<td>24.7</td>
</tr>
<tr>
<td>ZFP 33a</td>
<td>-115</td>
<td>-455</td>
<td>-670</td>
</tr>
</tbody>
</table>

Table 4.4: Verification by real-time PCR of fold differences between osteoclasts and macrophages as shown by macroarray analysis using the ATLAS human array system. The two sets of real-time PCR were performed on different preparations of cells, obtained from different donors (Table adapted from Day et al, 2004)
Treatment with cyclosporin was shown to cause a profound and dose dependent down
regulation of calcitonin receptor, while expression levels of cathepsin K and TRAP
remained unchanged. These data suggested that different mechanisms of regulation
are employed to control expression of cathepsin K, TRAP and calcitonin receptor, and
that NFATc1 is necessary for calcitonin receptor expression, but not cathepsin K or
TRAP expression (Figure 4.6).

The 20-fold induction of GM-CSF receptor (CSF2Rα) in osteoclasts led to further
investigations into the role of GM-CSF in osteoclastogenesis. Continuous exposure of
RANKL and M-CSF treated cells to GM-CSF blocked osteoclast differentiation in a
dose dependent manner. The expression of TRAP and cathepsin K was investigated in
cells treated with high levels of GM-CSF, where no visible osteoclasts had formed.
Cathepsin K and TRAP expression was examined using real-time PCR, which showed
that cells treated with GM-CSF, RANKL and M-CSF compared with cells treated
with RANKL and M-CSF had much lower levels of Cathepsin K and TRAP
expression (Figure 4.7).
Figure 4.4: Expression of transcription factors identified by ATLAS macroarray as being significantly regulated between osteoclasts and macrophages at three time-points of osteoclast differentiation: 3 days post-RANK-L addition, 1 week post-RANKL addition, and 3 weeks post-RANK-L addition.
Figure 4.5: Inhibition of osteoclast formation by Cyclosporin A. All pictures show cells treated with RANK-L and M-CSF, the osteoclast differentiation protocol used in this model. A) shows the effect of the addition of 10ng/mL Cyclosporin A., B) shows the effects of adding 50ng/mL Cyclosporin A, C) of adding 250ng/mL Cyclosporin A, and D) 1000ng/mL Cyclosporin A (From Day et al, 2004).
4.3 Discussion

From the overall microarray and macroarray data, the osteoblast and fibroblast cell populations used appear to be remarkably similar. Only a small number of genes were found to be significantly differentially regulated between osteoblasts and fibroblasts (75 out of 21352 genes assayed using both microarrays and macroarrays). When a gene known to be expressed by osteoblasts, osteonectin, was examined, it was found that across eight target samples, analysed data from all eight spots varied only by 0.8 fold. Although regulation of this gene was not found to be statistically significant, at least this point suggests that hybridization ratios from different target spots are reasonably reproducible. Unfortunately the exact expression levels of osteonectin in osteoblasts compared with fibroblasts are not known. It is, however, known that osteoblasts express high levels of osteonectin as a structural element of bone. The lack of significant regulation of this gene according to statistical analysis of our microarray data suggests several possibilities: Firstly, the microarray underestimates gene expression differences, and so the analysis methods used are too stringent to detect minor changes in fold regulation; or, that fibroblasts express osteonectin at higher levels than would be expected from what is known about osteonectin expression in bone. In any event, the behaviour of osteonectin suggests that hybridization data may be reproducible.
Figure 4.6: Effects of treatment with Cyclosporin A on expression of Cathepsin K, TRAP, and calcitonin receptor, as relative fold compared with M-CSF and RANKL treated control cells (osteoclasts). The hatched columns are cathepsin K (cross hatching) and TRAP (vertical hatching) expression levels, showing no repression in response to Cyclosporin A treatment. Calcitonin receptor expression levels are shown by the solid columns, and demonstrate profound repression of calcitonin receptor expression in response to Cyclosporin A treatment.
Figure 4.7: Inhibition of cathepsin K (CTSK) and TRAP expression by GM-CSF in the continuous presence of M-CSF and RANK-L for three weeks.

Expression levels of cells treated with GM-CSF are shown in grey, while expression levels of cells treated with M-CSF and RANKL alone are shown in black.
One of the more common criticisms of microarray experiments is directed at their sensitivity. This is regarded as a particular limitation when looking for transcription factors, as it is generally thought that biologically-significant changes in expression levels of transcription factors may not be detected as being statistically significant. While this may be true in the case of low-level expression of transcription factors, it is noteworthy that the experiments described here identified 15 known activators and repressor of nuclear transcription, not including translocation products. This is nearly half of all the genes identified as being significantly regulated, which suggests that as a first-pass experiment to identify transcription factor differences between cell types, arrays work reasonably well.

As can clearly be seen from the fold regulation differences, and the number of genes significantly regulated in the macroarray experiment comparing osteoclasts and macrophages, exposure to RANK-L has some dramatic effects on the gene expression. Amongst the genes found to be either switched on or off, or significantly up-regulated in this experiment are a number of growth factors and receptors, including GM-CSF receptor. Research performed in our laboratory following on from this observation has found that treating osteoclasts with RANK-L and GM-CSF restricts terminal differentiation of the osteoclasts, suggesting a possible feedback-regulation mechanism. This relationship between M-CSF, GM-CSF, and RANK-L is a target for further research. G-CSF receptor was also shown to be up-regulated in osteoclasts, suggesting that it may be another player in what could be a highly complicated network of cytokine-mediated differentiation controls. Examining the expression levels of these cytokine receptors and the appropriate ligands may yield some interesting information on the ability of an osteoclast cell to moderate the
activity of neighbouring osteoclasts *in vivo*, a process which must be regulated in order to avoid excessive bone resorption. Similarly, cell biology experiments on NFATc1 have confirmed that it plays a significant role in osteoclastogenesis, as indicated by the macroarray analysis. Inhibition of NFATc1 activity by cyclosporine A lead to a decrease in multinucleation, and a decrease in expression of the calcitonin receptor.

Another interesting observation yet to be investigated further is the up-regulation of ornithine decarboxylase antizyme (12-fold up-regulated in osteoclasts). This particular gene was also isolated from the osteoblast-enriched library described in chapter 3. It has been postulated that ornithine decarboxylase may fit into a targeting pathway mediated by spermidine to attract macrophages to damaged or infected cells for destruction, by restricting the ability of cells expressing ODC to respond to the targeting molecule. This may be one of the key genetic reasons macrophages are generalized cellular debris scavengers, while osteoclasts are restricted to degrading bone (see chapter 3 discussion for a brief review).

In addition to the genes outlined above, a number of genes involved in cell-cycle regulation and adhesion and migration are shown to be upregulated in osteoclasts. These observations are not surprising, given what is already known about these cells. Osteoclasts are multinucleated cells, therefore it is logical that higher levels of cell-cycle regulatory factors such as tankyrase and cdk7 would be required. Similarly, osteoclasts are motile- the cells main function is to adhere to bone, degrade it, detach,
and move on. The expression of high levels of mRNA for integrins, myosin, and calcium-sensitive signalling molecules may very well be a reflection of that purpose.

In addition to these candidate genes, a number of transcription factors, including zinc finger KOX31, and binding factors appear to be significantly regulated in osteoclasts. That the two critical regulators of bone cell differentiation identified thus far have been either transcriptional activators (CBFA1 in osteoblasts) or cytokines (RANK-L in osteoclasts) makes further investigation of regulated members of either group a logical step.

A further interesting observation to come out of this experiment is that alpha-synuclein is upregulated 16-fold in osteoclasts. This observation becomes more significant in light of the data that alpha-synuclein is involved in the pathogenesis of both Parkinson’s disease and Alzheimer’s disease, especially when another experiment described in this thesis implicated another gene involved in the pathogenesis of Alzheimer’s disease as being expressed at high levels in osteoblasts (Chapter 5).

Alpha-synuclein is a small protein highly expressed in the synapse and the nuclei of nervous tissue (Maroteaux et al, 1988; Jakes et al, 1994; Irizarry et al, 1996). It has been demonstrated to be the main non-Aβ component of plaques isolated from Alzheimer’s disease brains (Ueda et al, 1993), and a component of the Lewy bodies and Lewy neurites which are the characteristic lesions of the brain associated with Parkinson’s disease (Baba et al, 1998; Spillantini et al, 1997; Spillantini et al, 1998).
Transgenic mice expressing high levels of either wild type, or Parkinson’s disease mutation-coding alpha synuclein show lesions and develop other symptoms of Parkinson’s disease, suggesting that alpha-synuclein plaque formation may be a cause of the disease, rather than an effect (Masliah et al., 2000; Feany et al., 2000).

Alpha-synuclein is thought to be involved in maintaining the pool of distal synaptic vesicles (Murphy et al., 2000), but its exact function is yet to be determined, although this proposed regulatory role in synaptic transmission fits with the pattern of Parkinson’s disease. It is interesting, however, that in a study of model osteoclast cells, a gene, aberrant forms of which are implicated in two aging-related diseases, appears to be playing a role. Similarly, several other genes identified as significantly regulated in osteoblasts compared with fibroblasts have postulated roles in the pathogenesis of Alzheimer’s disease and Parkinson’s disease.

Overall, this series of experiments has lead to several observations regarding a number of candidate genes, but it also indicates that it may be worth considering the morphological similarity of the cells when deciding whether DNA array analysis is a suitable method for investigating gene expression differences between cell types. The osteoblast and fibroblast cells used in this comparison appear very similar morphologically, and indeed, left to grow in culture without adequate treatment, osteoblasts default back to a fibroblast-like cell type. A macroarray comparison of these two cell types yielded fewer differentially expressed genes than a replicate experiment comparing two cell types more distant both morphologically and developmentally- osteoclasts and macrophages default back to, and develop from, a
common precursor cell, the monocyte. This developmental and morphological
distance may be a useful marker when considering array experiments, especially as
the sensitivity of arrays as an experimental system is still under review.
CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

During the course of this project, a number of techniques for the comparative analysis of gene expression between cell types have been utilized, for the purposes of gaining a clearer understanding of the molecular differences between the two key bone modeling cell types and their nearest developmental relatives.

A modified technique of subtractive hybridisation was developed and is described here which enriched a foetal osteoblast phagemid library by removing phagemid containing transcripts common to foetal osteoblasts and dermal fibroblasts, thus resulting in identification of genes expressed highly in osteoblasts. The technique identified 65 genes that were expressed either highly or specifically in osteoblasts when compared with fibroblasts. Some of the genes identified were found in multiple library clones, such as collagen and fibronectin, both of which are key structural components of bone, abundantly expressed by osteoblasts. Expression of some other identified genes had not previously been detected in osteoblasts, making them interesting targets for further investigation. Interesting genes revealed using this technique included prohibitin, leptin-receptor gene related protein, ornithine decarboxylase antizyme, amyloid precursor peptide and connective tissue growth factor. The usefulness of the technique was verified by performing real-time PCR to confirm the expression of two of these genes either specifically or abundantly in osteoblast cells.
While the enrichment process of the fetal osteoblast library did not result in a complete subtraction of all common sequences between osteoblasts and fibroblasts, as evidenced by the presence on CTGF in both osteoblasts and fibroblasts when evaluated using real-time PCR, many of the more abundant common genes were not evident in the enriched library. And real-time PCR confirmed that APP was not present in cDNA isolated from fibroblasts, but was present in osteoblast cDNA, although the abundance of the transcript cannot be determined from these techniques.

The absence of ribosomal RNA sequences, and other ubiquitously expressed genes reduced the number of sequencing reactions that would otherwise have been required to obtain any useful information from the library in its original form. As it is, the technique appears to have yielded several interesting genes a number of times, which have already been verified as expressed at higher levels in osteoblasts, suggesting that other sequences obtained from the subtracted library may be equally interesting, if in lower abundance. As a technique for identifying completely novel sequences, the subtraction appears to have worked very well.

From the data presented in chapter 4, it would appear that the use of DNA arrays might be limited when the two cells types being compared are quite similar. From the array experiments comparing osteoblasts and fibroblasts, relatively few genes were found to be regulated. It is also interesting to note that arrays seem to be significantly less useful for detecting differences in rare transcripts than are the other methods used in this project. CTGF, APP, and osteonectin were all identified in a number of clones sequenced from the subtracted library described in chapter 3, suggesting that transcripts of these genes were quite abundant in the original starting library. Of these three genes, however, all of which were represented multiple times on the microarray
chip, and none were found to be significantly regulated. Osteonectin is recognized as being an abundant transcript in osteoblasts, CTGF less so. This suggests that low abundance of a gene within a microarray probe may prevent it from ever being detected by microarray, certainly when analysis of the array is performed under rigorous statistical criteria. The microarray comparison of fibroblasts and osteoblasts showed that the two cell types were very similar, with just 27 genes upregulated in osteoblasts, and 37 genes down regulated in osteoblasts, out of the 19,000 genes represented on the array.

The data yielded from the DNA macroarray experiment showed that 12 genes out of the 2352 genes on the array were differentially expressed between the two cell types, 8 upregulated in osteoblasts, and 4 downregulated in osteoblasts. Genes upregulated in osteoblasts included transcription factor Dp-2, cAMP response element binding protein 1, and transcription factor ATF2. Genes down-regulated in osteoblasts included teratocarinoma derived growth factor, thymosin beta-10, and transcription factor 3.

It is interesting to note at this point that the experiment comparing osteoclasts and macrophages yielded a considerably larger number of gene targets worthy of further investigation than did the experiment comparing osteoblasts and fibroblasts using the same set of macroarrays. It is, therefore, possible that frequency of regulation is a reflection of the extent of differences between the cell types. The two cell types used in the osteoblast/fibroblast experiment appear similar, while the osteoclast and macrophage cells are quite distinct in appearance- possibly differences in morphology.
are an indicator of how useful an array experiment is likely to be in identifying
differential gene expression between cell types. It should be noted at this point that
comparable amount of cDNA were used in both experiments, and that the probes used
were prepared in exactly the same way- overall, it seems likely that the differences
resulting from exposure to RANK-L are simply far greater than the differences
between osteoblasts and fibroblasts. This may be an interesting point to bear in mind
when using arrays to compare closely-related tissue types, such as healthy and
cancerous tissue from biopsies of the same patient.

This project has revealed a number of interesting candidate genes for further
investigation, including CTGF, APP, ornithine decarboxylase, GM-CSF and G-CSF,
NFATc, and Kox 31, and it has also demonstrated that in order to compare different
populations of cells, it may be necessary to try a number of different techniques- a
novel subtraction process performed on osteoblasts has yielded a lot of interesting
candidate genes not identified by the array-based experiments. Conversely, array
experiments suggested there were very few differences between osteoblasts and
fibroblasts, while a subset of the same arrays highlighted a number of differentially
expressed targets for further research in osteoclasts and macrophages. And as stated in
the introduction to this thesis, the aims of this project were two-fold- to identify genes
which may present suitable targets for further focused research, with a long-term view
to identifying therapeutic agents, and to evaluate a number of comparative gene
expression techniques.

Verification work done on genes identified as regulated in osteoclasts compared with
fibroblasts confirmed that macroarray data is reproducible. Follow up experiments
done to investigate two genes identified by the array, NFATc and GM-CSF receptor, showed that these genes are important in vivo, and play an important role in osteoclastogenesis.

Of the possible pathways for further research highlighted by this project, two in particular appear to be worth pursuing, as components of each have been found in both osteoblasts and osteoclasts.

The first of these is the ornithine decarboxylase antizyme pathway. Identified as being expressed by osteoblasts, and upregulated in osteoclasts, suggests that it may be playing some part in bone remodelling. Other evidence suggests that the spermine pathway is involved in targeting macrophages to damaged cells for destruction- it is possible that a similar targeting mechanism could be used to target osteoblast precursors or osteoclasts to sites of bone remodelling, and that antizyme could be upregulated to prevent polyamine accumulation in the cell from damaging the bone metabolizing cell once it reaches the target site.

The other interesting pathway to examine is the relationship between different aging-related diseases. In both osteoblasts and osteoclasts, Alzheimer’s and Parkinson’s disease related-genes have been identified- amyloid precursor peptide in osteoblasts, and alpha-synuclein in osteoclasts. Furthermore, other genes shown to be significantly upregulated in bone cells by these experiments have also been implicated either directly in processing amyloid precursor protein (Calpain- expressed in subtracted library, chapter 4, Chen et al, 2000) or other aspects of Alzheimer’s disease (CDK7-upregulated in osteoclasts, chapter 5, Zhu et al, 2000), or else are highly expressed in
neuronal cells. Little information about the coincidence of osteoporosis, the most common age-related disease of the skeleton, and neurological aging disorders is available, however when a group of 408 men and 1105 women with either Alzheimer’s disease or hip fracture as the variables were examined, Weller (2000) found that patients with Alzheimer’s disease were more likely to have osteoporosis than those without Alzheimer’s disease, and those who had hip fractures were more likely to have Alzheimer’s disease than those who did not have fractures. Data like this suggests that there is an association between these two types of aging-related illnesses, and possibly by examining the roles of proteins which, when mutated, cause neurological disorders, it may be possible to uncover the normal role of these proteins in both neurological and skeletal tissue.

As mentioned earlier, there are limitations to the techniques utilised here. The sensitivity of DNA microarrays is yet to be conclusively determined- certainly, from our observations, many of the previously identified abundantly expressed genes validated by other means are do not appear to be significantly regulated on the arrays used here, which suggest either that the arrays are not sensitive, and are a technology requiring further optimisation, or that the statistical analysis used are too stringent, and a lot of results are being discarded needlessly during analysis. However, verification using real-time PCR indicates that data from DNA macroarrays is reproducible, and reflects changes in transcription levels occurring in the cells.

A further important limitation of these techniques is that both focus on cDNA samples from cells, and in order to verify that these changes in transcription are having an
effect *in vivo*, work needs to be done at the protein level to confirm that increased transcription is resulting in increased protein expression of the candidate genes.

However, as a preliminary method for identifying candidate genes for time and resource consuming experiments which examine only one gene at a time, the techniques utilised here would seem to provide a useful foundation for further reseach.

Further research which could be carried out following on from these studies could include pursuing some of the candidate genes identified here at the protein level, and determining that increased translation is following on from increased transcription of these candidate genes. In particular, it would be interesting to follow the connection hinted at from this study between osteoporosis and Alzheimer’s and Huntington’s Diseases. So far the ‘normal’ function of APP has remained elusive- possibly by examining the effects of APP on bone, more insight could be gained.


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APPENDIX 1: Materials used and suppliers

General consumables

Eppendorf (Hamburg, Germany) supplied 1.5 ml tubes, while Corning (NY, USA) supplied 50 ml tubes. Sarstedt (Numbrecht, Germany) supplied 10ml tubes, and 10 and 20cm bacterial culture plates. BD Biosciences (USA) supplied T75 75cm² tissue culture flasks, 96-well plates and syringes, Swann-Morton (Sheffield, UK) supplied needles, and Millipore (Billeria, Massachusetts, USA) supplied syringe filters. Ultracentrifuge tubes were obtained from BD Biosciences (USA).

Chemicals, reagents and enzymes

Alpha phosphate- P32 dCTP and Ficoll-paque were purchased from Amersham (Uppsala, Sweden). Agarose and Tris were purchased from Astral (India). Sigma-Aldrich (St. Louis, USA) supplied Ampicillin, Bromophenol blue, Collagenase, Dnase, Hyaluronidase, ethidium bromide, ethylene diamine tetraacetic acid, Herring sperm DNA, tetracycline, xylene cyanol, and sodium dodecyl sulfate. Agar and NZY stock powder were purchased from BD Biosciences (USA), and Oxoid (Hampshire, UK) supplied LB stock powder. Minimal Essential Media, Dulbecco’s Minimal Essential Media, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, California, USA). Ajax (Sydney, Australia) supplied maltose, mahnesium sulfate and sodium chloride. Deoxynucleotide triphosphates and Taq polymerase were purchased from Promega (Wisconsin, USA). New England Biolabs (Beverly,
Massachusetts, USA) supplied Rsa I. Superscript II reverse transcriptase was purchased from Invitrogen (Carlsbad, California, USA). ATLAS Human 2.1 arrays 1,2,&3 were purchased from BD Biosciences-Clontech (USA). The Human 19k arrays, version 2 were obtained from The Clinical Genomic Centre, University Health Network, Toronto, Canada. SYBR green was purchased from Invitrogen-Molecular Probes (Carlsbad, California, USA), and F-cal was purchased from Bio-Rad (Hercules, California, USA). Cy3 and Cy5 fluorophores were purchased from Applied Biosystems (Foster City, California, USA). Reagent grade ethanol was purchased from BDH-AnalaR (UK).

All water used to make up solutions was autoclaved Milli-Q® filtered water (Millipore, Billeria, Massachusetts, USA) and all water used in for RNA work was DEPC-treated Milli-Q® filtered water. All solutions were sterilized by autoclaving or filtration through a 0.2µM filter prior to use.

**Oligonucleotide Primers**

All oligonucleotides used in this research were obtained from Geneworks (Adelaide, Australia). Primer sequences are given in Table 2.2.
**Plasmids**

The foetal osteoblast library used was cloned into the pAD-GAL4 phagemid vector (Stratagene, USA).

**Phagemids, bacterial strains and media**

The helper phage strain used to excise the library phagemids in chapter 4 was the ExAssist helper phagemid strain (Stratagene, USA). The bacterial host strains used for excision of the library, and for titration of the phagemid library were XL1Blue MRF’, and XLOLR, which also came from Stratagene (USA). The excised phagemids were transformed into DH5α cells. Glycerol stocks of all three strains, and any transformants, were stored at -80°. The genotypes of all strains used are shown in Table 2.2.

**Mammalian cell lines**

Mammalian cells used in the course of this project have all been primary cell lines. Osteoclasts and macrophages used were derived by treating monocytes isolated from blood obtained from a donor with M-CSF and RANK-L. Osteoblasts and fibroblasts
were primary cultures obtained from tissue samples by Dr Rebecca Mason, Department of Physiology, University of Sydney, Australia.

**Foetal osteoblast library**

The primary foetal osteoblast cDNA library was obtained from Dr Gary Leong, of the Garvan Institute, Sydney, Australia, and was made by Stratagene, USA.

**Molecular biology kits**

The library clones described in chapter 4 were prepared using the Eppendorf DirectBind plasmid purification kit, in the 96-well format, supplied by Eppendorf (Hamburg, Germany). The PCR purification kits used were the QIAquick PCR Purification kit, (Qiagen, UK) and the Wizard PCR Preps DNA purification Kit (Promega, Wisconsin, USA).

**Apparatus**

Sequencing of DNA was performed using an ABI 377 sequencer from Applied Biosystems (Foster City, California, USA). Agarose gel electrophoresis was performed using a tank supplied by Hoefer Scientific Instruments (San Francisco, California, USA), and a power supply from Bio-Rad (Hercules, California, USA). Gels were visualised using a UV light box and camera from UltraViolet Products.
(Cambridge, UK). The microarrays were scanned using an array scanner from Affymetrix (USA). Radioactive images were developed using Phosphorimager screens from Kodak (USA) and developing cassettes from Amersham (Uppsala, Sweden), and scanned using a Phosphorimager from Bio-Rad (Hercules, California, USA). A phosphorimager screen eraser from Bio-Rad (Hercules, California, USA) was used to clear screens after use. Real-time PCR was performed in a Bio-Rad I-Cycler. Nick translation columns used to separate unincorporated radionucleotides were obtained from Promega (Wisconsin, USA). PCR amplification and restriction digests and labelling reactions were performed in a Thermocycler from Corbett Research (Sydney, Australia). Ultracentrifugation was performed in an ultracentrifuge supplied by Beckman (BD-Biosciences, USA).

**Cell lines and media**

Osteoclasts and macrophages were cultured in Minimal Essential Media, supplemented with Earle’s salts, L-glutamine, and non-essential amino acids, without sodium bicarbonate, supplied in powdered form by GiboBRL(USA). Media was supplemented with Penicillin/streptomycin, supplied by the same company. Fibroblasts used to make the RNA used in the subtraction were cultured in Dulbecco’s Minimum Essential Medium, with high glucose, 25mM HEPES buffer, pyridoxine.HCl, without L-glutamine or sodium pyruvate, also supplied by GibcoBRL. Ficoll-Paque, used to isolate white blood cells, was obtained from Amersham (Uppsala, Sweden).
General reagents and buffers

TBE, TAE, TE, EDTA, Tris-Cl, DNA loading dye, and Denhardt’s reagent were all made up according standard protocols. Other chemicals used were standard items available from general chemical stockists. Other apparatus used were standard equipment found in any molecular biology research laboratory.
Appendix 2: Real-time PCR primers and location

Amyloid Precursor Protein Primers

Genomic chr21 (reverse strand):

Exons are marked in blue capital letters. APP primer 1 is shown in green. The complement of APP primer 2 is shown in purple. The cDNA and genomic DNA product of these primers is 181 bps. Introns in this gene are two large to enable intron-spanning primers to be used.

cctcattttt tattcctctct gtggttttcaaa aagaaaaatcctttgtctct 26346107
aagaaacgga aatgactccc ataaggctca aataacagtctgtaatatatttt 26346057
cattgtctacctttatgtctctggaattctctctcatttactcctatttatcactagtt 26346007
ggatgctctcagaaccttacatattttgtttgtgaaatatattttctttgtctttctttctct 26345957
ctcccagaaaa gccaaaagaccttcaattcagagaaattatctctattgacctgtaaac 26345907
tttcgattttcattggtctgcaagggataattttctttctctttctttctctctctctctct 26345857
attactctgcagacgactcagtttatttctttctttctttctttctttctttctttctttcttttt 26345807
ccatgtgagataaaaacttcaacttcaagtctgctggcattgtctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttc
Connective Tissue Growth Factor

Genomic chr6 (reverse strand):

Exons are marked in blue capital letters. CTGF primer 1 is shown in green. The complement of CTGF primer 2 is shown in purple. The cDNA product of these primers is 116 bps, and the genomic DNA product is 504 bps.

TGGCATCCCC TGTCGACGAT GGAATCTGCT CTGCCCAGCCC CTGACTGCC 13225097
TCTCCCCGAG AAGTTGAACC TGCCCCGAGA ATGTGCTGAG GAGTTGGGCT 13225047
GTGACGAGCC CAAGGAGGAA ACCCTGGTGG GGGCTCGGCT CGGAGCgtgag 13225197
tcagtctcct ctctaatgctct ggcgtgtgtat tctctcccaag gaggaggtct 132251947
ctaaactgac gcaggggacag ggggaatgac ctatcccagctcttgttatcat 132251897
tgtgtttggt tcctctgtct gcggagCTTA CCGACTGGAA GACACGTTGG 132251847
GCCCGACGTC AACTATGATG AGACCCAGCT GCCGCTGCTCA GACACAGAG 132251797
TGGAGGCTCT GTTGAGGAGT GGGATCTCTA CCCGGTTTAC 132251747
CAATGAGACAC CTCCTTGGCA GGCAGAGGAA GCAAGAGCCG CTGTCGATGG 132251697
CTAGGCCTTGG CGAAGCTGAC GCCCTGCTCA GACACGTTGG 132251647
ctctctttta ctcttttttca caggaanacc aagttgatatg acccaaactta 132251597
GACCCTTGGCA GAGGCTCTTGG CAGAACCAC ACCCTGCCTAC CTTTGCTGCCA 132251097
GGCGAGGTCA TGAAGAAGAA CATGATGTTC ATCAAGACCT GTGCCTGCCA 132251047
TTACAACTGT CCCCGAGACA ATGACATCTT TGAATCGCTG TACTACAGGA 132250997
AGATGATGAG AGACATGGCA tgaagccaga gaTGAGAGA CATTAACTCA 132250947
TTAGACTGGA ACTTGGAATG ATTCACATCT CATTTTTCCG TAAAAATGAT 132250897
TTCAGTAGCA CAAGTTATTT AATCTGTTT TTCTAACTGG GGGAAAAGAT 132250847
TCCCACCCAA TTCAAACAT TGTGCCATGT CAAACAAATA GTCTATCAAC 132250797
CCCAGACACT GGTTTGAAGA ATGTTAAGAC TTGACAGTGG AACTACATTA 132250747
GTACACAGCA CCAGAATGTA TATTAAGGTT TGCCCTTAGG AGCAGTGGGA 132250697
GGGTACCAGC AGAAAGGTTA GTATCATCAG ATAGCATCTT ATACGAGTAA 132250647
TATGCCTGCT ATTTGAAGTG TAATTGAGAA GGAAAATTTT AGCGTGCTCA 132250597
CTGACCTGCC TGTAGCCCCA GTGACAGCTA GGATGTGCAT TCTCCAGCCA 132250547
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Appendix 3: Primers used to verify gene expression data from macroarray, and product sizes (F is forward primer, R is reverse primer).

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