The control of bone formation by neuropeptide Y receptors

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The Control of Bone Formation by Neuropeptide Y Receptors

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ABSTRACT

Osteoporosis is a growing health concern, characterised by deterioration of bone and increased fracture incidence. Anabolic treatments for reversing bone loss are presently limited. A bone anabolic response was recently reported following deletion of hypothalamic neuropeptide Y2 receptors in mice. In contrast, no discernable bone phenotype was observed in Y4 receptor knockout ($Y4^{-/-}$) mice, revealing specificity between the Y receptors in their control of bone formation.

Studies in this thesis revealed a second anabolic response in the absence of another Y receptor subtype; the Y1 receptor. The potential interaction between the Y1 and Y2-anabolic pathways with each other and with Y4 was investigated through the generation of mouse models lacking multiple Y receptor subtypes. Interestingly, no synergistic elevation in bone volume was observed in $Y1^{-/-}Y2^{-/-}$ double knockout mice, indicative of shared mechanisms of action. In contrast, the synergistic elevation in bone volume of male $Y2^{-/-}$ $Y4^{-/-}$ mice was likely due to additive effects of leptin signalling. Consequentially, potential interaction between Y receptors and leptin was investigated by crossing the Y receptor knockouts onto the leptin deficient ob/ob background, revealing differential responses of the Y receptor pathways to leptin deficiency, with the anabolic response of the $Y2^{-/-}$ model retained in $Y2^{-/-}$/ob mice but abolished in $Y1^{-/-}$/ob mice compared to $Y1^{-/-}$. Differential responses of these two pathways were also revealed following gonadectomy of $Y1^{-/-}$ and $Y2^{-/-}$ mice. Importantly, these studies also demonstrated the ability of the central Y2-anabolic pathway to halt gonadectomy-induced bone loss.

Interestingly, cultured stromal cells from germline $Y2^{-/-}$ mice exhibited an enhanced ability to undergo mineralisation and adipocyte differentiation, associated with a greater number of mesenchymal progenitor cells present within the bone of $Y2^{-/-}$ mice, suggesting a potential mechanism for the greater mineralisation of the $Y2^{-/-}$ model *in vitro* and *in vivo*. Y1 receptor expression was also detected in stromal cells from wild type mice, but was nearly abolished in $Y2^{-/-}$ mice. Together these findings demonstrate an important therapeutic potential for these pathways in the treatment of osteoporosis and indicate that modulation of Y receptor signalling within the bone microenvironment may alter proportions of mesenchymal progenitor populations with effects on bone formation.

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CERTIFICATE OF ORIGINALITY

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

Signed …….
Susan J. Allison

Date …….

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ABBREVIATIONS

α-MEM	α-minimum essential medium
α-MSH	α-melanocyte-stimulating hormone
$1,25(OH_2)D_3$	$1\alpha,25$-dihydroxyvitamin D_3
AAV	Adeno-associated virus
ADD1	Adipocyte determination and differentiation-dependent factor 1
AdV/TV	Adipocyte volume
AgRP	*agouti*-related protein
ALP	Alkaline phosphatase
AP-1	Activating protein-1
APC	Allophycocyanin
APC	Antigen presenting cell
AR	Androgen receptor
BA	Bone area
β-AR	β-Adrenergic receptor
BAT	Brown adipose tissue
BFR	Bone formation rate
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenic protein
BMSC	Bone marrow stromal cell
BV/TV	Trabecular bone volume
C/EBP	CAAT/enhancer binding proteins
cAMP	3',5'-cyclic adenosine monophosphate
CART	Cocaine-amphetamine-regulated transcript
CatK	Cathepsin K
cDNA	Complementary DNA
CFU	Colony forming unit
CGRP	Calcitonin gene-related peptide

CKK	Cholecystokinin
CNS	Central nervous system
DBH	Dopamine β-hydroxylase
DEPC	Diethylpyrocarbonate
dLS	Double labeled surface
DMF	Dimethyformamide
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
DPPIV	Dipeptidyl peptidase IV
DXA	Dual X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
EGMEE	Ethylene glycol mono-ethyl ether
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblastic growth factor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-phosphate dehydrogenase
GH	Growth hormone
GX	Gonadectomy
HEPES	Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPA	Hypothalamic-pituitary adrenal
HRT	Hormone replacement therapy
ICV	Intracerebroventricular
IGF-1	Insulin-like growth factor-1
IGFBP	IGF binding protein
IL-1	Interleukin-1
Ip	Polar moment of inertia
LHA	Lateral hypothalamic area

MACS	Magnetic activated cell sorting
MAR	Mineral apposition rate
MC4-R	Melanocortin-4 receptor
MCH	Melanin-concentrating hormone
M-CSF	Macrophage colony stimulating factor
MMA	Methyl methacrylate
MMP	Matrix metalloproteinase
MS	Mineralising surface
MSC	Mesenchymal stem cell
MSG	Monosodium glutamate
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
Oc.S	Osteoclast surface
OPG	Osteoprotegerin
ORX	Orchidectomy
OVX	Ovariectomy
OXM	Oxyntomodulin
PACAP	Pituitary adenylate cyclase-activating peptide
PBM	Peripheral blood monocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Phycoerythrin
Peri	Bone perimeter
PFA	Paraformaldehyde
PNS	Peripheral nervous system
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PPAR	Peroxisome proliferator-activated receptors
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone

PTHrP	Parathyroid hormone related protein
PVN	Paraventricular nucleus
PYY	Peptide YY
RANK	Receptor activator of NF-κβ
RANKL	Ligand for receptor activator of NF-κβ
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
Sca-1	Stem cell antigen-1
SERMs	Selective estrogen receptor modulators
SHBG	Sex hormone-binding globulin
sLS	Single labeled surface
SNS	Sympathetic nervous system
SOCS3	Suppressor of cytokine signalling 3
SP	Substance P
SREBP1	Sterol regulatory element binding protein 1
STAT3	Signal transducers and activators of transcription
TA	Total area
Tb.N	Trabecular number
Tb.Th	Trabecular thickness
TGFβ	Transforming growth factor-β
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
VIP	Vasoactive intestinal peptide
VMH	Ventromedial hypothalamus
WAT	White adipose tissue
WGA	Wheat germ agglutinin

Chapter 1

Introduction

1.1 BONE – STRUCTURE AND FUNCTION

1.1.1 Bone function

Bone is a highly specialised form of connective tissue with a mineralised extracellular matrix providing strength and rigidity to the skeleton; properties that are required for the skeletons' role as an internal mechanical support system and its protective role physically shielding soft inner organs and bone marrow. Moreover, the skeleton also provides sites for the attachment of muscle, allowing locomotion, and acts as a reservoir of ions for metabolic processes, including the maintenance of calcium and phosphate homeostasis (Baron, 2003; Rodan, 1992).

Bone is a dynamic, metabolically active tissue, which in the normal adult is constantly remodelled. Remodelling is a process involving the resorption and subsequent formation of new bone, and is regulated by numerous factors such as mechanical loading, locally acting factors, and systemically derived hormones, together maintaining whole body mineral homeostasis while maintaining the structural integrity of the skeleton.

1.1.2 Bone structure

1.1.2.1 Development of long and flat bones

Anatomically, the skeleton comprises two types of bone; flat bones; such as in the skull and mandible, and long bones; such as the tibia and femur. These types of bone not only differ structurally, but are also derived from separate developmental processes under the influence of cytokines and local growth factors including parathyroid hormone-related protein (PTHrP), hedgehogs, the bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), as well as various transcription factors such as the Sox genes and Runx2 (Baron, 2003; de Crombrugghe et al., 2001; Olsen et al., 2000).

Flat bones arise from the process of intramembranous ossification. Here, proliferation of mesenchymal cells forms a condensation within a highly vascularised area of embryonic

connective tissue, which then differentiates into bone matrix-synthesising osteoblasts (Olsen et al., 2000).

Long bone is structurally different from flat bone, comprising two epiphyses at the extremities, and a midshaft or diaphysis region comprising the cylindrical centre. The epiphyseal and diaphyseal regions are separated by the metaphysis. In the developing long bone, the epiphysis and metaphysis are separated by a layer of proliferative cells and cartilage, called the growth plate (Figure 1.1). This layer is responsible for the longitudinal growth of bones through a process called endochondral ossification (Olsen et al., 2000).

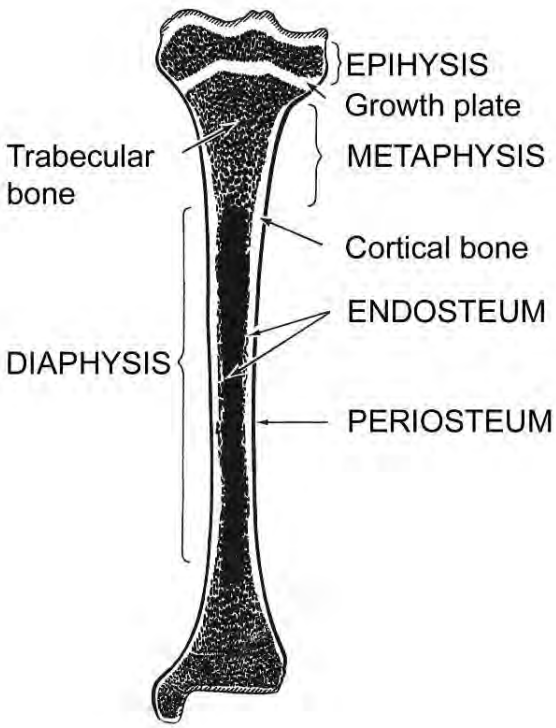

Figure 1.1 Schematic diagram of a longitudinal section through a long bone.
Adapted from Baron, 2003.

Endochondral ossification involves the differentiation of mesenchymal cells into prechondroblasts and then into chondroblasts, which secrete a cartilaginous matrix in which they are subsequently embedded as chondrocytes. At the periphery of the cartilage, or the

perichondrium, appositional growth occurs by the continual proliferation and differentiation of mesenchymal cells. Chondrocytes embedded within the matrix maintain their ability to proliferate, producing columns of chondrocytes called isogenous groups, each derived from a single parental chondroblast. These cells progressively enlarge, becoming hypertrophic before undergoing apoptosis. At this point mesenchymal-derived osteoblasts together with osteoclasts and blood vessels invade the region in which the hypertrophic chondrocytes reside. Osteoclasts then resorb the cartilaginous matrix while osteoblasts use the partially degraded matrix as a scaffold for the formation of an osteoblast-specific matrix which is subsequently calcified (de Crombrugghe et al., 2001; Olsen et al., 2000).

Growth in the diameter of the shaft results from the deposition of bone on the outer or periosteal surface of the bone, and is a process which continues throughout life. Simultaneously, removal of bone occurs on the inner or endosteal surface (Mundy et al., 2003). Osteoclasts continually resorb the lower part of the metaphysis, gradually transforming it into the narrower cylindrical diaphysis (Baron, 2003).

1.1.2.2 Cortical and trabecular bone

Within the skeleton, there are two morphologically different types of bone; cortical bone, comprising around 80% of skeletal mass, and trabecular bone, comprising the remaining 20% (Figure 1.1) (Baron, 2003).

Cortical bone is the thick, dense layer surrounding the long bone and houses the central marrow cavity. It comprises two surfaces, the exterior periosteal, and the interior endosteal, which contacts the trabecular bone and bone marrow. Around 80-90% of the volume of cortical bone is calcified, accounting for its considerable density (Baron, 2003), with added strength provided by the organisation of collagen matrix (Martin et al., 1996). Cortical bone is thickest in the diaphysis of long bones, where it surrounds the bone marrow. Further towards the metaphysis and epiphysis it becomes thinner, and encases the network of trabeculae.

Trabecular bone is the porous, sponge-like bone located within the cortical shell. In long bones, it is primarily found in the epiphysis and metaphysis. Trabecular bone is less dense than cortical bone, with only 15-25% being calcified (Baron, 2003). However, the lattices of individual trabeculae are interconnected in a network providing maximal mechanical strength (Biewener et al., 1996; Huiskes et al., 2000). This network also results in a substantially larger surface area per unit bone compared with cortical bone, consistent with a higher rate of metabolic activity and remodelling (Rodan, 1992).

1.1.2.3 Bone composition

Bone is composed of an organic matrix and an inorganic, mineralised component. The major protein in organic matrix is comprised primarily of type I collagen (Robey and Boskey, 2003). The orientation of the collagen fibres alternates between layers, resulting in a lamellar structure (Martin et al., 1996), and allowing the highest density of collagen per unit volume of tissue.

The remaining 10% of the organic matrix consists of proteoglycans, for example decorin, and other non-collagenous proteins including glycoproteins, such as osteonectin, and γ-carboxylated (gla)-proteins, such as osteocalcin. Moreover, growth factors have been identified in bone matrix and include transforming growth factor β (TGFβ), insulin-like growth factor I and II (IGF-1, IGF-II), and bone morphogenic proteins (Robey and Boskey, 2003). The precise role of all these non-collagenous components within bone is not fully understood, however it is likely they have diverse roles, with some serving primarily structural roles influencing the organisation of the matrix, while others have a more functional role influencing the mineralisation process, bone metabolism, and bone cell activity through both direct interactions with bone cells and indirectly by modulating the expression and activity of bone-acting factors (Brekken and Sage, 2000; Delany et al., 2000; Ducy et al., 1996; Hocking et al., 1998; Hunter and Goldberg, 1993; Luo et al., 1997; Robey and Boskey, 2003). Some glycoproteins within bone matrix contain a cell attachment consensus sequence that binds to the integrin class of cell-surface molecules, and are proposed to play a role in the regulation of bone cell function, for example

osteopontin supports the attachment of osteoclasts to bone, and has been proposed to play a mechanistic role in the coupling of bone resorption and formation (Denhardt et al., 2001; Robey and Boskey, 2003).

The inorganic mineralised component makes up the majority of the dry weight of bone, and is primarily composed of calcium and phosphorous in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] (Posner, 1985). Hydroxyapatite crystals are orientated parallel to the long axis of the collagen fibres to provide rigidity and mechanical strength (Bonar et al., 1983; Weiner and H.D., 1998; White et al., 1977). Other trace elements such as carbonate, magnesium, aluminium, and fluoride act as a reservoir for potentially toxic ions, and also enable bone to contribute to the maintenance of mineral homeostasis (Broadus, 2003; Bronner and Stein, 1995).

1.2 BONE – CELL DYNAMICS

1.2.1 Bone remodelling

Bone cell activity varies considerably throughout life in the developing and in the mature skeleton, including bone formation and resorption in both the process of *de novo* bone growth, and in bone remodelling, in which skeletal integrity is maintained. There are fundamental differences between the development of the growing skeleton and remodelling of the adult skeleton. Throughout growth, new bone formation occurs with growth in the length and diameter (section 1.1.2.1). Following the cessation of growth, the adult skeleton is maintained in a dynamic state by the continuous removal and replacement of bone through the actions of bone resorbing osteoclasts and bone forming osteoblasts in a tightly controlled process known as remodelling, allowing the mature skeleton to respond and adapt to mechanical stress, repair damaged bone, and participate in the maintenance of mineral homeostasis (Figure 1.2) (Huiskes et al., 2000; Mundy et al., 2003).

Although the remodelling process differs between cortical and trabecular bone (Mundy et al., 2003), in both types the process of bone remodelling occurs through the actions of

osteoclastic and osteoblastic cells, beginning with the activation and formation of osteoclasts that resorb bone in discrete pockets. The resorption phase is followed by invasion of the resorption pit by preosteoblasts which differentiate into osteoblasts, and deposit extracellular matrix which is subsequently mineralised. The resorption phase is estimated to last around 10 days in humans, with the following repair process lasting around three to four months (Mundy et al., 2003).

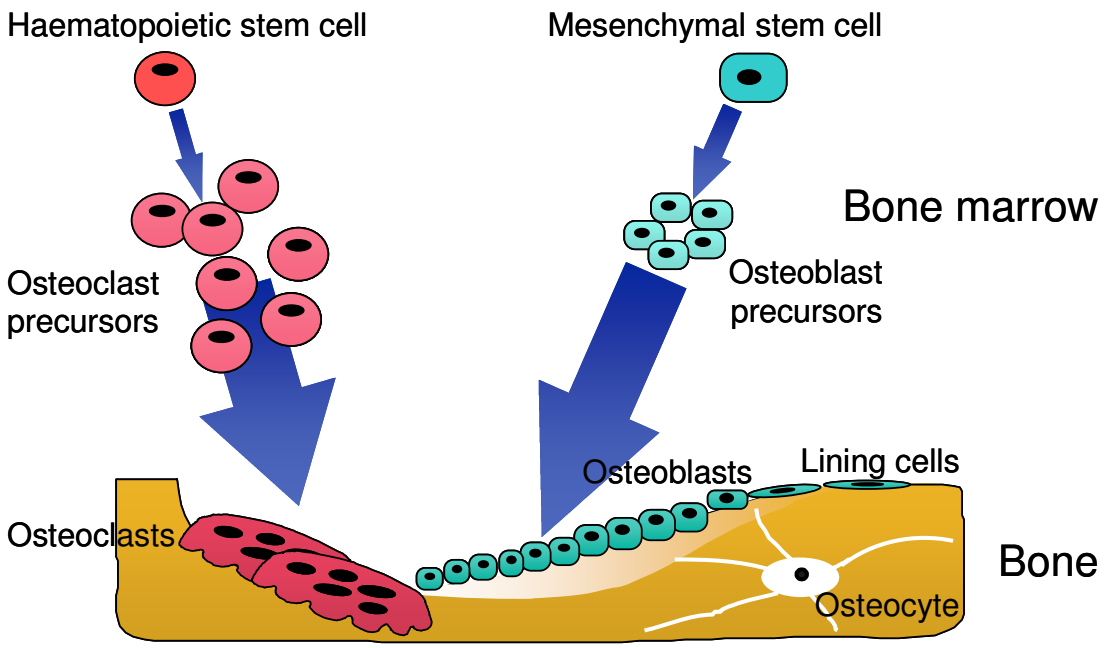

Figure 1.2 The process of bone remodeling.
Bone resorption occurs through the actions of osteoclastic cells, which are derived from haematopoietic precursor cells. Bone formation is achieved by osteoblasts, derived from mesenchymal stem cells. Following the formation of bone, osteoblasts either become embedded within bone as osteocytes or become bone lining cells. Adapted from P. Baldock, thesis 2001.

Under physiological conditions the process of bone remodelling is coupled, or in equilibrium, such that localised resorption occurring in discrete areas is followed by an equal amount of bone formation, maintaining a constant bone mass. Under pathological conditions, changes in bone mass caused by an imbalance between the amount of bone resorbed and the amount of bone formed, result in either a net gain in bone, or a loss of bone at the remodelling site. The mechanisms that determine the remodelling site or the initiation of remodelling or the signals coupling bone formation to the preceding resorption

phase are not yet known. It has been proposed that coupling is mediated by factors released from the matrix during resorption, for example IGF-1 or TGFβ, and these stimulate the recruitment of osteoblasts to the resorption pit (Dallas et al., 2002; Locklin et al., 2003; Mundy et al., 1982; Pfeilschifter et al., 1990). However there are numerous hormones, cytokines, and other factors that regulate the activity of osteoclasts and osteoblasts, and which together are likely to modulate the remodelling process. Structural proteins such as collagen or osteocalcin also have chemotactic effects and may therefore also be involved in the recruitment of osteoblasts (Mundy et al., 1982). Moreover, osteoclastogenesis is highly dependent on the presence of osteoblasts, with essential factors for osteoclast proliferation and differentiation released from, and presented on the surface of osteoblasts. Some of these factors are described briefly below.

1.2.2 The osteoclast

The osteoclast is a giant bone-specific cell type containing numerous mitochondria, Golgi apparatus, and transport vesicles containing lysosomal enzymes. These multinucleated cells are derived from the differentiation of haematopoietic precursor cells of the marrow, and are responsible for the resorption of mineralised matrix (Suda et al., 1992).

The differentiation of osteoclast precursors is regulated by a number of local factors. Two haematopoietic factors essential for inducing osteoclast differentiation are the ligand for receptor activator of NF-κβ (RANKL), a member of the tumor necrosis factor (TNF) family of cytokines, and macrophage colony stimulating factor (M-CSF) (Yasuda et al., 1998). M-CSF and RANKL alone are sufficient to induce the proliferation and differentiation of haematopoietic precursor cells into osteoclasts (Matsuzaki et al., 1998; Yasuda et al., 1998). Together these factors regulate terminal osteoclast development and osteoclast function, and are required to induce the expression of genes such as those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK), calcitonin receptor, and the β3-integrin, that identify the osteoclast (Lacey et al., 1998; Tanaka et al., 1993).

M-CSF is produced by stromal cells, and its expression is correlated with osteoclast formation (Hofstetter et al., 1995; Stanley et al., 1994). Osteoclast precursors and mature osteoclasts express transcripts encoding the M-CSF receptor (Hofstetter et al., 1992; Hofstetter et al., 1995), and are able to directly bind M-CSF (Hofstetter et al., 1995), however, M-CSF alone is not sufficient to cause osteoclast differentiation from precursors.

RANKL is a type II transmembrane protein that exists both as a soluble factor and as a membrane-associated protein in osteoblasts/stromal cells (Hofbauer and Heufelder, 2001; Walsh and Choi, 2003). RANKL is involved in both osteoclastogenesis and in regulating the activity of mature osteoclasts (Fuller et al., 1998; Jimi et al., 1999; Lacey et al., 1998). *In vivo* studies have demonstrated recombinant soluble RANKL to be a potent inducer of bone resorption, stimulating the formation and development of osteoclasts from bone marrow precursors in the presence of M-CSF by binding to its receptor RANK (receptor activator of NF-κβ) expressed on the surface of haematopoietic osteoclast progenitor cells (Lacey et al., 1998). RANKL also activates mature osteoclasts through activation of RANK, regulating osteoclast survival, activity and number of resorption cycles (Burgess et al., 1999; Jimi et al., 1999). Mice lacking RANKL have severe osteopetrosis due to a lack of multinucleated osteoclasts. Interestingly, these mice do however possess osteoclast progenitors, demonstrating a requirement for RANKL in osteoclast differentiation (Kong et al., 1999b).

The effects of RANKL are attenuated by osteoprotegerin (OPG), a soluble secreted protein of the TNF receptor superfamily which shares structural similarity in its N-terminal region with that of RANK, thereby acting as a decoy receptor competing for binding of RANKL (Boyle et al., 2003; Hofbauer and Heufelder, 2001; Matsuzaki et al., 1998; Walsh and Choi, 2003). Thus, while RANKL acts as a positive regulator of osteoclastogenesis, OPG acts as a negative regulator. OPG also directly inhibits the function of mature osteoclasts (Burgess et al., 1999; Lacey et al., 2000; Udagawa et al., 2000), mediated in part by actions on cell

survival, demonstrating actions of OPG on both the formation and the activity of mature osteoclasts.

The systemic hormones parathyroid hormone (PTH), 1α,25-dihydroxyvitamin D_3(1,25$(OH)_2D_3$), and calcitonin, also influence osteoclast activity. PTH stimulates bone formation when administered intermittently but stimulates bone resorption when administered continuously. 1,25$(OH)_2D_3$ also stimulates the differentiation and fusion of osteoclast progenitors (Roodman et al., 1985). Osteoclasts do not express receptors for PTH or 1,25$(OH)_2D_3$, thus the actions of both PTH and 1,25$(OH)_2D_3$ are mediated indirectly by osteoblastic cells through alteration of RANKL expression (Atkins et al., 2003; Tsukii et al., 1998; Yasuda et al., 1998), again confirming the important role of osteoblasts in osteoclast formation. The effects of PTH are mediated in part through Runx2, a transcription factor important for osteoblast development (Hofbauer and Heufelder, 2001; Krishnan et al., 2003; Locklin et al., 2003). 1,25$(OH)_2D_3$ can also alter the expression of various cytokines, some of which have effects on bone resorption (discussed below), and thus the effects of 1,25$(OH)_2D_3$ on the osteoclastic lineage appear to be multiple and complex.

Local factors and cytokines are also potent regulators of osteoclastic bone resorption. Interleukin-1 (IL-1) is released by activated monocytes and osteoblasts and stimulates osteoclast differentiation and activation through a RANKL-mediated mechanism (Boyce et al., 1989; Tsukii et al., 1998; Walsh and Choi, 2003). Addition of IL-1 to purified osteoclasts increases osteoclast formation and survival, an action attenuated by IL-1 antagonists (Jimi et al., 1999). TNFα also stimulates bone resorption by increasing osteoclast proliferation and differentiation (Kobayashi et al., 2000; Tashjian et al., 1987). IL-6 is expressed and secreted in response to osteotropic hormones such as PTH, TNFα, and IL-1 (Feyen et al., 1989; Ishimi et al., 1990), and can also stimulate osteoclast differentiation (Tamura et al., 1993).

TGFβ is produced by immune cells but is also synthesised by stromal cells, stored in bone, and released with resorption. The effects of TGFβ on bone resorption are complex and vary between species with stimulation of osteoclast differentiation and resorption in some systems and inhibition of resorption in others (Chenu et al., 1988; Dieudonne et al., 1991; Hughes et al., 1996; Kaneda et al., 2000; Sells Galvin et al., 1999). The ability of TGFβ to inhibit osteoclast formation in some systems is likely due to a stimulation of OPG production by osteoblastic cells (Walsh and Choi, 2003).

Other factors such as FGF, IL-15, IL-17, IL-18, TGFα, prostaglandins, sex hormones, and glucocorticoids (Horwood et al., 1998; Ibbotson et al., 1986; Ogata et al., 1999; Takahashi et al., 1986; Van bezooijen et al., 1999) are also involved in the regulation of osteoclast formation, activation, and function, thus the regulation of bone resorption involves mediation at multiple levels by different factors together acting to control the resorptive process.

1.2.3 Mechanism of osteoclast action

Following proliferation of osteoclast precursors in the bone marrow, mononuclear osteoclasts migrate to the bone surface, where they fuse together forming multinuclear osteoclastic cells.

The active osteoclast is highly polarised (Watanabe et al., 1995). Activation of RANK results in a series of internal alterations including rearrangements of the actin cytoskeleton and formation of a sealing zone and ruffled border (Burgess et al., 1999). The sealing zone is the area of the osteoclast by which it attaches to the bone surface, facilitated by integrins creating a tight attachment to the bone surface and effectively isolating the region in which resorption will occur (Nesbitt et al., 1993). The sealing zone encircles the motile ruffled border. It is this region, at the basal surface of the osteoclasts plasma membrane, which is responsible for the secretion of acid and bone resorbing enzymes and the resorptive activity of the osteoclast (Rodan, 1992).

Complete resorption of bone requires both dissociation of the inorganic mineral component and degradation of the protein matrix. The former is enabled by acidification through the export of hydrogen ions generated by an ATP6i complex (Baron et al., 1985; Chatterjee et al., 1992; Li et al., 1999). The acidic environment causes the solubilisation and removal of bone hydroxyapatite crystals, dissolving the mineral phase of the bone. Degradation of organic components such as type I collagen is enabled by secretion of lytic enzymes such as TRAP and CatK from the ruffled border (Bossard et al., 1996; Delaisse et al., 2000; Drake et al., 1996; Votta et al., 1997). The expression of matrix metalloproteinases 9 (MMP-9) has been demonstrated *in vivo* and in isolated osteoclasts, consistent with a proposed role in facilitating the degradation of bone matrix proteins (Blavier and Delaisse, 1995; Tezuka et al., 1994). The degradation products are then internalised for processing, and are released at the basolateral domain into circulation via endocytosis of transcytotic vesicles (Nesbitt and Horton, 1997; Salo et al., 1997).

1.2.4 The osteoblast

The osteoblast is a highly specialised secretory cell involved in the process of bone formation. It is responsible for both the synthesis of the matrix, and the mineralisation process. The osteoblast is derived from the pluripotent mesenchymal stem cells (MSCs) in the bone marrow. These precursors are proliferative, and are also able to give rise to chondrocytes, myocytes, fibroblasts, and adipocytes under the influence of specific factors (Short et al., 2003). MSCs and the interrelationship between osteoblasts and adipocytes are discussed in more detail later in the chapter (section 1.3).

The progression of osteoblast maturation requires expression and activation of various signalling proteins and transcription factors, with specific roles in the commitment and differentiation of osteoblasts. The runt homology domain protein Runx2/Cbfa1 (Runx2) is an osteoblast-specific transcription factor, essential for the commitment of mesenchymal progenitors to the osteoblastic lineage. It is the earliest of the osteoblast differentiation markers currently known, and was originally identified for its ability to bind and

subsequently transactivate an osteoblast-specific *cis*-acting element, OSE2 on the osteocalcin promoter (Banerjee et al., 1998; Ducy et al., 1997). It is also able to bind and regulate the expression of other genes expressed in osteoblasts, including α1(I) collagen, bone sialoprotein, and osteopontin (Ducy et al., 1997). The mechanisms and transcription factors that act upstream of Runx2 to control its expression are not yet understood, however it is known that Runx2 is able to regulate its own expression by binding to its own promoter (Drissi et al., 2000; Ducy, 2000), suggesting the possibility of an autoregulatory feedback signalling system throughout the osteoblast life-cycle.

Expression of Runx2 in non-osteoblastic cell lines induces osteoblast-specific gene expression (Ducy et al., 1997; Gori et al., 1999; Lian and Stein, 2003), while transgenic mice overexpressing Runx2 exhibit osteoblast maturational defects (Liu et al., 2001). Mice deficient in this transcription factor lack osteoblasts, and are unable to form mineralised matrix due to impaired osteoblast differentiation, supporting a critical role for Runx2 in osteoblastic differentiation (Ducy et al., 1997; Komori et al., 1997). Importantly, Runx2 deficiency also affects osteoclastogenesis, supporting the proposal that osteoclast formation is dependent on the presence of functional osteoblasts. However, while Runx2 is necessary for the osteoblast differentiation process, further studies indicate that this factor alone is not sufficient to support this process (Lee et al., 1999; Wang et al., 1999), suggesting a requirement for additional co-regulatory factors.

The zinc finger transcription factor Osterix is also essential for osteoblast differentiation. Mice deficient in Osterix do not form bone due to a deficit in osteoblast differentiation (Nakashima et al., 2002). However, while these Osterix-null mesenchymal cells express Runx2, Osterix is not expressed in Runx2-deficicent mice, indicating a role for Osterix in later stage osteoblast differentiation (Nakashima et al., 2002).

The helix-loop-helix proteins, for example, Id and Twist, are also expressed in proliferating progenitor cells and are important for the expansion of osteoprogenitors. These proteins are negative regulators of osteoblast differentiation and are down-regulated at the initiation of

differentiation down the osteoblastic lineage (Murray et al., 1992; Ogata and Noda, 1991). Members of the activating protein (AP-1) family of transcription factors are involved in diverse physiological processes, including the regulation of osteogenesis. For example, *c-fos* is expressed in osteoprogenitor cells and periosteal tissues but not mature osteoblasts (Machwate et al., 1995), while Fra-2 is expressed in mature osteoblasts and increases matrix formation and expression of bone specific genes (McCabe et al., 1996). Over-expression of Fra-1 leads to increased bone formation and an osteosclerotic phenotype in mice (Jochum et al., 2000). In contrast, transgenic mice over-expressing c-fos develop osteosarcomata (Grigoriadis et al., 1993). Further evidence for an important role for AP-1 related proteins was revealed with characterisation of mice over-expressing a naturally occurring truncated form of FosB present in osteoblasts (ΔFosB), resulting in increased bone formation (Sabatakos et al., 2000). Interestingly, ΔFosB concurrently inhibited adipocyte differentiation through alteration of adipose-related transcription factors, indicating a role for ΔFosB in the transcriptional regulation of adipocyte-osteoblast differentiation.

IGF-1, as well as circulating from hepatic synthesis, is produced in bone and inhibits the degradation of collagen most likely through inhibition of a collagen-degrading protease (Canalis et al., 1995). It also prolongs osteoblast lifespan by preventing apoptosis (Hill et al., 1997). Mice lacking IGF-1 have decreased bone formation. However, analyses of bone microarchitecture from IGF-1 knockout mice demonstrated deletion of IGF-1 signalling does not reduce trabecular parameters (McCarthy and Centrella, 2001). Conversely, transgenic mice over-expressing IGF-1 have increased bone formation (Bikle et al., 2002; Zhao et al., 2000). Furthermore, hepatocyte-specific deletion of IGF-1 synthesis caused an 80% reduction in serum IGF-1 levels but did not alter somatic growth (Sjogren et al., 1999), revealing distinct actions of local- and systemically-produced IGF-1 on cortical and trabecular bone types. Bone cells also secrete IGF binding proteins (IGFBPs), affecting the lifespan and biological activity of IGF-1 (Devlin et al., 2002; Hwa et al., 1999).

Bone morphogenic proteins (BMPs) are potent regulators of osteoblast differentiation and bone formation. To date, more than 15 BMPs have been cloned, primarily belonging to the TGFβ superfamily. Members of the BMP family are co-ordinately regulated throughout bone development, with expression of specific BMP subtypes in discrete localisations (Yamaguchi et al., 2000). BMP receptors are expressed in bone (Abe et al., 2000), and downstream signalling through Smad proteins induces the expression of transcription factors including Runx2, thereby inducing the differentiation and activation of osteoblasts (Ducy et al., 1997; Hughes et al., 1992; Thies et al., 1992; Yamaguchi et al., 2000).

Recent studies have now also revealed an important role for Wnt proteins in the regulation of bone formation. These proteins bind seven-transmembrane frizzled proteins or single transmembrane LDL-receptor related proteins 5 or 6 (LRP5/6) (Huelsken and Birchmeier, 2001), resulting in gene transcription by β-catenin (Nishita et al., 2000). LRP5 is expressed by osteoblasts and its expression is stimulated by BMPs (Gong et al., 2001). Activating mutations in LRP5 result in a high bone mass phenotype (Little et al., 2001), whereas inactivating mutations results in osteoporosis (Gong et al., 2001; Kato et al., 2002).

Systemic hormones also modulate bone formation. PTH stimulates the growth of osteoprogenitor populations, and induces synthesis of IGF-1 by osteoblasts (McCarthy and Centrella, 2001). The steroid hormone $1,25(OH)_2D_3$ and sex steroids also regulate gene transcription of osteoblast-related genes, by both direct actions and through interaction with other local bone-acting factors and cytokines, together co-ordinately regulating the control of bone formation. The effects of sex steroids are discussed in more detail in section 1.5.

1.2.5 Mechanisms of osteoblast action

Osteoblastic bone formation requires initial recruitment of osteoblast precursors to the resorption pit, a function most likely mediated by local factors produced during the resorption process such as TGFβ, IGF-1, platelet-derived growth factor (PDGF), and collagen (Dallas et al., 2002; Mundy et al., 2003). This is followed by proliferation of

osteoblast precursors and their differentiation into mature osteoblasts. The active osteoblast contains Golgi apparatus and a well developed rough endoplasmic reticulum, consistent with its role in protein secretion (Robey and Boskey, 2003). Secretory vesicles between the nucleus and bone surface secrete type I collagen and non-collagenous proteins of bone matrix producing non-calcified matrix called osteoid. Osteoid tissue is present due to a time-lag between matrix formation and mineralisation. Osteoblasts have receptors for PTH as well as steroid receptors for estrogen and $1,25(OH)_2D_3$. In addition, they express receptors for adhesion molecules and cytokines as well as actually expressing cytokines on their membrane surface, for example RANKL.

The expression of osteoblast-associated genes is associated with various stages of matrix production and the mineralisation process. The initial phase is characterised by the production of type I collagen, and alkaline phosphatase, which increases transiently prior to the progression of mineralisation. Expression of the non-collagenous proteins osteopontin, bone sialoprotein, and osteocalcin are associated with the mature osteoblast and the onset of mineralisation, and have a proposed role in regulating the mineral deposition process and the formation of hydroxyapatite crystals (Aubin, 1998a; Candeliere et al., 2001).

Increased bone formation may occur due to an increase in progenitor proliferation, differentiation, or lifespan (Erben et al., 1997; Jilka et al., 1999). Following the completion of bone matrix synthesis, osteoblasts either become embedded within the matrix to become osteocytes, while others, bone lining cells, become flattened cells on the surface of the bone. It is thought that the remaining osteoblasts, like osteoclasts, undergo apoptosis (Weinstein and Manolagas, 2000).

1.2.6 The osteocyte

Osteocytes are mature osteoblasts embedded throughout the mineralised matrix in lacunae (Baron, 2003). They are stellate shaped cells, with numerous extended processes rich in microfilaments which extend through fluid-filled channels, enabling osteocytes to contact each other, and also contact surface osteoblasts and lining cells via gap junctions (Civitelli

et al., 1993), allowing direct communication between osteocytes, and with cells on the bone surface.

Osteocytes are thought to play a primary mechanosensory role, by sensing and transducing stress signals to biological activity, enabling bone to adapt to the direction of primary mechanical forces (Burger and Klein-Nulend, 1999; Skerry et al., 1989). Between the osteocyte plasma membrane and the bone matrix is the periosteocytic space, filled with extracellular fluid. It is proposed that osteocytes identify mechanical load through strain-mediated fluid flow (Klein-Nulend et al., 1995c), that initiates cellular responses, such as facilitating nutrient transport, and the production of various bone active compounds such as prostaglandins and nitric oxide as well as Runx2 transcription (Burger and Klein-Nulend, 1999; Jiang and Cheng, 2001; Klein-Nulend et al., 1995a; Klein-Nulend et al., 1995b; Ziros et al., 2002). Rapid flux of calcium across gap junctions is thought to facilitate transmission of information between osteoblasts on the bone surface, and osteocytes (Jorgensen et al., 2003).

Osteocytes can remain in the bone matrix until the region they occupy is resorbed or alternatively they can undergo apoptosis (Noble et al., 1997; Tomkinson et al., 1997). This process is of particular interest, as disruption of the osteocyte network may compromise skeletal strength. Estrogens inhibit osteocyte apoptosis whereas glucocorticoids induce it, possibly contributing to the development of osteoporosis commonly associated with menopause or glucocorticoid use (Tomkinson et al., 1997; Weinstein et al., 1998).

1.3 MESENCHYMAL PROGENY: THE OSTEOBLAST AND THE ADIPOCYTE

The osteoblast and the adipocyte share a common mesenchymal stem cell progenitor, and the transcriptional mechanisms which determine the differentiation down either of these lineages are of much interest. A mouse model of accelerated aging suggests that aging is associated with decreased osteoblastogenesis and increased numbers of adipocytes (Jilka et

al., 1996). These findings are consistent with those of a number of studies suggesting a change in the ratio of adipocyte progenitors and osteoblast progenitors with age (Gimble et al., 1996) and can be extended to clinical observations in humans, where the number and size of bone marrow adipocytes increases with age and in osteoporotic patients compared with control subjects (Gimble, 1990; Meunier et al., 1971; Rozman et al., 1989; Verma et al., 2002). Interestingly, co-culture studies of primary human osteoblastic cells have demonstrated the ability of mature adipocytes and polyunsaturated fatty acids to inhibit osteoblast proliferation (Maurin et al., 2000; Maurin et al., 2002), indicating the ability of adipose-secreted factors to negatively influence osteoblast formation.

These findings suggest that the differentiation of adipocytes occurs at the expense of the osteoblast however, little is known about the factors determining lineage fate *in vivo*. Two different possibilities underlying lineage commitment of mesenchymal stem cells have been proposed; firstly is the possibility of an expanding hierarchy of increasingly restricted progeny. The second is that there is a non random, single step process, whereby multipotential progenitors become exclusively restricted to a single lineage (Aubin, 1998b). It is estimated that only a small percentage (15%) of proliferating cells obtained from flushed marrow have multipotential stem cell-like properties, with only a proportion of these able to undergo osteoblastic differentiation (Aubin, 1999; Wu et al., 2000). Studies using clonal cultures of human bone marrow-derived fibroblasts have demonstrated a limited ability of these cells to differentiate into the three primary mesenchymal lineages (osteoblastic, adipocytic, chondrocytic), with up to 80% of clones displaying an osteo/chondro potential (Muraglia et al., 2000). However, cell types have been identified in which both osteoblast and adipocyte markers are present simultaneously (Dorheim et al., 1993; Rickard et al., 1996). Hence, while these findings contrast with the study in which an osteo/chondro progenitor was favoured, together they suggest the possibility of a bipotential progenitor, consistent with a lineage hierarchy in which cells are increasingly restricted in their differentiation capacity (Aubin, 1998b).

The numbers of adipocytes present in the whole body is not fixed, clearly demonstrated by the expansion of adipocyte cell number or hypertrophic growth that occurs with

overfeeding. Interestingly, high fat feeding in rats has been associated with an increase in the percentage of bone marrow fat due to increased adipocyte size, but not number (Gevers et al., 2002). Differential adipose deposition also occurs in differing locations in males and females (Bjorntorp, 1991), suggesting that adipocyte differentiation is regulated in response to different levels of regulatory hormones or nutrient signalling.

A number of transcription factors involved in the regulation of adipogenesis have been identified. Of these, the CAAT/enhancer binding proteins (C/EBPα, β, δ) and the peroxisome proliferator-activated receptors (PPARα, γ2, δ) have been the most extensively studied. Both C/EBP and PPAR genes are expressed in mesenchymal progenitor cells of the bone marrow, and are induced early during adipocyte differentiation (Cao et al., 1991; Gimble et al., 1992). Transfection of these transcription factors promotes adipogenesis from fibroblasts or preadipocytes (Shao and Lazar, 1993; Umek et al., 1991; Yeh et al., 1995). Binding sites for C/EBP have been identified in the promoter regions of several genes that are expressed preferentially in adipocytes, and their transactivation by C/EBP has been demonstrated *in vitro* (Spiegelman and Flier, 1996). Interestingly, PPARγ-deficient embryonic stem (ES) cells cannot form adipocytes, but spontaneously differentiate into osteoblasts (Akune et al., 2004). Mice heterozygous for PPARγ knockout have high bone mass due to increased osteoblastogenesis (Akune et al., 2004), supporting an inverse relationship between osteoblast and adipocyte formation.

Other transcription factors include the adipocyte determination and differentiation-dependent factor 1 (ADD1), a transcription factor present in rats, and sterol regulatory element binding protein 1 (SREBP-1) in humans, which is up-regulated during adipocyte differentiation and regulates transcription of the low-density lipoprotein receptor gene (Tontonoz et al., 1993; Yokoyama et al., 1993).

Observations in rat and mouse stromal cells have described the association of enhanced expression of markers of adipogenesis with decreased expression of markers of osteoblastogenesis (Beresford et al., 1992; Dorheim et al., 1993), further suggesting that an

increase in one pathway results in a decrease of the other. For example, over-expression of PPARγ2 inhibits osteoblastogenesis and Runx2 expression and stimulates adipocyte differentiation in a murine bone marrow-derived cell line (Lecka-Czernik et al., 1999). Several studies have also demonstrated the ability of transcription factors to induce transdifferentiation of committed phenotypes to other phenotypes (Jeon et al., 2003; Lecka-Czernik et al., 1999; Nuttall et al., 1998; Park et al., 1999). The formation of adipocytes from a bone marrow cell line was reversible with the ability of these cells to convert into matrix mineralising cells under osteogenic-promoting conditions (Lecka-Czernik et al., 1999). Osteoblasts also, even once matured to the stage of expressing the marker osteocalcin, can be induced down an adipogenesis pathway in the presence of PPARγ2 (Nuttall et al., 1998), suggesting some plasticity of stromal progenitor cell types.

Steroid hormones are known to play a role in osteoblastic and adipocytic differentiation. Estrogen stimulates osteoblastogenesis while inhibiting adipogenesis, providing a potential explanation for the increased levels of marrow adipocytes seen in post-menopausal osteoporotic patients. Dexamethasone also promotes osteoblastogenesis *in vitro* (Beresford et al., 1994; Leboy et al., 1991; Rickard et al., 1996), and its actions are enhanced by $1,25(OH)_2D_3$ (Locklin et al., 2003). However *in vivo* glucocorticoid excess causes osteopenia. Glucocorticoids including dexamethasone have also been shown to promote adipocyte differentiation *in vitro* increasing the expression of C/EBPδ, and PPARγ (Gimble et al., 1990), and these effects can be antagonised by the presence of $1,25(OH)_2D_3$ (Beresford et al., 1992; Kelly and Gimble, 1998), suggesting interaction between multiple steroid hormones in the control of lineage differentiation.

Growth hormone (GH) may also be involved in the regulation of marrow adiposity. GH secretion is reduced with age (Corpas et al., 1993), and its deficiency is associated with increased adiposity and decreased bone mass (de Boer et al., 1995; Snel et al., 1995; Vandeweghe et al., 1993), which can be reversed by GH treatment. Rats lacking GH also have decreased trabecular bone volume and increased marrow adiposity, which again was reversed by GH treatment (Gevers et al., 2002; Kassem et al., 1993). BMPs may also play a

role in regulating lineage determination. Over-expression of a constitutively active BMP receptor type 1A (BMPR-1A) in a mouse calvarial-derived cell line induced formation of mature adipocytes with increased expression of adipsin and PPARγ, while overexpression of the constitutively active receptor type 1B (BMPR-1B) induced formation of mineralised bone matrix, with increased expression of Runx2, and markers of osteoblast differentiation (Chen et al., 1998). These data together indicate that BMPs may play regulatory roles throughout osteoblast and adipocyte differentiation while the expression of different receptor isoforms may also be important for commitment of progenitors to a particular lineage.

Therefore, while it is clear that the osteoblast and adipocyte are derived from a common progenitor cell, with many studies suggesting that the greater marrow adiposity concurrent with decreased bone mass with age or in osteoporotic patients is the result of a pathological switch in the differentiation potential of the progenitor, many aspects of this concept are not understood, and therefore this is an area which requires further investigation before the potential to alter the lineage switch for treatment of bone disease could be achieved.

1.4 OSTEOPOROSIS

Disturbances in the balance between bone resorption and bone formation result in a change in net bone turnover and is responsible for many bone diseases including osteoporosis. Osteoporosis is a common disease in which low bone mass and deterioration of bone microarchitecture lead to increased risk of fracture with minimal trauma (Cooper, 2003), resulting in significant morbidity and mortality in both men and women (Center et al., 1999; Chrischilles et al., 1991; Cooper et al., 1993).

During a lifetime, women and men lose on average 30-40% and 20-30%, respectively, of their peak bone mass, affecting both cortical and trabecular bone (Mundy et al., 2003). Loss of cortical bone is thought to begin at around age 40, with an acceleration of cortical bone loss occurring for 5-10 years following menopause in women. This accelerated bone loss

continues for about 15 years, and then slows (Mundy et al., 2003), but continues indefinitely throughout life (Jones et al., 1994). Cortical bone loss is the major predisposing factor for hip and wrist fractures. It is thought that loss of trabecular bone occurs earlier than cortical bone loss, and loss of this bone type is a predisposing factor for spinal fracture. The incomplete replacement of bone by osteoblasts relative to the amount resorbed results in decreased trabecular width. With excessive bone resorption or inadequate formation, complete perforation of trabecular structures can occur, leaving no template for bone to build on and resulting in the disconnected bone microarchitecture observed in osteoporotic bone (Mundy et al., 2003). Current treatments are primarily antiresorptive and therefore cannot drive the formation of new bone, and thus the process of trabeculae perforation and consequential fracture is largely irreversible. Furthermore, osteocyte viability decreases with age, and is likely to compromise the effective repair of bone structures (Dunstan et al., 1993).

Post-menopausal osteoporosis is the most common form of osteoporosis in women, occurring with accelerated loss of bone following loss of estrogen. The loss of protective estrogenic effects on bone either with natural or surgical menopause, following ovariectomy, results in increased bone turnover, with elevations in both osteoclast and osteoblast activity. While the mechanisms behind this loss of bone are complex and involve many factors, the loss of bone results from an increase in the prevalence of osteoclasts through increased proliferation and differentiation of progenitors, increased support of osteoclast formation through increased osteoblastic numbers, and a decrease in osteoclastic apoptosis (section 1.5). Thus, despite an apparent increase in osteoblastic activity, the increase in osteoclast activity exceeds the increase of the osteoblastic population, and an overall net increase in bone resorption ensues resulting in ongoing and at times, rapid bone loss.

Bone loss also occurs as a result of aging in both men and women. Compared to post-menopausal bone loss, this type of bone loss is a slower process, and is thought to be due predominantly to a decline in the number of osteoblasts recruited to the remodelling site. A threefold decrease in osteoblast progenitors was reported at 3-4 months of age in the

SAMP6 mouse model of accelerated senescence/aging and osteopenia, corresponding to a decrease in bone formation and decreased bone mineral density (BMD) (Jilka et al., 1996).

While sex hormone deficiency and aging are the two causes of osteoporosis that are the focus of this thesis, other factors are also implicated in the aetiology of osteoporosis. These include genetic factors, increasing PTH levels with age, vitamin D deficiency and decreased calcium absorption resulting in hyperparathyroidism, glucocorticoid therapy, excessive alcohol intake, and inflammation (Kelepouris et al., 1995; Lukert, 2003; Riggs et al., 1998).

1.5 SEX HORMONES AND BONE

The incidence of osteoporotic fracture is 2-3 fold greater in women compared with men of the same age (Orwoll and Klein, 1995), due to a rapid loss of bone that occurs with loss of ovarian function and estrogen following menopause in women, with low circulating estradiol levels at the time of menopause (Eastell, 2003). Although not as rapid as the effects seen in females, an age-related decline of androgen levels is associated with a gradual decline in bone mass in males (Alexandre, 2005; Orwoll and Klein, 1995; Rochira et al., 2006). Decreased BMD is also observed in those men with rare genetic disorders affecting sex hormone metabolism and action, such as an inactivating mutation of the genes encoding the estrogen receptor (Smith et al., 1994), or the enzyme which converts androgens to estrogens, aromatase (Morishima et al., 1995). These rare mutations demonstrate an important protective role for estrogen also in men. In older men, estrogen levels correlate more closely with BMD than testosterone levels (Greendale et al., 1997; Khosla et al., 1998; Slemenda et al., 1997).

Thus, both estrogens and androgens have important roles in the regulation of bone turnover, with effects on the rate of proliferation, formation, activity, and lifespan of both osteoclastic and osteoblastic cells. Receptors for estrogen (ERα and ERβ) and androgens (AR) have been found in chondrocytes, bone marrow stromal cells, osteoblasts, and osteoclasts and

their progenitors (Abu et al., 1997; Bellido et al., 1993; Bellido et al., 1995; Benz et al., 1991; Chen et al., 2005; Colvard et al., 1989; Kameda et al., 1997; Komm et al., 1988; Pederson et al., 1999), consistent with direct effects of sex steroids on bone cell function.

Estrogen and testosterone can inhibit bone resorption, although estrogen appears to be dominant in this role in both genders (Falahati-Nini et al., 2000; Leder et al., 2003). The inhibition of bone resorption is provided in part by the direct induction of osteoclast apoptosis, thereby reducing the osteoclast lifespan (Hughes et al., 1996; Kameda et al., 1997). Both estrogens and androgens can also suppress the activity of mature osteoclasts through direct, receptor interactions (Falahati-Nini et al., 2000; Hughes et al., 1996; Leder et al., 2003; Oursler et al., 1994; Pederson et al., 1999; Weinstein and Manolagas, 2000).

The inability of the elevated rate of bone formation to match the elevated bone resorption in the absence of sex hormones has been suggested by some to indicate an association of sex hormone deficiency with a defect in bone formation (Syed and Khosla, 2005). Evidence suggests both estrogen and testosterone inhibit osteoblast apoptosis by non-genotropic pathways, thereby increasing osteoblast lifespan (Kousteni et al., 2001; Tomkinson et al., 1997). Up-regulation of mesenchymal progenitor number following ovariectomy was taken as an indication of a role for estrogen in the regulation of osteoblastogenesis (Jilka et al., 1998). However, studies investigating the effects of estrogen on osteoblast proliferation, differentiation, mineralisation, and synthesis of bone matrix genes *in vitro* have yielded varied results, depending on the model system used, stage of osteoblastic differentiation, and expression of ER isoform (Waters et al., 2001), with an inhibitory effect of estrogen on osteoblast proliferation observed in osteoblastic cell lines expressing ERα (Kassem et al., 1996; Monroe et al., 2003; Robinson et al., 1997), while in cells expressing ER-β, estrogen had no effect (Monroe et al., 2003). In the osteosarcoma cell line MG-63, ER-β mediated the synthesis of bone matrix proteins (Cao et al., 2003b), while estrogen administration to a murine bone marrow cell line and primary mouse bone marrow cells stimulated osteoblast differentiation and inhibited adipocyte differentiation (Dang et al., 2002; McCarthy et al., 2003). Thus, despite some result differences, these data together support the concept that

estrogens directly regulate osteoblast proliferation and differentiation, although this does differ by species differences, cell system heterogeneity and differentiation stage, ER isoform expression and receptor concentration. Some evidence also suggests that estrogens may target osteocytes, with estrogen administration inhibiting osteocyte apoptosis induced by pro-apoptotic stimuli (Kousteni et al., 2001). The role of osteocytes in mechanosensing suggests the loss of these protective effects may impair the skeletal response to loading, and may contribute to the reduction of bone mass seen in sex hormone deficiency. Therefore, loss of sex steroid function not only prevents apoptosis of osteoclasts, but also results in the loss of the protective anti-apoptotic effects on osteoblasts and osteocytes, contributing further to the increased bone resorption and net loss of bone.

1.5.1 Sex hormones and cytokines

The protective actions of sex hormones on bone are also mediated by their ability to regulate the synthesis of various bone-acting factors and cytokines, which in turn affect bone turnover. Therefore, loss of sex hormones disrupts the regulatory control of cytokines in the bone marrow, contributing to the changes in bone remodelling and deterioration of bone microarchitecture.

Estrogens inhibitory actions on osteoclastic cells are mediated in part by its ability to suppress the production of RANKL by osteoblastic, T- and B-cells, and stimulate the production of OPG (Eghbali-Fatourechi et al., 2003; Hofbauer and Heufelder, 2001; Hofbauer et al., 1999; Kawano et al., 2003)). *In vitro* studies have also demonstrated androgens to suppress RANKL production by primary murine osteoblastic cells (Kawano et al., 2003). Furthermore, both estrogen and androgen can suppress RANKL-induced osteoclast differentiation by blocking RANKL-induced AP-1 dependent transcription by altering the expression and phosphorylation status of c-jun (Huber et al., 2001; Shevde et al., 2000; Srivastava et al., 2001).

Recent evidence suggests that bone-resorbing cytokines such as IL-1, IL-6, TNFα, M-CSF, and prostaglandins may mediate the deterioration of bone following loss of estrogen

(Girasole et al., 1992; Horowitz, 1993; Jilka et al., 1992; Kimble et al., 1996; Kimble et al., 1994; Kitazawa et al., 1994; Manolagas and Jilka, 1995; Pacifici et al., 1991). Some, but not all studies have reported increased production of IL-1, TNFα, and IL-6 in peripheral macrophages or marrow cells of estrogen-deficient or postmenopausal compared with premenopausal women (Bismar et al., 1995; Cohen-Solal et al., 1993; Hogasen et al., 1995; Kassem et al., 1996; Pacifici et al., 1991). The involvement of these cytokines in the process of bone resorption and the pathogenesis of osteoporosis is supported by the ability of specific cytokine inhibitors to IL-1, IL-6, and TNFα to effectively reduce the bone resorbing activity of peripheral blood monocyte (PBM) supernatants from postmenopausal women (Cohen-Solal et al., 1993). It is now thought that regulation of these cytokines by estrogen probably plays a pivotal role in mediating postmenopausal bone loss. An interesting and functionally important mechanism of cytokines, particularly in the case of IL-1, IL-6, and TNF, is their interdependence and ability to regulate their own and each others' synthesis. Thus, if a moderate change in the synthesis of one cytokine occurs, a cascade effect could be initiated resulting in synergistic effects on all three cytokines.

IL-1 is one of the most potent inducers of bone resorption *in vitro*. Its expression is suppressed in human macrophages following administration of estrogen (Kitazawa et al., 1994), and conversely is increased in PBM with loss of estrogen (Horowitz, 1993). In addition, IL-1 stimulates osteoclast production by increasing expression of other cytokines including RANKL, M-CSF, IL-11, and IL-6 which in turn act to stimulate osteoclast progenitor differentiation and inhibit osteoclast apoptosis. IL-1 also stimulates the expression of adhesion molecules such as osteopontin which may facilitate interactions between osteoblastic cells and osteoclasts to further support osteoclast formation (Jin et al., 1990).

IL-6 is also an important regulator of bone homeostasis, promoting haematopoiesis and osteoclastogenesis. Its production is suppressed by estrogen and androgen which blocks activation of the IL-6 promoter (Jilka et al., 1995). The two subunits of the IL-6 receptor (IL-6Rα and gp130) are also suppressed by estrogen (Galien et al., 1996; Girasole et al.,

1992; Kurebayashi et al., 1997; Manolagas and Jilka, 1995). IL-6 enhances osteoclast formation in *ex vivo* marrow cultures and in trabecular bone (Jilka et al., 1992), effects which are attenuated by administration of estrogen or a neutralising antibody to IL-6, suggesting that the increase in bone resorption associated with postmenopausal osteoporosis may be partly due to the loss of estrogen-mediated regulation of IL-6. This hypothesis is further supported by the demonstration that administration of a neutralising antibody to IL-6 or deletion of IL-6 in mice prevents the increased osteoclast formation in trabecular bone, and protects against loss of bone following gonadectomy (Bellido et al., 1995; Jilka et al., 1992; Poli et al., 1994).

TNFα is also a potent inducer of bone resorption, which also acts to stimulate the secretion of M-CSF and IL-6 from osteoblastic cells. Estrogen exerts inhibitory actions on TNFα most likely through inhibition of the AP-1 binding to the TNF promoter (Kimble et al., 1997; Kitazawa et al., 1994), and loss of estrogen results in increased TNFα secretion from PBM (Horowitz, 1993; Pacifici et al., 1991).

Estrogen-regulated cytokines are also involved in the regulation of bone cell apoptosis. *In vitro* studies have shown inhibition of osteoclast apoptosis by IL-1, IL-6, and TNF (Hughes and Boyce, 1997; Hughes et al., 1996). It is therefore possible that increased levels of IL-1, IL-6, and TNF secondary to lack of estrogen, could potentiate the negative effects of estrogen loss on bone by prolonging the lifespan of osteoclasts. Therefore sex steroids protect bone by both direct effects, and indirectly by suppressing the production of bone-resorbing cytokines. In an estrogen deficient state, this level of regulation is lost, allowing for increased production of cytokines, leading to elevated osteoclast formation and increased bone resorption. Thus, the loss of sex hormones results in a loss of bone mass in both males and females.

1.6 OSTEOPOROSIS TREATMENTS

Treatments for osteoporosis can either be anti-catabolic to inhibit further bone loss, or anabolic to increase bone mass. The majority of currently available treatments are anti-catabolic or antiresorptive, and are therefore designed to inhibit bone resorption and prevent further bone loss.

1.6.1 Antiresorptives

1.6.1.1 Bisphosphonates

Bisphosphonates are synthetic compounds characterised by two C-P bonds (Fleisch, 1998). Several bisphosphonates are currently available, each with their own biological and pharmacological profile. Importantly, they all target the osteoclast, inhibiting osteoclast recruitment, adhesion, activity, and reducing osteoclast lifespan (Fleisch, 1998). They are widely used for the treatment of osteoporosis, inhibiting bone resorption and therefore decreasing bone turnover and bone loss, and are able to effectively reduce fracture incidence and improve quality of life in osteoporotic patients.

1.6.1.2 Calcitonin

Calcitonin also inhibits bone resorption to reduce bone loss, and decrease fracture incidence (Chesnut et al., 2000; Delmas, 2002). Calcitonin is produced by the thyroid gland and interacts with its specific receptor on the osteoclast (Findlay and Sexton, 2004), to decrease the recruitment and function of osteoclasts. Like bisphosphonates, calcitonin also inhibits osteoblast and osteocyte apoptosis both *in vitro*, and *in vivo* (Plotkin et al., 1999). However, the benefits of calcitonin are lost with continued treatment, likely due to the down-regulation the calcitonin receptor (Takahashi et al., 1995).

1.6.1.3 HRT and SERMs

Administration of estrogen to postmenopausal osteoporotic women decreases the development of osteoclast progenitors and also promotes osteoclast apoptosis.

Consequentially, hormone replacement therapy (HRT) has been widely prescribed to early postmenopausal women with low BMD (Syed and Khosla, 2005). However, while effective in preventing bone loss in postmenopausal women, the associated risk of breast cancer and cardiovascular events recently reported with HRT use has resulted in a marked reduction in the number of patients on HRT therapy (Beral and Collaborators., 2003; Chlebowski et al., 2003; Rossouw et al., 2002; Warren and Halpert, 2004).

Selective estrogen receptor modulators (SERMs) were designed to create synthetic tissue-specific, partial estrogen receptor agonists that act as agonists in bone and in the cardiovascular system, without effects in reproductive tissue. Raloxifene, for example, acts as estrogen to prevent bone loss while reducing serum cholesterol levels, but without stimulating uterine hyperplasia in ovariectomised rats (Turner et al., 1994).

1.6.1.4 Other Antiresorptives

Cathepsin K is abundantly expressed in osteoclasts, and plays a critical role in the degradation of bone. Targeted inhibition of Cathepsin K activity is therefore a promising avenue for osteoporosis treatment, and is supported by studies in which oral administration of a Cathepsin K inhibitor prevented bone loss in ovariectomised rats (Kim et al., 2006; Yamashita and Dodds, 2000).

1.6.2 Anabolic therapies

Currently available anti-resorptive therapies are effective at preventing further bone loss, but are limited in their ability to restore lost bone mass. At best, they reduce the incidence of osteoporotic fracture by 50% (Boivin and Meunier, 2002). Therefore there is a requirement for novel anabolic treatments which promote the formation of new bone by osteoblasts (Rosen and Rackoff, 2001), with the potential to induce greater increments in bone density and greater reductions in fracture risk compared with anti-resorptive therapies.

1.6.2.1 PTH 1-34

Recombinant parathyroid hormone PTH 1-34 is the only anabolic agent currently approved for the treatment of osteoporosis. Intermittent administration of PTH 1-34 induces anabolic effects on bone, increasing bone formation and BMD at the lumbar spine and hip, and decreasing risk of vertebral and non-vertebral fracture (Bradbeer et al., 1992; Finkelstein et al., 1998; Neer et al., 2001). The anabolic effects are likely due in part to inhibition of osteoblast apoptosis, resulting in an increased rate of bone formation (Jilka et al., 1999).

The success of PTH demonstrates the clear value of anabolic therapies for osteoporosis and highlights the necessity for the development of further anabolic agents.

1.6.2.2 Other anabolic agents

Growth factors have also been investigated as potential anabolic agents for bone formation. Local administration of various growth factors, for example TGFβ, IGF-1, FGFs, and BMPs induces bone formation (Rosen and Rackoff, 2001), supporting a potential use for these factors for the treatment of osteoporosis or for improving fracture healing.

1.6.2.3 Strontium renelate

Strontium renelate has been shown in clinical trials to increase BMD of the lumbar spine and femoral neck, and reduce risk of vertebral and non-vertebral fractures in postmenopausal osteoporotic women (Meunier et al., 2004; Reginster et al., 2005b). This compound appears to have a unique dual mechanism of action; reducing bone resorption by direct inhibition of osteoclast formation and activity, and stimulating bone formation by increasing the proliferation of pre-osteoblastic cells and increasing matrix synthesis by mature osteoblasts (Reginster et al., 2005a), suggesting this to be a potentially rewarding avenue for osteoporosis treatment.

1.7 BODY WEIGHT AND BONE

Bone mineral density is closely related to body weight in adult men and women throughout the entire skeleton (Felson et al., 1993; Ravn et al., 1999; Reid et al., 1992a). Obesity has protective effects on bone, and is associated with a reduction in the prevalence of osteoporosis. The protective effects of body weight on bone mass was primarily thought to result from mechanical loading effects, with the consequential increase in bone volume secondary to a requirement to provide a support for a greater weight. In line with this, lean body status is a known risk factor for low BMD, and thus for fracture risk and the development of osteoporosis. Interestingly, several studies investigating the contribution of lean mass versus fat mass have reported fat mass to be a major correlate of bone density (Reid, 2002; Reid et al., 1992a; Reid et al., 1994; Reid et al., 1992b). However, the strong correlation between fat mass and non-weight bearing parts of the skeleton indicated the relationship between fat mass and bone mass was not entirely due to mechanical loading, leading to the hypothesis that adipose itself, or an adipose-released factor may influence bone mass.

Increased adipose is associated with lower levels of sex hormone binding globulin (SHBG), resulting in increased levels of circulating sex steroids, which may act to conserve bone mass (Reid, 2002). Interestingly, in post menopausal women, the primary source of estrogen is produced by aromatisation of androstenedione to estrone in adipose tissue (Frisch et al., 1980). Thus, postmenopausal women with low adipose mass have a reduced source of estrogen (Reid, 2002), consistent with a greater risk of osteoporosis in leaner subjects. However, while adipocytes are a major source of estrogen production in post-menopausal women, the relationship between fat and bone mass remains in premenopausal women, where only a small amount of estrogen production is dependent on adipose stores (Reid et al., 1992b), suggesting the production of estrogen by adipose is not likely to be the mechanism for the protective effects of adipose on bone mass.

A number of adipokines such as insulin, amylin, and leptin are released from adipocytes, and circulate at increased concentrations in obese subjects (Considine et al., 1996; Enoki et

al., 1992; Hanabusa et al., 1992). Recent evidence now also suggests an important role for these factors in the regulation of bone cell activity. Insulin receptors have been identified on osteoblast-like cells, and administration of insulin stimulates a proliferative response (Hickman and McElduff, 1989; Pun et al., 1989). Furthermore, local injection of insulin over one hemicalveria of adult mice increased indices of bone formation, including osteoid area, and osteoblast number and area, suggesting a direct anabolic effect of insulin on bone (Cornish et al., 1996). Amylin is co-secreted with insulin from the pancreatic β-cell, and can also directly stimulate osteoblast proliferation *in vitro* and *in vivo*. Unlike insulin, amylin is also able to inhibit bone resorption, therefore resulting in greater increases in mineralised bone area (Alam et al., 1993; Cornish et al., 1995). Recent studies have also indicated a role for the adipocyte-derived hormone adiponectin in the regulation of bone turnover. Unlike other adipokines, adiponectin levels are inversely correlated with adipose mass and BMD (Lenchik et al., 2003). While adiponectin is primarily excreted from adipocytes, it is also produced by primary human osteoblasts which also express adiponectin receptors (Berner et al., 2004; Luo et al., 2005). Administration of adenovirus expressing adiponectin into the jugular vein of mice results in increased trabecular bone mass and reduced parameters of bone resorption (Oshima et al., 2005). These findings are supported by *in vitro* evidence demonstrating the ability of adiponectin to stimulate proliferation and mineralisation in human osteoblasts and murine osteoblast cell lines respectively, and inhibit osteoclastogenesis in cultures of mouse bone marrow macrophages and human peripheral blood mononuclear cells (Luo et al., 2005; Oshima et al., 2005), demonstrating the ability of adiponectin to directly modify bone cell activity. However another recent study using adiponectin-deficient ($Ad^{-/-}$) and liver-specific adiponectin over-expressing models, suggests the control of bone formation by adiponectin involves opposing positive and negative effects of adiponectin signalling mediated by a direct autocrine/paracrine pathway, and an indirect endocrine pathway (Shinoda et al., 2006). These findings, together with the observation that adiponectin can also enhance insulin signalling to stimulate bone formation (Shinoda et al., 2006), suggests the control of bone formation by adiponectin to be very complex.

Secretion of leptin has been demonstrated by extramedullary adipose tissue and by marrow adipocytes (Laharrague et al., 1998), indicating it may directly affect bone cell activity by local interactions within the bone microenvironment. Indeed, several lines of evidence suggest this peptide is able to directly modulate the activity of osteoblasts, osteoclasts, and chondrocytes, and therefore may play an important role in bone remodelling. However, the actions of leptin in the regulation of energy homeostasis are mediated primarily through receptors located in the hypothalamus, and via a series of downstream effectors which together modulate the multiple effects of leptin on food intake and energy balance. Recent evidence suggests these centrally-located receptors may also modulate the actions of leptin on bone cell activity, indicating the actions of leptin on bone physiology may be more complex than initially thought. Therefore, before the role of leptin in bone remodelling is discussed further, it is important to understand the role of leptin and its interaction with key players in the regulation of energy homeostasis.

1.8 CENTRAL CONTROL OF ENERGY HOMEOSTASIS

The regulation of food intake and energy homeostasis is fundamental for sustaining life, and involves regulation by both short-term factors which regulate processes on a meal-to-meal basis, and long-term factors which are involved in the neuro-hormonal control of long-term energy homeostasis. Together these processes are able to maintain body weight within a narrow range over time in response to changing caloric intake and metabolic requirements. Short-term factors include cholecystokinin (CKK), peptide YY (PYY), and oxyntomodulin (OXM), released from endocrine intestinal cells, and ghrelin, released from the gastrointestinal tract. These factors respond rapidly to alterations in nutrient levels and are involved in the regulation of short-term satiety or the stimulation of feeding behaviour, and digestive processes following food intake (Batterham et al., 2002; Chelikani et al., 2005; Konturek et al., 2004). It is now well established that the central nervous system (CNS) also plays an important role in the regulation of energy homeostasis by receiving and processing a variety of responses to peripheral metabolic and nutritional information, thereby co-ordinating a series of interactions to maintain energy balance on a longer-term

basis. The primary CNS site controlling food intake and energy homeostasis is the hypothalamus (Elmquist et al., 1999).

1.8.1 Leptin

Leptin is small polypeptide hormone secreted into the circulation by adipocytes. Serum leptin levels are proportional to body adiposity, and are sensed by the leptin receptor in the hypothalamus to play a pivotal role in the regulation of whole body energy homeostasis (Friedman and Halaas, 1998; Zhang et al., 1994). Leptin inhibits feeding behaviour and stimulates energy expenditure by modulating sympathetic activity, thermogenesis, oxygen consumption, and locomotor activity. More importantly however, down-regulation of leptin expression with food restriction triggers a series of physiological responses to conserve energy. Consequentially, mice with mutations in the *obese* gene (ob/ob) encoding leptin are hyperphagic, morbidly obese, hyperglycemic, and hyperinsulinemic (Friedman and Halaas, 1998; Zhang et al., 1994). A similar phenotype has also been observed in humans carrying an inactivating mutation of the *obese* gene (Farooqi et al., 1999; Montague et al., 1997; Ozata et al., 1999; Strobel et al., 1998). Leptin deficiency also results in a number of other starvation-associated responses, for example, reduced fertility and thyroid activity, and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Ahima et al., 1998; Ahima et al., 1996; Guo et al., 2004; Legradi et al., 1997; Schwartz et al., 1995), all responses which conserve energy. The HPA axis is responsible for glucocorticoid production, and it would be expected that increased glucocorticoid levels during starvation would stimulate hepatic gluconeogenesis, providing the brain with glucose in a situation of depleted nutrients. Increased glucocorticoid levels would also be expected to induce a stress-response, possibly increasing chances of survival. Suppression of the thyroid axis reduces metabolic rate via several mechanisms, for example reduction of thermogenesis by uncoupling proteins in brown adipose tissue, and by altering lean body mass. These abnormalities are normalised by central administration of leptin, causing a dose-dependent reduction in body weight in control and ob/ob mice, and partially correcting the obesity, body temperature, glucose, and insulin levels of ob/ob mice (Ahima et al., 1996; Campfield et al., 1995; Chehab et al., 1996; Halaas et al., 1995; Pelleymounter et al., 1995), illustrating the important role of

central leptin in modulating neuro-endocrine and metabolic responses to maintain energy homeostasis during starvation. The significance of leptin in this role is supported by studies in which leptin administration during food restriction prevented many of the starvation-induced neuroendocrine changes, including activation of the HPA axis, and suppression of the reproductive, growth hormone, and thyroid axes (Ahima et al., 1996; Chan et al., 2003; Legradi et al., 1997).

Leptin's actions are mediated by the long form of its receptor Ob-Rb, a member of the class-1 cytokine receptor superfamily (Tartaglia, 1997; Tartaglia et al., 1995). Mice lacking functional leptin receptors (db/db) therefore have a similar obese phenotype to the ob/ob mouse, but cannot be corrected by exogenous leptin (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996). Mutations in the Ob-Rb receptor in humans are also associated with massive obesity, failure to undergo puberty, and decreased levels of growth hormone and thyroid hormone (Clement et al., 1998). However, the majority of obese humans have high levels of circulating leptin, correlating with their greater adiposity, yet do not have mutations in the leptin gene or receptor. It has been proposed that similar to insulin resistance, these subjects have developed a resistance to leptin and are therefore unable to respond to circulating leptin, irrespective of highly elevated leptin levels, possibly due to saturation of the leptin transport system resulting in defective transport of leptin across the blood-brain barrier from the periphery to the CNS. This proposal is supported by findings that high levels of serum leptin observed in obesity are not paralleled by proportional levels in cerebrospinal fluid leptin (Caro et al., 1996; Oh-I et al., 2005). Alternatively, leptin resistance may arise due to defects in downstream targets of leptin within the CNS, or due to antagonism of the central actions of leptin, for example, with increased levels of the IL-1 receptor antagonist (IL-1a) concurrent with obesity (Arch et al., 1998; Meier et al., 2002; Sahu, 2004).

Ob-Rb is most highly expressed in the hypothalamus, with the highest density of leptin receptors located in the arcuate nucleus, the ventromedial hypothalamus (VMH), and the dorsomedial hypothalamus (DMH), areas which surround the median eminence (Elmquist et al., 1998b; Fei et al., 1997; Mercer et al., 1996b; Schwartz et al., 1996b). Neuron-

specific, but not hepatocyte-specific disruption of the leptin receptor was associated with obesity, indicating that the nervous system is a primary target for the energy-homeostatic effects of leptin (Cohen et al., 2001). Leptin signalling results in activation of STAT3 (signal transducers and activators of transcription), a protein messenger downstream of the Ob-Rb receptor, activating immediate-early genes such as c-fos, and inducing the inhibitor of leptin signal transduction, SOCS3 (suppressor of cytokine signalling 3) (Bjorbaek et al., 1999; Elmquist et al., 1998a). Identification of neurons expressing c-fos following leptin administration has revealed regions that respond to activation by leptin, including the arcuate nucleus, VMN, DMH, and paraventricular nucleus (PVN) (Elias et al., 1998a; Elmquist et al., 1998a; Elmquist et al., 1997). Importantly, the actions of leptin on food intake and modulation of the gonado-, cortico-, somato-, and thyrotropic axes are mediated by several downstream effectors, including neuropeptide Y (NPY) and the melanocortin system.

1.8.2 Neuropeptide Y

NPY is an established potent stimulator of food intake (Blomqvist and Herzog, 1997; Hokfelt et al., 1998; Inui, 1999; Stanley et al., 1986). Expression of this 36 amino acid neuropeptide is widely distributed thoughout the peripheral nervous system (PNS) and the CNS including the arcuate nucleus (Allen et al., 1983; Chronwall et al., 1985), where some NPY-ergic neurons co-express the leptin receptor (Mercer et al., 1996a). Expression of NPY is elevated in response to fasting, when leptin levels are low (Schwartz et al., 1995; Spanswick et al., 1997; Spiegelman and Flier, 1996), and in ob/ob mice (Wilding et al., 1993). Administration of leptin to ob/ob mice reduces the elevated levels of NPY (Schwartz et al., 1996a; Stephens et al., 1995), while central injection of NPY produces physiological effects similar to those observed in leptin deficiency, including hyperphagia, hyperinsulinemia, decreased thermogenesis, and the development of obesity (Billington et al., 1994; Stanley et al., 1986; Stanley and Leibowitz, 1985), supporting a role for NPY as a key downstream effector of leptin's actions on body weight. This is supported by studies in which deletion of NPY partially corrected the obesity phenotype of ob/ob mice (Erickson et

al., 1996b). However, some neuroendocrine abnormalities typical of the ob/ob mouse remain in the double knockout, indicating the involvement of other signalling pathways.

NPY also activates the HPA axis, however, the effects of starvation such as activation of the HPA axis, with suppression of reproduction and the thyroid axes occur normally in NPY knockout ($NPY^{-/-}$) mice, (Erickson et al., 1997; Erickson et al., 1996b), again suggesting the involvement of other factors in the regulation of the neuroendocrine starvation response. In support of this, body weight and feeding behaviour in $NPY^{-/-}$ mice is normal, despite the known role of NPY in the regulation of feeding and energy homeostasis, indicating the presence of compensatory factors and suggesting that NPY is not the sole downstream effector for leptin actions on body weight (Stephens, 1996).

1.8.3 The melanocortin system

The melanocortin system involves a series of peptides which are cleaved from pro-opiomelanocortin (POMC), and within the CNS are located in the arcuate nucleus and the nucleus of the solitary tract (NTS) (Broberger et al., 1998; Elias et al., 1998b). One of the POMC products, α-melanocyte-stimulating hormone (α-MSH), is also significantly involved in the regulation of energy homeostasis, mediating several of the physiological effects of leptin. A high percentage of POMC-expressing neurons within the arcuate express the leptin receptor, Ob-Rb (Cheung et al., 1997). POMC expression in the arcuate is also induced by leptin administration (Schwartz et al., 1997), and conversely is markedly reduced in ob/ob mice and fasted rodents (Mizuno et al., 1998b; Thornton et al., 1997). α-MSH is an agonist for the melanocortin-4 receptor (MC4-R) (O'Rahilly et al., 2004), and administration of a synthetic MC4-R agonist suppresses food intake (Fan et al., 1997), indicating an important role for these receptors in regulation of body weight and metabolism. Mutation or deletion of the MC4-R results in obesity in rodents and humans (Farooqi et al., 2003; Farooqi et al., 2000; Huszar et al., 1997; O'Rahilly et al., 2004; Vaisse et al., 1998; Yeo et al., 1998). A naturally occuring MC4-R antagonist, *agouti*-related protein (AgRP), is produced in the arcuate nucleus (Broberger et al., 1998; Elias et al., 1998b; Mizuno and Mobbs, 1997; Ollmann et al., 1997), where it colocalises with a high

percentage of NPY neurons (Hahn et al., 1998). It acts to blocks the anorexic actions of α-MSH on melanocortin receptors, and over-expression of either AgRP in mice using a transgenic approach, or another MC4-R antagonist, agouti, in the yellow agouti (A^y) mouse produces an obese phenotype similar to the MC4-R knockout model (Fan et al., 1997; Graham et al., 1997; Ollmann et al., 1997; O'Rahilly et al., 2004). In contrast to POMC, expression of AgRP is increased in ob/ob mice, and during fasting (Hahn et al., 1998; Mizuno and Mobbs, 1997). The distribution of MC4-R mRNA and axon terminals containing α-MSH and AgRP in specific subdivisions of the paraventricular nucleus which innervate parasympathetic and sympathetic preganglionic neurons, are consistent with a role for the melanocortin system also in the regulation of autonomic activity by leptin (Kishi et al., 2003; Kishi and Elmquist, 2005; Mountjoy et al., 1994). Consistent with this, antagonism of MC4-R suppresses the sympathetic-mediated expression of uncoupling protein-1 by leptin (Satoh et al., 1998). Melanocortins are also involved in the regulation of the HPA and the thyrotropic axis (Fekete et al., 2000; Lu et al., 2003). Therefore, melanocortin-mediated signaling is one of the downstream targets of leptin action, mediating multiple effects on energy balance and neuro-endocrine function to maintain energy balance.

1.8.4 Melanin-concentrating hormone

AgRP and α-MSH fibers also project to the lateral hypothalamic area (LHA), a region in which melanin-concentrating hormone (MCH) is produced (Bittencourt et al., 1992; Qu et al., 1996). Levels of MCH are also elevated during fasting, when leptin levels are reduced, and in ob/ob mice, while central administration of MCH stimulates feeding behaviour. Mice lacking both MCH and leptin receptors have reduced fat mass compared with ob/ob mice, (Segal-Lieberman et al., 2003), demonstrating a role for MCH downstream of leptin to modulate its effects on feeding and energy homeostasis.

1.8.5 Cocaine- amphetamine-regulated transcript

POMC neurons of the arcuate nucleus also co-express cocaine- amphetamine-regulated transcript (CART), a neuropeptide widely distributed throughout the CNS which inhibits both normal and starvation-induced feeding when administered centrally into rodents (Kristensen et al., 1998; Lambert et al., 1998). Like POMC, CART is also a target of leptin, as CART levels are decreased with low levels of leptin, for example with starvation or in ob/ob mice, and are restored with administration of leptin (Kristensen et al., 1998). CART peptide-immunoreactive terminals innervate central autonomic sites, for example, sympathetic preganglionic neurons in the spinal cord (Elias et al., 1998a), and may therefore also regulate sympathetic activity by leptin (Fenwick et al., 2006).

1.8.6 Insulin

In addition to leptin, insulin is also thought to act as a negative feedback signal to the brain (Schwartz et al., 1992). Receptors for insulin are expressed in the brain and plasma insulin can access these via a receptor-mediated saturatable transport system (Schwartz et al., 1991). Insulin levels are increased in the fed state and insulin secretion reflects body adiposity. However, the expression of insulin is also regulated by leptin, which acts to reduce glucose-induced insulin secretion (Mizuno et al., 1998a; Zhao et al., 1998). Central administration of insulin reduces food intake and body weight in rodents (Chavez et al., 1995; Sipols et al., 1995). Interestingly however, administration of insulin to fa/fa rats with defective leptin signalling does not reduce food intake, and while this may be simply due to insulin-resistance, it may also suggest that intact leptin signalling is required for insulin to exert its central effects (Ikeda et al., 1986).

Therefore, the actions of leptin are likely to be mediated by simultaneous activation of multiple catabolic effectors and suppression of anabolic effectors present in the hypothalamus, which together interact in what appears to be a complex system to maintain energy homeostasis.

1.9 ACTIONS OF LEPTIN ON BONE – CENTRAL AND PERIPHERAL EFFECTS

The above sections discussed briefly the strong correlation between body weight and bone mass and the recent demonstration that several hormones released from adipocytes, or associated with the regulation of energy homeostasis including insulin, amylin, and adiponectin, can modulate bone cell activity.

1.9.1 Central antiosteogenic actions of leptin

The finding that ob/ob mice had increased trabecular bone formation and a high trabecular bone mass phenotype within the proximal tibiae and lumbar vertebrae, despite their hypogonadism and hypercorticism was unexpected (Ducy et al., 2000). Hypogonadism would be expected to cause an increase in bone resorption, resulting in bone loss. The ob/ob mice did in fact have increased numbers of osteoclasts associated with increased parameters of bone resorption, and correction of the hypogonadism phenotype by subcutaneous implantation of either testosterone or estradiol pellets normalised resorption resulting in further increases in trabecular bone volume (Ducy et al., 2000). Hypercorticism also favours the development of osteoporosis. However, the ob/ob mice were found to have a nearly two-fold greater trabecular bone volume compared to their wild type littermates, associated with an increased rate of bone formation. Similar observations in the db/db mouse led to the conclusion that leptin signals through its known receptor to control bone mass. Intracerebroventricular (icv) infusion of leptin into the third ventrical resulted in a loss of bone in both ob/ob and wild type mice, demonstrating leptin to act via the central nervous system to inhibit bone formation (Ducy et al., 2000). The absence of detectable leptin in the serum of icv-administered ob/ob mice was consistent with a central mechanism of leptin action. A key role for hypothalamic leptin in regulating bone formation was confirmed using a parabiosis model in which only one ob/ob mouse of a parabiosed pair received icv leptin. Correction of the ob/ob skeletal phenotype by loss of bone mass was only achieved in leptin-recipient, and not the contralateral mouse (Takeda et al., 2002),

clearly demonstrating that similar to its control of energy homeostasis by central mediation, the antiosteogenic actions of leptin are also regulated through a similar hypothalamic relay.

Studies using other mouse models of obesity not related to leptin signalling demonstrated the control of bone formation by leptin in mice to be independent of the presence of fat (Ducy et al., 2000). A high trabecular bone mass phenotype was also observed in the A-ZIP/F-1 mouse which also has reduced leptin levels due to a lack of white adipose tissue, and in heterozygous $ob^{+/-}$ mice, or young ob/ob mice fed a low fat diet, demonstrating the increase in bone formation was due to an absence of leptin signalling, and not secondary to changes in fat mass (Ducy et al., 2000).

The regulation of bone formation by leptin however, appears to be more complex than these initial investigations suggest. In the first instance, as mentioned above (section 1.7), leptin levels are highly elevated in obese individuals, which would appear to contradict the findings from the ob/ob mouse studies described above. It is possible that part of this apparent contradiction arises with the development of leptin resistance with excessive adiposity, possibly due to altered transport of leptin across the blood brain barrier. This proposal is supported by findings from Elefteriou et al, 2004, in which a transgene-induced increase in serum leptin reduced trabecular bone volume and osteoblast activity to a similar extent, regardless of whether serum leptin was increased by 4-fold or 200-fold (Elefteriou et al., 2004), suggesting that while levels of serum leptin is a determinant of bone mass, sensitivity of the hypothalamic neurons to leptin's antiosteogenic actions appear to get abrogated at a particular level (Elefteriou et al., 2004).

It is also possible that differences in osteoblastic responses to leptin signalling are the result of species or strain differences. Similar to the db/db mouse, leptin-resistant Zucker (fa/fa) rats have an inactivating mutation in their Ob-Rb leptin receptor gene (Takaya et al., 1996), but in contrast to the ob/ob or db/db models, these rats have reduced femoral bone mineral density (BMD) and calcium content, and decreased measurements of trabecular bone volume (Mathey et al., 2002; Tamasi et al., 2003). Recent studies have also demonstrated that leptin action in cortical bone may be distinct from its antiosteogenic effects in

trabecular bone, with reduced bone mineral content (BMC), and BMD, associated with reduced cortical thickness in 4-week old, and in skeletally mature ob/ob mice (Baldock et al., 2006b; Hamrick et al., 2004; Steppan et al., 2000), which in young mice was reversed following intraperitoneal administration of leptin (Steppan et al., 2000). Some studies have also reported reduced femoral length in ob/ob mice (Hamrick et al., 2004; Steppan et al., 2000), however, this finding is not consistent across all studies (Baldock et al., 2006b), and may relate to strain differences. Discrepancies in femur length may also relate to actions of leptin at the growth plate rather than on osteoblastic activity, as studies have demonstrated the ability of leptin to stimulate the proliferation and differentiation of growth plate chondrocytes *in vitro*, and to increase growth plate thickness *in vivo* (Cornish et al., 2002; Maor et al., 2002; Nakajima et al., 2003). Interestingly, some studies have also reported reduced trabecular bone volume within the distal and proximal femur of ob/ob mice (Hamrick et al., 2004; Steppan et al., 2000), contradicting findings from other studies which reported greater trabecular bone volume in the distal femur, proximal tibiae, and lumbar vertebrae of ob/ob mice (Baldock et al., 2005; Ducy et al., 2000). The reason for these inconsistencies is not clear but may relate to differences in the methods used to measure trabecular bone volume. One study demonstrating reduced trabecular bone volume within the distal femur of ob/ob mice used peripheral quantitative computerised tomography (pQCT) to measure trabecular bone within a 1mm cross-section (Steppan et al., 2000). Other studies used histomorphometry, however one study identified trabecular bone by hematoxylin and eosin staining on decalcified sections and would therefore not be able to distinguish mineralised bone from osteoid (Hamrick et al., 2004), while others measured mineralised trabecular bone volume by von Kossa staining of un-decalcified sections (Baldock et al., 2005; Ducy et al., 2000). Discrepancies between these studies could also relate to the sampling differences, with reduced trabecular bone volume reported in the proximal femur (Hamrick et al., 2004), but elevated in the distal femur of ob/ob mice (Baldock et al., 2006b). Of note, the study by Hamrick et al, reporting reduced femoral BMC and BMC in ob/ob mice also reported that while cortical thickness of lumbar vertebrae was reduced, vertebral length and lumbar BMC and BMD was increased (Hamrick et al., 2005), suggesting distinct actions of leptin not only on cortical and trabecular compartments, but also differential responses to leptin signalling between

appendicular and axial skeletal sites, with decreased trabecular bone volume within long bones but increased trabecular bone volume within the vertebral bones of leptin deficient ob/ob mice (Hamrick et al., 2005). It has been proposed this may be due to differences in the innervation of these regions, or secondary to muscle mass, mechanical loading, or differing proportions of marrow adipocytes, contributing to an altered response of marrow cells to differentiation stimulus (Hamrick et al., 2005). However, evidence from other studies demonstrating a greater trabecular bone volume within long bones including tibiae and femur, as well as vertebrae of ob/ob mice, suggest differences between these studies are not entirely the result of differential effects of leptin deficiency at different skeletal sites (Baldock et al., 2005; Ducy et al., 2000). These studies emphasise the importance of sampling multiple skeletal regions to assess a phenotype, however they appear to remain contrasting with those of Ducy et al, in which greater trabecular bone volume was reported in both long bones and vertebrae of ob/ob mice.

The increase in trabecular bone formation reported in the ob/ob and db/db mice suggests an important antiosteogenic role for leptin acting through a centrally-mediated pathway. However, substantial evidence also exists for direct actions of leptin within the periphery to positively modulate osteoblast activity. Peripheral administration of leptin is associated with reduced ovariectomy-induced bone loss in rats (Burguera et al., 2001), and increased bone strength in mice (Cornish et al., 2002). The contradiction between these findings and the central antiosteogenic actions of leptin reported in the ob/ob mouse could result from the effects of positive peripheral leptin signaling at elevated leptin levels dominating the antiosteogenic effects of central leptin signaling (Khosla, 2002).

1.9.2 Leptin in humans

Genetic evidence in humans regarding the role of leptin is so far limited with mixed findings. An osteogenic effect of leptin is supported by the report that one of four individuals in a family carrying a missense mutation in the leptin gene exhibited low bone mass despite morbid obesity, however a high degree of consanguinity and associated endocrine defects means caution must be taken when interpreting these findings (Ozata et

al., 1999). In another study supporting an osteogenic effect of leptin, administration of leptin to a young patient with a leptin mutation was associated with loss of adipose and an increase in whole body BMD (Farooqi et al., 1999). These findings contrast with findings from one report in which indirect evidence suggested an antiosteogenic effect of leptin, with advanced bone age in a leptin-deficient child and in patients with lipodystrophy (Elefteriou et al., 2004). This is supported by another study with the identification of a polymorphism in the human leptin-receptor gene associated with reduced leptin-binding affinity, which was not associated with altered body weight or serum leptin, but was associated with higher lumbar BMD in young men, although these effects were dependent upon genotype at the estrogen receptor-α locus and linkage disequilibrium has not been excluded (Koh et al., 2002). However, calorie restriction in anorexia nervosa is associated with low BMD, and although these patients also exhibit low levels of IGF-1 and amenorrhea, these findings suggest that unlike the ob/ob model, depleted leptin levels in humans cannot override the consequences of low IGF-1 and hypogonadism (Miller et al., 2004; Soyka et al., 1999). Furthermore, leptin treatment in patients with hypothalamic amenorrhea due to strenuous exercise or low body weight resulted in increased levels of both IGF-1 and bone formation markers (Welt et al., 2004). Interpretation of these studies however is clouded due to multiple alterations in neuroendocrine function and reproductive effects in these subjects. Studies looking at association between leptin and bone mass in subjects with normal leptin signalling have also produced mixed results. In cross-sectional studies, serum leptin levels and BMD are correlated in pre- and post-menopausal females in some studies (Thomas et al., 2001; Yamauchi et al., 2001), but not others (Rauch et al., 1998). A positive correlation between leptin levels and BMC was observed in a group of healthy nonobese women (Pasco et al., 2001), while a weak correlation was observed in postmenopausal osteoporotic women but not controls (Odabasi et al., 2000). However, studies in males have reported either no association (Thomas et al., 2001), or a negative association (Sato et al., 2001), between leptin and BMD, suggesting gender specificity or interaction of leptin with sex hormones in its control of bone formation. In a further study however, while a positive association between leptin and BMD was observed, no association between leptin and biochemical markers of bone resorption or formation were

observed, concluding that leptin may not play a direct role in regulating bone cell activity (Goulding and Taylor, 1998). Thus the role of leptin in human bone biology is still a matter for debate.

1.9.3 Peripheral effects of leptin on bone

Evidence for direct peripheral effects of leptin on bone cell activity is supported by findings from numerous *in vitro* studies. Evidence for the presence of the leptin receptor Ob-Rb, or leptin-binding sites have been detected on ossifying fetal cartilage (Hoggard et al., 1997), marrow stromal cells and osteoblasts, chondrocytes, and some but not all osteosarcoma cell lines (Cornish et al., 2002; Enjuanes et al., 2002; Lee et al., 2002; Reseland et al., 2001; Steppan et al., 2000; Thomas et al., 1999). Studies have also demonstrated the production of leptin by primary human and rat osteoblasts, and by human primary bone marrow adipocytes, further supporting a role for direct regulation of bone cell activity by leptin (Laharrague et al., 1998; Morroni et al., 2004; Reseland et al., 2001). Several studies have also demonstrated direct effects of leptin on chondrocytes and osteoblasts. Leptin stimulates the proliferation and differentiation of cultured growth plate chondrocytes (Nakajima et al., 2003), and induces differentiation of osteoblasts while suppressing adipogenesis in a human stromal cell line (Thomas et al., 1999). Leptin also stimulates the proliferation, differentiation, and function of osteoblastic cultures from human and rat (Cornish et al., 2002; Gordeladze et al., 2002). Leptin can also inhibit osteoclastogenesis *in vitro* (Cornish et al., 2002; Holloway et al., 2002), supporting a role for leptin in multiple aspects of bone remodelling. However, one clearly contradictory study failed to detect expression of leptin or the leptin receptor in osteoblasts, and did not observe any effect of leptin administration on the formation of mineralised nodules in primary mouse osteoblastic cultures (Ducy et al., 2000). These contrasting data could be due to species differences, or to the sensitivity of the methods used. However, the majority of *in vitro* evidence supports a model in which the direct peripheral actions of leptin to modulate bone cell activity. Inconsistencies in the literature may therefore in part be explained by competing effects of positive peripheral actions and antiosteogenic central actions of leptin on bone cell physiology.

1.9.4 Control of bone remodelling by the sympathetic nervous system

Recent studies have revealed the central antiosteogenic actions of leptin are mediated via the sympathetic nervous system (SNS). A mutant mouse strain deficient in dopamine β-hydroxylase (DBH), an enzyme necessary to produce the catecholamine ligands for adrenergic receptors; noradrenaline and adrenaline, was also found to have high bone mass (Takeda et al., 2002), which importantly, was not reduced by icv infusion of leptin, indicating the requirement for functional sympathetic signalling for leptin antiosteogenic function. This study was supported by studies using β-adrenergic receptor agonists and antagonists. Sympathetic tone is reduced in leptin deficient ob/ob mice (Bray and York, 1998). Administration of the non-selective β-adrenergic receptor antagonist propranolol increased bone mass in vertebrae and long bones of ob/ob and wild type mice, and prevented bone loss following ovariectomy (Takeda et al., 2002). Conversely, administration of the β-adrenergic agonist isoproterenol restored sympathetic activity in ob/ob mice, and decreased bone mass in both ob/ob and wild type mice without affecting body weight (Takeda et al., 2002), demonstrating that modulation of SNS activity can affect bone remodelling. These findings are supported by findings from some, but not all studies from other groups. One study found that treatment with propranolol increased bone strength in intact rats and enhanced bone formation in the repair of surgically introduced bone defects (Minkowitz et al., 1991), while another study reported that administration of the specific β2-adrenegic agonist clenbuterol prevented a reduction in mineralisation of bone caused by sectioning of the sciatic nerve in rats (Zeman et al., 1991). However, changes in skeletal muscle following clenbuterol treatment may also have contributed to the inhibition of bone mass observed in this study.

Supporting an involvement of β-adrenergic signalling in the control of bone formation, β-adrenergic receptors (β-AR) have been identified on osteoblasts and in osteoblast-like cell lines. β1- and β2-ARs are expressed in human osteoblast-like cells lines (Kellenberger et al., 1998), and β2-ARs have been identified on rat osteoblast-like cells, and in mouse

primary osteoblast cultures (Moore et al., 1993; Takeda et al., 2002), suggesting modulation of sympathetic activity might modulate osteoblast function through direct receptor interactions. Administration of the β2-AR agonist formoterol induced cAMP levels and expression of the immediate early gene *c-fos* in a human osteosarcoma cell line (Kellenberger et al., 1998), while administration of a specific β2-AR antagonist inhibited *c-fos* expression, demonstrating that β2-ARs in osteoblasts are coupled to functional intracellular signalling pathways. Further studies have demonstrated that administration of adrenaline to an osteoblast cell line stimulated expression of RANKL (Takeuchi et al., 2000), and the ability of noradrenaline to stimulate bone resorption in mouse calvariae organ cultures (Moore et al., 1993), demonstrating the ability of adrenergic signalling to also alter osteoclast activity, possibly by an indirect osteoblast-mediated pathway.

Mice lacking functional β2-ARs (*Adrb2*$^{-/-}$) have normal fat mass and are fertile, but have an even greater trabecular bone volume compared with ob/ob mice, or with wild type mice receiving β-receptor antagonists (Elefteriou et al., 2005). Transplantation of non-adherent bone marrow cells from wild type mice normalised bone formation in *Adrb2*$^{-/-}$ mice, whereas the reciprocal transplant led to increased bone formation, supporting a role for osteoblasts in mediating the SNS control of bone remodelling (Elefteriou et al., 2005). In addition to increased bone formation however, *Adrb2*$^{-/-}$ mice also had significantly decreased bone resorption, and were therefore resistant to gonadectomy-induced bone loss (Elefteriou et al., 2005). Not surprisingly, central administration of leptin to *Adrb2*$^{-/-}$ mice was unable to reduce bone mass, but was also unable to correct the resorption phenotype, indicating that SNS signalling mediates not only the antiosteogenic activity of leptin, but together with the above *in vitro* data suggests that leptin also regulates bone resorption via sympathetic signalling (Elefteriou et al., 2005). Interestingly, transplantation of wild type bone marrow cells restored normal levels of bone resorption in *Adrb2*$^{-/-}$ mice, while the reciprocal transplant reduced parameters of resorption, consistent with the control of bone resorption by sympathetic signalling occurring via cells of the osteoblastic lineage (Elefteriou et al., 2005). Furthermore, the β-agonist isoproterenol did not increase osteoclast formation in co-cultures of wild type bone marrow macrophages with osteoblasts

from *Adrb2*$^{-/-}$ mice, while an increase did occur in co-cultures using wild type osteoblasts (Elefteriou et al., 2005). Isoproterenol did increase RANKL expression in cultured wild type but not *Adrb2*$^{-/-}$ osteoblasts, via a mechanism involving the osteoblast-specific transcription factor ATF4, supporting indirect sympathetic regulation of resorption via cells of the osteoblastic lineage.

Thus, *in vitro* and rodent model studies support a role for the regulation of bone remodelling by sympathetic activity. However studies investigating the effects of β-adrenergic antagonists commonly used for the treatment of cardiovascular disease on bone turnover, bone mineral density, and fracture risk, in human population-based studies have produced mixed findings. β-blocker use was associated with a reduction in fracture risk and increased BMD at the hip and forearm in women over 50 years of age (Pasco et al., 2004), and reduced fracture risk in women and men between 30 and 79 years of age (Schlienger et al., 2004), consistent with the rodent model data. However, a third observational study contradicts these findings, with β-blocker use associated with a three-fold increase in fracture risk and reduced serum osteocalcin in perimenopausal women (Rejnmark et al., 2004), together indicating that placebo-controlled randomised clinical trials may be necessary to more effectively assess the effects of β-blocker use on bone turnover in humans.

1.9.5 Role of CART in bone remodelling

The finding that *Adrb2*$^{-/-}$ mice were resistant to gonadectomy-induced bone loss due to reduced resorption was unexpected, as they were considered to be a phenocopy of the hypogonadal ob/ob mice, in which bone resorption is significantly elevated (Elefteriou et al., 2005). The contradicting findings from these two genetic models were resolved with the finding that CART, which is reduced in ob/ob mice, is able to inhibit bone resorption. Cart$^{-/-}$ mice have low bone mass associated with increased bone resorption, with an enhanced resorptive response to icv leptin infusion (Elefteriou et al., 2005), demonstrating a role for CART in the inhibition of bone resorption, and the ability of leptin-mediated sympathetic

regulation of bone mass to occur in the absence of CART. Transcripts for CART were not detected in bone cells, however, up-regulation of RANKL expression in Cart$^{-/-}$ bones suggests indirect regulation of osteoclast function through the osteoblast lineage (Elefteriou et al., 2005).

1.9.6 Role of the melanocortin system in bone remodelling

Chemical ablation of hypothalamic structures has also been used to identify the set of neurons responsible for leptins antiosteogenic action. Using this method it was concluded that neurons within the ventromedial hypothalamic nucleus mediate leptin antiosteogenic actions, while neurons within the arcuate nucleus mediate leptin actions on body weight, but not bone mass. Therefore, it appears that the regions controlling leptins antiosteogenic and anorexigenic networks differ (Takeda et al., 2002). These data are supported by characterisation of the A^{y} mouse. As discussed earlier (section 1.8), these mice have reduced melanocortin signaling and develop a late-onset obesity phenotype. Interestingly, while these mice were resistant to the anorexigenic actions of leptin, they were not resistant to leptin antiosteogenic action, with decreased bone volume observed with long-term icv leptin infusion (Takeda et al., 2002). Furthermore, administration of the melanocortin receptor agonist, MTII to ob/ob mice significantly decreased body weight, but did not affect bone mass (Takeda et al., 2002), further indicating that the pathways by which leptin regulates body weight and bone formation are distinct, and suggesting that melanocortin signalling is not involved in the regulation of bone remodelling.

However, while MC4-R$^{-/-}$ mice have been reported to have normal bone mass at 3 months of age (Takeda et al., 2002), at 6 months of age they have a high bone mass phenotype associated with increased hypothalamic expression of CART and reduced osteoclast number (Elefteriou et al., 2005). These latter findings support findings in human studies, in which bone density is increased in subjects lacking MC4-Rs (Farooqi et al., 2000), associated with decreased serum markers of bone resorption (Elefteriou et al., 2005).

Evidence also exists suggesting direct actions of POMC-derived peptides on bone cell activity. *In vitro* studies have demonstrated the ability of α-MSH to increase proliferation of cultured osteoblasts and chondrocytes, and stimulate chondrogenesis in mouse bone marrow cultures (Cornish et al., 2003), suggesting α-MSH acts to increase bone turnover. These findings are further supported by *in vivo* studies, with the demonstration that systemic administration of α-MSH decreases bone (Cornish et al., 2003). Expression of the five known subtypes of melanocortin receptors have been identified in bone cells, with expression of MC4-R mRNA so far identified in murine osteoclast cell lines and rat primary osteoclasts, rat and human primary osteoblasts and osteoblast-like cell lines, and associated with periosteal bone and growth plate chondrocytes in mouse bone tissue (Dumont et al., 2005; Zhong et al., 2005). However, other recent studies suggest that hypothalamic elevation of CART is the sole cause for the high bone mass observed in MC4-R$^{-/-}$ mice, acting to indirectly reduce parameters of bone resorption (Ahn et al., 2006). These findings from the *in vivo* and *in vitro* studies may therefore suggest that similar to the actions of leptin, the regulation of bone metabolism by POMC-derived peptides may also involve the actions of both central and peripherally-mediated effects.

While the precise mechanism by which MC4-Rs and CART regulates bone resorption are not known, these findings together clearly demonstrate that multiple neural factors with known interactions in the regulation of energy homeostasis are also involved in the regulation of bone remodelling, and may therefore participate in the mechanism by which increased body weight protects against bone loss in humans.

1.10 NEUROPEPTIDE Y AND THE Y RECEPTORS

1.10.1 NPY system ligands

The neuropeptide Y (NPY) system involves the actions of 3 ligands; NPY, peptide YY (PYY), and pancreatic polypeptide (PP), which are produced in specific distributions, but all consist of a 36 amino acid peptide with a carboxy-terminal amide (Tatemoto et al.,

1982). NPY has a high degree of sequence identity with PYY (70%) and PP (50%) (Larhammar, 1996a).

NPY is produced by neurons of the central and peripheral nervous systems, and is present in both sympathetic and parasympathetic nerve fibres, particularly around blood vessels where it has a well-established role in vasoconstriction, in addition to enhancing the action of other pressor agents (Morris, 1994; Parker and Herzog, 1999; Pernow et al., 1987). NPY-ergic neurons are also abundant in the brain, with high levels in the arcuate nucleus and ventromedial hypothalamus of the hypothalamus, as well as in the cerebral cortex, the hippocampus, the septum, and the brainstem (Chronwall, 1985; Hokfelt et al., 1998; Lindefors et al., 1990). Central NPY action is associated with the regulation of food intake, cardiac and respiratory activity, and the release of pituitary hormones (Wettstein et al., 1995). Of note, NPY has also been found in non-sympathetic neurons outside the CNS, for example in the gastrointestinal tract, salivary and thyroid glands, pancreas, urinogenital system, and the heart, and is expressed by non-neuronal cells, for example rat megakaryocytes (Ericsson et al., 1987; Silva et al., 2002).

Human NPY is formed by proteolytic processing of the precursor pre-pro-NPY to remove a 28 amino signal peptide at the N-terminal end, followed by cleavage of a dibasic site by prohormone convertases (Silva et al., 2002). Further truncation at the C-terminal end and conversion of the carboxyl-terminal glycine into the amide function leads to the biologically active amidated NPY (Blomqvist et al., 1992). The amide moiety is essential for the activity of NPY and prevents degradation by carboxypeptidases. At least two proteolytic processing sites are found within the mature NPY, which are cleaved by the cell surface enzymes dipeptidyl peptidase IV (DPPIV), and aminopeptidase P, to produce NPY_{3-36}, and NPY_{2-36}, respectively (Silva et al., 2002).

PYY is produced primarily from the endocrine L cells of the gastrointestinal tract with some expression in the pancreatic islets, and its function is related to satiety control and gastrointestinal regulation (Lundberg et al., 1984; Tatemoto, 1982). PYY expression has also been reported in brainstem neurons, although the functional significance of this

localisation is not known (Ekblad and Sundler, 2002). PP is produced by endocrine islet cells of the pancreas in response to stimuli such as ingestion of food, hypoglycaemia, and regulates endocrine functions of the pancreas as well as satiety (Ueno et al., 1999).

1.10.2 The Y receptors

The NPY system mediates its actions through activation of 5 Y receptor subtypes; Y1, Y2, Y4, Y5, and in the mouse and rabbit y6 (Blomqvist and Herzog, 1997; Lin et al., 2005). All the Y receptor subtypes are seven-transmembrane receptors coupled to inhibitory G proteins, making it one of the most complex families of G-protein coupled receptors known (Blomqvist and Herzog, 1997). The Y receptors therefore mediate inhibition of cAMP synthesis; however, Y1 receptors can also couple to phospholipase C to induce release of Ca^{2+} from intracellular stores (Herzog et al., 1992; Selbie et al., 1995). While NPY and PYY exhibit high similarity in primary and tertiary structure, the Y receptor subtypes show low sequence identity, for example Y1, Y2, and Y5 are only 30% identical (Blomqvist and Herzog, 1997; Larhammar, 1996b). NPY and PYY have equal affinity for all Y receptor subtypes, however PP has the highest affinity for the Y4 receptor (Bard et al., 1995; Blomqvist and Herzog, 1997).

Y1 and Y2 receptors are located at the post- and presynaptic terminals of the neuroeffector junction, respectively. They are both abundant in neural tissue, with hypothalamic expression of Y1 in several brain sites important for energy balance, including the paraventricular nucleus, with significant expression of Y2 in the arcuate nucleus (Kishi et al., 2005; Kopp et al., 2002; Naveilhan et al., 1998). Y1 expression has also been detected in the periphery on small arteries and arterioles of many organ systems, including the lymphatic system, the gastrointestinal duct, the kidneys, the reproductive and endocrine systems, as well as on the islets of Langerhans and cardiovascular smooth muscle cells (Matsuda et al., 2002; Pedrazzini, 2004). Activation of Y1 receptors located post-junctionally on blood vessels results in potent vessel vasoconstriction.

As mentioned above, the Y2 receptor is also abundantly expressed within the CNS, with particularly high levels within the arcuate nucleus, an area which surrounds the median eminence and is therefore accessible to circulating peripheral factors (King et al., 2000). The predominantly pre-synaptic Y2 receptor is thought to act as an autoreceptor (King et al., 2000), and is also known to suppress the synthesis and release of neurotransmitters including the production of NPY by Y2 expressing NPY-ergic neurons (King et al., 2000), in addition to regulating sympathetic activity by modulating the release of noradrenaline (Brain and Cox, 2006). In the mouse and in other species, NPY activates Y2 receptors on the parasympathetic nerve terminal to inhibit parasympathetic activity to the heart (Smith-White et al., 2002b). The Y4 receptor is widely distributed, with abundant expression in specific brain stem nuclei such as the area postrema, an area with an incomplete blood-brain barrier (Parker and Herzog, 1999; Trinh et al., 1996), and in peripheral tissues including the colon, heart, lung, pancreas, thyroid gland, and small intestine. Although PP has the highest affinity for the Y4 receptor, NPY and PYY are also able to activate Y4 at high concentrations (Herzog, 2003). The Y5 receptor is expressed in the CNS including the PVN and the dentate gyrus (Gerald et al., 1996), and also in the periphery such as in the intestine, ovaries, testes, spleen, pancreas, liver, and heart (Silva et al., 2002). Interestingly, the Y5 receptor is co-localised with the Y1 receptor on chromosome 4, with the two genes transcribed in opposite directions from a common promoter region (Herzog et al., 1997), suggesting the two receptors may be co-ordinately expressed. The y6 receptor is most likely a pseudogene in humans but generates a functional receptor in the mouse and rabbit (Gregor et al., 1996; Rose et al., 1997; Starback et al., 2000), although the role of the y6 receptor in these species is as of yet unknown (Mullins et al., 2000).

1.10.3 Physiological roles of specific Y receptor subtypes – insights from knockout models

Because of the multiplicity of Y receptors and the range of their physiological targets, attempts to design receptor-specific agonists and antagonists have been made with the aim of dissociating the various effects of NPY. However the major limiting factor in understanding the role of different Y receptors is the availability of suitable

pharmacological tools, with only a few selective agonists and antagonists for the pharmacologically similar Y receptors available (Balasubramaniam, 2003). Furthermore, problems with solubility, toxicity, and inability to cross the blood-brain barrier have limited the potential utility of nonpeptidergic ligands for *in vivo* research and potential clinical application. Therefore the recent development of knockout and transgenic mouse models for the NPY family and their receptors have provided alternative approaches to the use of pharmacological antagonsists and agonists to elucidate the physiological roles of this complicated system. To date, over-expressing lines for NPY, PYY, and PP have been reported. Knockout models have also been generated for NPY, PYY, and various Y receptor subtypes, enabling significant insight into the specific physiological roles of the complex NPY family of ligands and receptors. Descriptions of the different Y receptor knockout models generated to date are summarised in Table 1-1, and are outlined below.

1.10.3.1 Y1 receptor deletion

Several laboratories have generated Y1 knockout ($Y1^{-/-}$) models (Howell et al., 2003; Kushi et al., 1998; Naveilhan et al., 2001; Pedrazzini et al., 1998). $Y1^{-/-}$ mice are viable and have normal food intake, however, a role for Y1 receptors in the regulation of feeding is supported by reduced fasting-induced refeeding and a blunted feeding response of $Y1^{-/-}$ mice to administration of NPY and PYY (Kanatani et al., 2000; Pedrazzini et al., 1998). $Y1^{-/-}$ mice also develop hyperinsulinemia and late onset obesity, particularly in females (Kushi et al., 1998; Sainsbury et al., 2006). Interestingly, $Y1^{-/-}$ mice do not respond to alterations in blood pressure induced by NPY but show a normal response to noradrenaline (Pedrazzini et al., 1998), and exhibit hypersensitivity to various pain stimuli (Naveilhan et al., 2001), implicating a role for the Y1 receptor in the regulation of blood pressure and nociception. The $Y1^{-/-}$ models have also revealed roles for Y1 in the control of aggression and in alcohol intake, with an increased territorial aggression phenotype and increased anxiety-like behaviours and with reduced sensitivity to the sedative effects of alcohol (Karl et al., 2006; Karl et al., 2004; Thiele et al., 2002).

An important role for the Y1 receptor in the regulation of the immune system was also revealed using the $Y1^{-/-}$ model (Wheway et al., 2005). T cells from $Y1^{-/-}$ mice were intrinsically hyper-responsive to activation, but $Y1^{-/-}$ mice were found to have reduced numbers of effector T cells due to a defect in the antigen presenting cell (APC) population, revealing an important regulatory role for the Y1 receptor in the inhibition of T cell activation and in the regulation of APC function (Wheway et al., 2005). The regulation of T cell activity by the Y1 receptor is of particular interest in terms of bone biology given the recently emerged role of the immune system in the regulation of bone remodelling and the pathophysiology of osteoporosis. Findings from *in vivo* and *in vitro* studies have demonstrated that estrogen deficiency results in an increase in adaptive immune function leading to increased production of TNFα by activated T cells, and may significantly contribute to the greater osteoclastogenesis and resorption characteristic of post-menopausal osteoporosis (Weitzmann and Pacifici, 2005). These studies therefore indicate that alteration of Y1 receptor signalling may have indirect effects on bone cell activity through actions on the immune system. These possibilities are discussed later in this thesis (section 8.2.2).

1.10.3.2 Y2 receptor deletion

Two different Y2 receptor ($Y2^{-/-}$) knockout lines have been reported (Naveilhan et al., 1999; Sainsbury et al., 2002a), with conflicting results regarding food intake and body weight. This may be due to differences in the background strain on which these mice were generated, or result from differences in the design of the targeting construct. The first $Y2^{-/-}$ model used a mixed 129SvJ/Balb/c background and disruption of the Y2 coding sequence left the translation initiation codon intact (Naveilhan et al., 1999). These mice had increased body weight, food intake and fat deposition, but normal NPY-induced food intake and starvation-induced re-feeding responses. The $Y2^{-/-}$ model generated by our laboratory used a mixed 129SvJ/C57BL/6 background and resulted in deletion of the entire Y2 receptor gene coding sequence (Sainsbury et al., 2002a). These mice had decreased body weight and adiposity, with increased food intake in female mice. Also in contrast to the first model, starvation-induced re-feeding was strongly increased in both males and females. A role for

the Y2 receptor in mediating the actions of leptin on energy balance was demonstrated in $Y2^{-/-}$/ob double knockout mice, in which deletion of Y2 receptors on the leptin-deficient ob/ob background reduced the obese phenotype of the ob/ob model, concurrent with reduced serum insulin and glucose levels (Sainsbury et al., 2002b).

Mice lacking Y2 receptor signalling also have behavioural alterations, but unlike the $Y1^{-/-}$ model, have reduced anxiety-like behaviours and an improved ability to cope with stress (Redrobe et al., 2003; Tschenett et al., 2003). Further behavioural studies have revealed deficits in learning and memory tasks (Redrobe et al., 2004), together suggesting an important role for the Y2 receptor in memory processing and in mediating the anxiolytic effects of NPY.

1.10.3.3 Y4 receptor deletion

Only one model of Y4 receptor knockout ($Y4^{-/-}$) has so far been described (Sainsbury et al., 2002c). $Y4^{-/-}$ mice are lean, with reduced body weight and food intake, associated with elevated plasma PP levels, supporting an important role for PP in the regulation of energy balance via Y4 receptors (Sainsbury et al., 2002c). A role for the Y4 receptor in reproduction is also highlighted with increased testosterone in male $Y4^{-/-}$ mice, and advanced mammary gland development in female $Y4^{-/-}$ mice (Sainsbury et al., 2002c). Furthermore, deletion of Y4 receptor signalling in leptin deficient ob/ob mice rescues the fertility of the ob/ob model, further supporting a role for the Y4 receptor in the regulation of the gonadotropic axis (Sainsbury et al., 2002c).

$Y4^{-/-}$ mice also have impaired cardiovascular function, with reduced basal blood pressure and a slower heart rate compared to wild type most likely due to reduced sympathetic activity (Smith-White et al., 2002a), suggesting a role for the Y4 receptor in maintaining autonomic balance in the cardiovascular system.

1.10.3.4 Y5 receptor deletion

The Y5 receptor is abundantly expressed in areas of the hypothalamus such as the paraventricular nucleus and the lateral hypothalamus, consistent with its proposed role in the regulation of feeding behaviour (Gerald et al., 1996). Targeted deletion of the Y5 receptor does not result in an abnormal feeding or adipose phenotype; however $Y5^{-/-}$ mice develop late-onset obesity, characterised by increased food intake and adiposity (Marsh et al., 1998). $Y5^{-/-}$ mice also respond normally to leptin administration and exhibit a normal fasting-induced re-feeding response, but have a reduced food intake response following central NPY administration (Marsh et al., 1998), demonstrating a role for the Y5 receptor in the regulation of food intake by NPY.

1.10.4 NPY in bone tissue

Early studies provided evidence that NPY-immunoreactive fibres were present in bone tissue, in particular associated with blood vessel walls, leading to the proposal of a primarily vasoregulatory role in bone, rather than the regulation of bone cell activity (Ahmed et al., 1993; Hill et al., 1991; Lindblad et al., 1994; Sisask et al., 1996). The report that NPY was produced by megakaryocytes within bone marrow supported this proposed role (Ericsson et al., 1987). However, NPY-immunoreactive nerve fibres were also identified associated with bone lining and marrow cells, and in the synovium and bone marrow of ankle joints of arthritic rats, while studies using RIA demonstrated the presence of NPY in rat periosteum, bone tissue and bone marrow (Ahmed et al., 1995; Ahmed et al., 1994; Hill et al., 1991). Subsequent functional studies demonstrated that NPY treatment in osteoblastic cell lines inhibited the cAMP response to PTH and noradrenaline (Bjurholm, 1991; Bjurholm et al., 1992), consistent with the known inhibitory action of NPY on the cAMP response in other systems and revealing a possible role for NPY in the regulation of osteoblast activity, through direct interaction with Y receptors on bone cells. However, reports of Y receptors in bone are contradictory. Expression of a Y receptor corresponding in sequence to the Y1 receptor was detected in human osteoblastic and human osteosarcoma-derived cell lines (Togari et al., 1997), while another study reported the expression of the Y1 receptor in mouse bone marrow cells (Nakamura et al., 1995).

However, our laboratory did not detect transcripts for any of the Y receptors in primary murine osteoblastic cultures or whole long bone preparations using RT-PCR (Baldock et al., 2002), therefore the presence of Y receptors on bone cells remains a matter for debate.

1.10.5 Y2 receptor deletion effects on bone

A role for the Y2 receptor in the regulation of bone mass was initially investigated because of the known co-localisation of Y2 and leptin receptors on neurons within the arcuate nucleus (Baskin et al., 1999; Broberger et al., 1997), and because of the emerging evidence of the central antiosteogenic action of leptin. Analysis of bone sections from the distal femur of germline $Y2^{-/-}$ mice revealed a two-fold greater trabecular bone volume in both male and female $Y2^{-/-}$ compared with wild type mice, associated with significant increases in both trabecular number and thickness (Baldock et al., 2002). Tetracycline-based dyes incorporated into newly mineralised bone were used to obtain a dynamic measurement of osteoblast activity following fluorescence-based microscopy, revealing that the increase in bone volume observed in $Y2^{-/-}$ mice resulted from a greater rate of bone formation due to elevated osteoblast activity, demonstrating this pathway to be anabolic. Parameters of bone resorption were unchanged except for a modest elevation in osteoclast number. Importantly, the bone formation and bone volume response seen in germline $Y2^{-/-}$ mice was achieved to a similar extent within just 5 weeks following conditional deletion of hypothalamic Y2 receptors in adult mice, revealing the potency by which this pathway modulates osteoblast activity, and demonstrating a role for central Y2 receptors in this process (Baldock et al., 2002). The increase in bone volume observed in both the germline and conditional $Y2^{-/-}$ mice occurred in the absence of measurable changes in the concentration of IGF-1, free T4, calcium, leptin and testosterone, with corticosterone levels increased only in conditional $Y2^{-/-}$ mice compared with control (Baldock et al., 2002). These findings suggested that the anabolic effects of Y2 receptor deletion on bone were not mediated indirectly by these known effectors of bone turnover, providing strength to the hypothesis that the Y2-mediated anabolic pathway acts via a previously un-described neuronal mechanism.

As PP levels are elevated in plasma of $Y2^{-/-}$ mice, the role for elevated PP in the regulation of bone cell activity was investigated in a PP over-expressing transgenic mouse model (Ueno et al., 1999). Over-expression of PP however, did not affect cortical or trabecular bone, effectively ruling out elevated PP as the sole source of the Y2-associated increase in bone formation (Sainsbury et al., 2003).

Another potentially important change noted in $Y2^{-/-}$ mice was a significant elevation in expression of hypothalamic NPY (Sainsbury et al., 2003). The role of elevated NPY in mediating the Y2-associated anabolic response is discussed further below (section 1.10.7) and in chapter 4 (section 4.4), however, to date, a role for elevated NPY in the anabolic response of the $Y2^{-/-}$ model has not been ruled out.

1.10.6 Y2Y4 receptor deletion effects on bone

In contrast to the $Y2^{-/-}$ model, which has a significantly greater trabecular bone mass, deletion of Y4 receptors did not alter bone mass from wild type levels (Sainsbury et al., 2003). Interestingly, while $Y4^{-/-}$ mice also had elevated plasma levels of PP, hypothalamic NPY expression was not altered, supporting a potential role for elevated NPY, but not PP, in the control of bone mass (Sainsbury et al., 2003).

As deletion of Y4 receptors did not result in a discernible bone phenotype, it was a surprise therefore when analysis of $Y2^{-/-}Y4^{-/-}$ double knockout mice revealed a synergistic three-fold increase in trabecular bone volume, compared with the two-fold increase in $Y2^{-/-}$ mice (Sainsbury et al., 2003). As in the $Y2^{-/-}$ model, this was associated with a greater rate of mineral apposition and bone formation, and was again concurrent with a marked elevation in hypothalamic NPY (Sainsbury et al., 2003). Importantly, the synergistic effect of $Y2^{-/-}$ $Y4^{-/-}$ in the control of bone was only evident in male mice, although both male and female $Y2^{-/-}Y4^{-/-}$ mice were lean, with body weight and white adipose tissue mass reduced compared to either $Y2^{-/-}$ or $Y4^{-/-}$ mice. Of particular note, was the observation that plasma leptin was significantly reduced by around 60% in male, but not female $Y2^{-/-}Y4^{-/-}$ mice (Sainsbury et al., 2003), suggesting that the synergistic increase in bone volume observed

in males could be the result of additive effects of the Y2- and leptin-associated antiosteogenic pathways in the absence of Y4 receptor signalling.

1.10.7 Interaction between leptin and Y2-regulated pathways in bone

The actions of leptin and NPY to regulate energy homeostasis within the hypothalamus are known to be linked (section 1.8), with NPY acting downstream of leptin to mediate its actions on feeding, reproduction, hormone release, and energy balance (Erickson et al., 1996b; Stephens et al., 1995). As mentioned previously, Y2 and leptin receptors are co-expressed on NPY-ergic neurons in the arcuate nucleus, and the Y2 receptor is thought to act as an autorecpetor, modulating the expression and secretion of NPY (King et al., 2000).

Several lines of evidence suggest that leptin and Y2 receptors interact or share a common regulatory pathway in the regulation of bone. Firstly, hypothalamic expression of NPY is elevated in both leptin deficient ob/ob and $Y2^{-/-}$ mice, indicative of a common mechanism which may modulate the activity of bone cells. The presence of NPY in sympathetic nerve fibres and the known ability of the Y2 receptor to modulate the release of adrenaline, also suggests the Y2 receptor may be involved in the modulation of adrenergic signalling in osteoblasts by leptin.

However, several studies also suggest the modulation of bone cell activity by leptin and the NPY system to occur by distinct pathways. Continuous administration of NPY into wild type mice for 28 days has been shown to actually decrease bone volume, suggesting that NPY and leptin might use different pathways to control bone mass and energy homeostasis (Ducy et al., 2000). Central administration of NPY would however, result in the development of obesity and therefore indirectly increase leptin signalling which may also be responsible for the loss of bone mass observed in these studies. As mentioned in section 1.9.6, studies using chemical ablation of hypothalamic structures to demonstrate that regions of the hypothalamus controlling leptins antiosteogenic and anorexigenic networks differ, also indicated that the antiosteogenic effects of leptin are distinct from NPY, as administration of monosodium glutamate (MSG) to ablate NPY-sythesising neurons and

arcuate nucleus structures attenuated the ability of leptin to reduce body weight, but did not affect bone mass or the ability of leptin to reduce bone mass. In contrast, ablation of gold thioglucose-sensitive neurons within the ventromedial hypothalamic nucleus resulted in increased bone formation which was not reversible by leptin administration (Takeda et al., 2002), further suggesting that leptin and NPY might use different pathways to control bone mass and body weight.

Recent studies in our laboratory also support a model in which the Y2-mediated anabolic pathway may be distinct from the antiosteogenic actions of leptin, with the demonstration that $Y2^{-/-}$ mice maintain their anabolic phenotype in the presence of elevated NPY and leptin (Baldock et al, 2005). These findings are discussed in further detail later in this thesis together with further studies using genetic models to investigate possible interaction between Y receptor signalling and leptin action on bone cell activity (chapter 4).

1.11 THESIS OBJECTIVES

The identification of NPY nerve fibres within bone, and recent evidence from the $Y2^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ knockout models suggests an important role for Y receptor signalling in the control of bone formation. These findings are supported by an increasing number of additional models, in which peptides, transmitters, and hormones, including CART, α-MSH and insulin, known to interact with NPY in the regulation of energy homeostasis, have now also been demonstrated to modulate bone cell activity. Of particular interest is the recently described antiosteogenic effects of leptin, mediated by receptors which colocalise with the NPY Y2 receptor subtype within the hypothalamus.

The aims of this thesis therefore were to firstly investigate the potential regulation of bone remodelling by another Y receptor subtype; the Y1 receptor, and to investigate possible interaction between the different Y receptor subtypes in their control of bone cell activity using a combination of genetic models. As leptin and NPY are known to interact in the regulation of energy homeostasis, the potential interaction between these different Y

receptor knockout models with the leptin antiosteogenic response was also investigated using a genetic approach.

The applicability of these Y receptor knockout models was then assessed in two models of osteoporosis; sex hormone deficiency induced bone loss, and aging induced bone loss, to investigate whether activation of the anabolic response in the absence of specific Y receptor signalling would provide resistance to bone loss in either the absence of sex hormones, or with age. The unique approach in which Y2 receptors can be deleted solely from the hypothalamus of adult mice was employed to investigate whether activation of the anabolic response could repair bone following the occurrence of significant bone loss due to either sex hormone deficiency or age.

Finally, the downstream mechanism of the Y2 anabolic response was investigated using stromal cell culturing techniques and by assessment of populations of mesenchymal and progenitor cells within the bones of $Y2^{-/-}$ mice. The findings from these studies led to re-assessment of the presence of Y receptor subtypes within bone, to investigate the potential for direct regulation of bone cell activity by Y receptor signalling.

Gene deletion	Phenotype	Reference
Y1 receptor	Increased body weight and fat mass	(Kushi et al., 1998; Pedrazzini et al., 1998; Sainsbury et al., 2006)
	Reduced fasting-induced re-feeding	(Pedrazzini et al., 1998)
	Reduced NPY and PYY-induced re-feeding	(Kanatani et al., 2000)
	Attenuated blood-pressure response to NPY	(Pedrazzini et al., 1998)
	Reduced antinoiception	(Naveilhan et al., 2001)
	Increased territorial aggressive behaviour	(Karl et al., 2004)
	Increased anxiety-like behaviour	(Karl et al., 2006)
	Increased alcohol consumption	(Thiele et al., 2002)
	Hyperactive T cell response	(Wheway et al., 2005)
	Increased fertility	(Pedrazzini et al., 1998)
	Increased neurogenesis	(Howell et al., 2003)
Y2 receptor	Decreased body weight and fat mass	(Sainsbury et al., 2002a)
	Increased fasting-induced re-feeding	(Sainsbury et al., 2002a)
	Increased trabecular bone volume	(Baldock et al., 2002)
	Reduced anxiety-like behaviours	(Redrobe et al., 2003)
	Improved ability to cope with stress	(Tschenett et al., 2003)
	Reduced learning and memory	(Redrobe et al., 2004)
Y4 receptor	Reduced body weight and adiposity	(Sainsbury et al., 2002c)
	Reduced food intake	(Sainsbury et al., 2002c)
	No alteration in bone mass	(Sainsbury et al., 2003)
	Enhanced mammary development (females) and increased testosterone (males)	(Sainsbury et al., 2002c)
	Reduced cardiovascular function	(Smith-White et al., 2002a)
Y5 receptor	Normal body weight and feeding	(Marsh et al., 1998)
	Late-onset obesity	(Marsh et al., 1998)
	Reduced NPY-induced re-feeding	(Marsh et al., 1998)

Table 1-1 Major phenotypes of Y receptor knockout mice.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemical/reagents and suppliers

Ethanol, chloroform, formaldehyde solution (40%), sodium thiosulphate, lithium carbonate, sodium nitrite, tartaric acid, glacial acetic acid, glycerol, chromium III potassium sulphate 12-hydrate, and toluidine blue were from BDH Laboratory Supplies, Poole, England. Diethylpyrocarbonate (DEPC) was from Qiagen Pty Ltd., VIC, Australia. Calcein, tris HCl, trypan blue, fast-blue RR, napthol AS-BI phosphate, acidified basic fuschin, silver nitrate, isopropanol, tris-buffer, sodium bicarbonate, sodium acetate, dibutyl phthalate, granulated gelatine, ethidium bromide, ethylene glycol mono-ethyl ether (EGMEE), bromophenol blue, haematoxylin [C.I. 75290; Natural Black 1], eosin, demeclocycline, and Oil Red-O were from Sigma Chemical Company, St Louis, MO, USA. Paraformaldehyde (PFA) was from Merck, Darnstadt, Germany. Dimethyformamide (DMF) was from Aldrich Chemical Co., Milwaukee, WI, USA. DNA molecular weight markers *Eco*RI digested bacteriophage SPP-1, and *Hpa*II restricted pUC19 were from Bresatec, Adelaide, South Australia. Ethylenediaminetetraacetic acid (EDTA) was from Boehringer Mannheim, Mannheim, Germany. DNA grade agarose was from Progen Industries, Brisbane, QLD, Australia. Trizol® Reagent was from Invitrogen, Australia Pty Ltd, Mount Waverly, VIC, Australia. Worthington collagenase type 1 was from ScimaR, Templestowe, VIC, Australia. Methyl methacrylate (MMA), acetone, and xylene were from APS Chemicals, Sydney, Australia. Perkadox 16 was from Swift and Company Ltd., Sydney, Australia.

2.1.2 Media for mammalian cell culture

α-MEM (powdered stock), sodium bicarbonate solution (7.5%w/v), L-glutamine solution (200mM), hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (1M), penicillin/streptomycin (5000IU/ml, 5000μg/ml), trypsin solution (0.25%) were from Gibco BRL, Invitrogen, Life Technologies, Mount Waverly, VIC, Australia. Fetal bovine serum (FBS; Lot #: E06133-500) for bone marrow stromal cell (BMSC) cultures and for washing buffer was from Thermo Electron Corporation, Melbourne, Australia. FBS for culturing of

mesenchymal stem cells (MSCs) and progenitor cells isolated by FACS was from Hyclone, Logan, UT, USA. Ethylenediaminetetraacetic acid (EDTA (2% w/v) was from ICN Biomedicals Inc., Costa Mesa, CA, USA. Gentamycin (40mg/ml, 40000U/ml) was from Pharmacia and Upjohn, Perth, Australia. Phosphate buffered saline (PBS) tablets were from Astral Scientific Pty Ltd., Gymea, NSW, Australia. Ascorbic acid, β-glycerol phosphate, and dexamethasone were from Sigma, St Louis, MO, USA. Insulin (Actrapid® Penfill®, 100IU/ml) was from Novovordisk Pharmaceuticals Ltd., Baulkham Hills, NSW, Australia.

2.1.3 Antibodies

Purified antibodies against B220, Gr-1, Mac-1, CD4, CD8, CD3, CD5, and TER119 were from Pharmingen, Franklin Lakes, NJ, USA, as were fluorescein isothiocyanate- (FITC)-conjugated Sca-1, and phycoerythrin- (PE)- conjugated CD45 antibodies. Biotinylated-conjugated CD51, fluorescein phycoerythrin- (PE)- conjugated CD31, fluorescein allophycocyanin- (APC)-conjugated streptavidin, and 7AAD were from BD Biosciences, San Jose, CA, USA.

2.1.4 Enzymes and Serum Biochemistry Kits

IGF-1 radioimmunoassay kit was from Biclone Australia PTY. Ltd., Marrickville, NSW, Australia. Corticosterone radioimmunoassay kit was from MP Biomedicals, Orangeburg, NY, USA. Osteocalcin mouse osteocalcin EIA kit was from Biomedical Technologies Inc., Stoughton, MA, USA.

Superscript™ III First-Strand Synthesis System was from Invitrogen Australia Pty, Ltd Mount Waverly, VIC, Australia.

Taq DNA polymerase and 10x PCR buffers were from Roche Molecular Systems, Inc., Alameda, CA, USA.

2.1.5 Surgical Solutions

Ketamine and Xylazine were from Mavlab, Slacks Creek, QLD, and Ilium Veterinary Products, Smithfield, NSW, Australia, respectively. Povidone-iodine antiseptic was from Orion laboratories Pty. Ltd. Balcatta, Australia, and buprenorphine was from Cenvet Pty. Ltd., Artamon, Australia. Methoxyfluorane was from Medical Developments Australia, Springvale, VIC, Australia.

2.2 METHODS

2.3 ANIMALS

2.3.1 Generation of germline $Y1^{-/-}$, $Y2^{-/-}$, and $Y4^{-/-}$ mice

Germline deletion of Y1, Y2, and Y4 receptor genes was achieved as previously described (Howell et al., 2003; Sainsbury et al., 2002a; Sainsbury et al., 2002c), by crossing Y1, Y2, and Y4 receptor floxed mice ($Y1^{lox/lox}$, $Y2^{lox/lox}$ and $Y4^{lox/lox}$) with oocyte-specific Cre-recombinase-expressing C57/BL6 mice (Schwenk et al., 1995), resulting in the removal of the entire coding region of the Y1, Y2, or Y4 gene. The successful deletion was confirmed by Southern Blot Analysis. All mice generated were maintained on a mixed C57/BL6-129/SvJ background.

2.3.2 Animal Maintenance

Mice were group housed unless otherwise stated, and fed with standard chow *ad libitum*. Mice were kept on a 12 hour light: dark cycle. Animal experiments were approved by the Garvan Institute of Medical Research Animal Research Authority and were conducted in accordance with relevant guidelines and regulations.

2.3.3 Flurochrome labelling

Animals used in the first studies (compound Y receptor mutants and Y receptor knockout mice crossed onto the leptin-deficient ob/ob background) were labelled with the fluorescent acetomethoxy compound calcein 10 days prior to collection and the fluorescent tetracycline related compound demeclocycline 3 days prior to collection. Due to problems with fading of the demeclocycline label in archived samples, subsequent studies used double calcein labelling 10 days and 3 days prior to collection. All solutions were prepared on the day of administration. Demeclocycline was prepared as a 3mg/ml solution in sterile saline and dissolved by stirring for 2 hours. The supernatant was decanted and used for injection. Calcein was prepared as a 4mg/ml solution in sterile saline and adjusted to approximately neutral pH with 8mg/ml sodium bicarbonate, and dissolved by vigorous shaking. Both solutions were administered at a final dose of 20mg/kg by i.p injection to fluorescently label newly formed bone.

2.3.4 Tissue collection

Mice were killed by cervical dislocation and trunk blood was collected. Serum was separated by centrifugation and stored at -20^{0}C. For measurements of tissue weights, interscapular brown adipose tissue (BAT), and white adipose tissue (WAT) deposits (right inguinal, right retroperitoneal, and mesenteric) were collected and weighed.

Vertebrae and both femora and tibia were excised as indicated and fixed in 4% PFA in PBS at 4^{0}C for 16 hours. Following fixation, bones were transferred to a solution of 70% ethanol before undergoing processing.

2.3.5 Bone processing for histomorphometry

Following fixation, femora and/or lumbar vertebrae (L4) were cleaned of muscle. The femoral shaft mid-point was calculated using a Digimatic Outside Micrometer (Mitutoyo Corporation, Tokyo, Japan), and right femora were subsequently bisected transversely at the midpoint of the shaft using a slow speed circular saw (Struers Minitom, Radiometer

Pacific, Sydney, Australia) with a diamond tipped blade (Moppes-IDP Ltd., Gloucester, England). Distal femur halves and vertebrae were then dehydrated in graded acetone (2x 70%, 1x 90%, 2x 100%) for a minimum of 2 hours each at 4^0C. A final incubation in 100% acetone was performed at room temperature. The samples were then transferred to MMA at 4^0C for 4 to 7 days. Bones were then transferred to an embedding mix containing 94.75% MMA, 5% dibutyl phthalate, and 0.25% Perkadox 16 in amber glass bottles (Edwards Instrument Co., Narellan, NSW, Australia). Bottles were tightly capped and placed in a waterbath at 28^0C for 24 hours, then at gradually increasing temperatures to 30^0C over 4 days for complete polymerisation. The embedded samples were removed by smashing the glass bottles, and fixed to aluminium block holders (Bio-Rad, Sydney, Australia) in correct orientation with Selleys Araldite® Strength epoxy glue (Selleys, Sydney, Australia).

2.3.6 Bone sectioning

Bone samples were sectioned in a way to ensure sampling of a standardised region throughout the studies. Following embedding, the samples were trimmed to expose the sample area by removing and discarding 10µm sections with a Leica microtome (Jung RM2055; Leica Instruments GmbH, Germany) using a tungsten carbide blade (Reichert-Jung; Leica, Germany). For sectioning of distal femora, the sample area was defined as the level at which the greater condyle of the epiphysis was bisected, reaching its maximal area (Figure 2.1). The diaphyseal region was orientated such that the midline was sectioned. For sectioning of lumbar vertebrae, the sample area was defined as the level at which both vertebral processes were removed at an equal distance into the vertebral disk on a coronal plane. After exposing the correct sample area, the block surface was moistened with 50% ethanol, and 6 consecutive 5µm sections were cut. Sections were placed on gelatin-coated (solution of 1.5% gelatin with 0.05% chromium III potassium sulphate) glass slides (superfrost; Menzel-Glaser GmbH, Braunschweig, Germany), and immersed in a mixture of 50% ethanol and 30% EGMEE in water, heated to 70^0C and gently stretched to flatten with forceps. The section was then covered with polyethylene plastic, and cartridge paper,

clamped, and annealed to the slide overnight at 37^0C. Prior to staining, MMA was removed from sections by 2x immersions in 100% acetone for 10 minutes each.

2.3.7 Staining methods

2.3.7.1 Von Kossa staining for identification of calcified tissue

Following removal of MMA with acetone (section 2.3.6), sections were transferred to a 1% silver nitrate solution in water and exposed to UV light for 30 minutes. Sections were then gently washed in distilled water for 10 minutes and transferred to a 2.5% solution of sodium thiosulphate in water for 30 seconds. The appropriate endpoint was dark brown stained mineralised tissue (Figure 2.1). In order to obtain well defined trabecular surfaces, overstaining was avoided. Following staining, sections were dehydrated by immersing twice in 100% ethanol, and then transferred to 100% xylene. Washes in Xylene were repeated 3 times. Sections were then mounted in the xylene based mountant Eukitt (Kinder GmbH and Co., Freiburg, Germany).

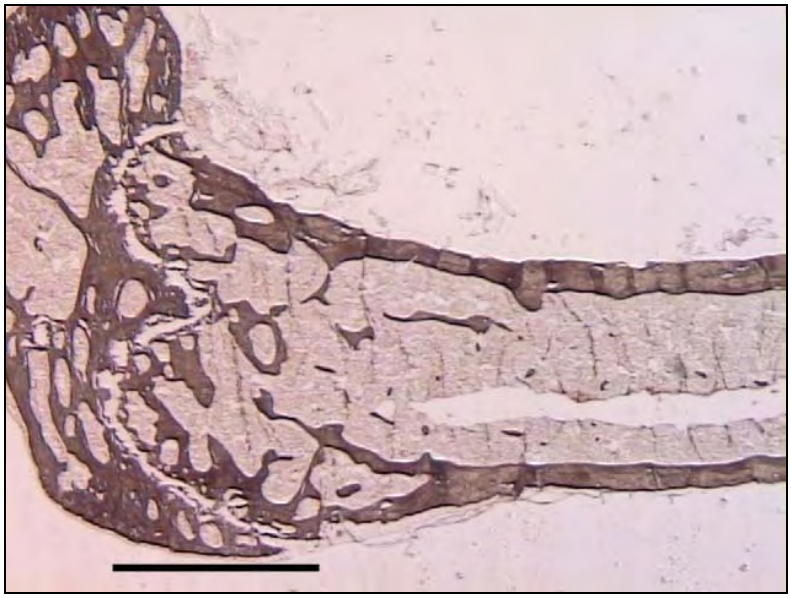

Figure 2.1 Sagittal section of the mouse distal femur stained by the von Kossa method for mineralised tissue. Mineralised tissue is stained dark brown while non-mineralised tissue remains unstained (Original magnification 2.5X). Bar represents 1mm.

2.3.7.2 Enzyme localisation of acid phosphatase

Following removal of MMA with acetone (section 2.3.6), sections were incubated in 1M Tris buffer (pH9.4) at 37^0C for 1 to 2 hours to reactivate the enzyme. Sections were then removed from the buffer and transferred to staining solution at 37^0C for 1 hour. The staining solution was prepared fresh by dissolving 40mg of the enzyme substrate, napthol ASBI phosphate in 2ml DMF, and 0.1ml of the chromophore, acidified basic fuschin in 0.1ml 4% aqueous sodium nitrite solution. The final staining solution was prepared by adding 35ml sodium acetate buffer (pH5) containing 35mg tartaric acid to the substrate solution, then adding the basic fuschin, sodium nitrite mixture. The staining solution was filtered prior to use using a 0.2µm filter (Nalgene Labware, Rochester, NY, USA). Following staining, sections were rinsed with distilled water for 15 minutes, then counterstained to identify cell nuclei with 0.5% haematoxylin for 30 seconds. Sections were rinsed again, transferred to saturated lithium carbonate for 1 minute, then rinsed in tap water again. Sections were mounted in aqueous mounting medium (DakoFaramount, Botany, Australia) (Figure 2.2).

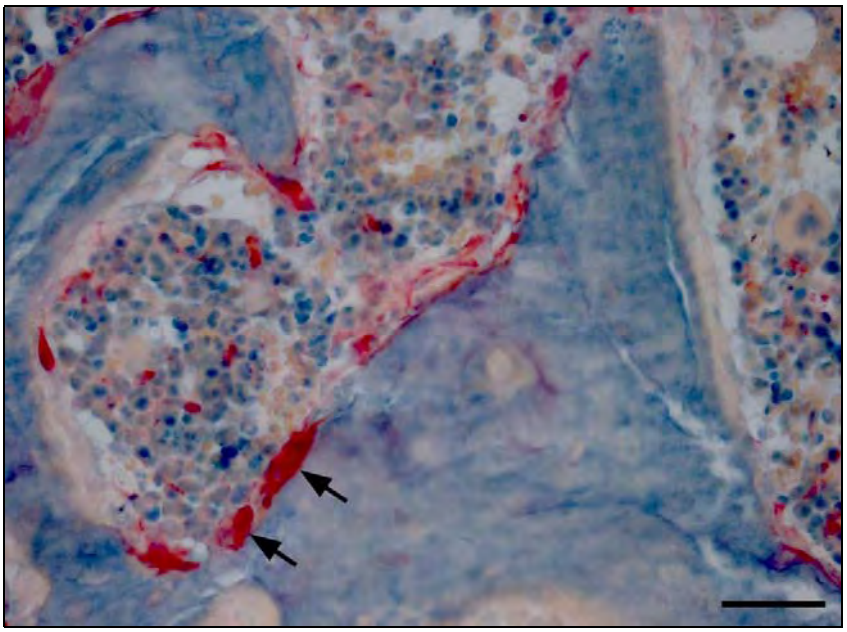

Figure 2.2 Identification of osteoclasts using enzyme localisation of acid phosphatase.
Osteoclasts (arrows) were identified by their red stained cytoplasm, and localisation adjacent to bone surfaces. Haematoxylin counterstain enables visualisation of cell nuclei (Original magnification 40X). Bar represents 10µm.

2.3.7.3 Haematoxylin and eosin staining

This stain was used to identify sinusoids and blood vessels from circular voids in the bone marrow made by adipocytes, removed during processing. Following removal of MMA with acetone (section 2.3.6), sections were briefly immersed in 70% ethanol, then transferred to 0.5% haematoxylin for 8 minutes to ensure adequate staining of any nuclei structures. Sections were then rinsed in tap water, and transferred to saturated lithium carbonate for 1 minute. Sections were rinsed again, stained in 0.1% eosin for 1 minute, then rinsed in water (Figure 2.3). Sections were then dehydrated by graduating concentrations of ethanol (50%, 90%, and 100%), and then transferred to 100% xylene. Washes in Xylene were repeated 3 times. Sections were then mounted in xylene based mountant Eukitt (Kinder GmbH and Co., Freiburg, Germany).

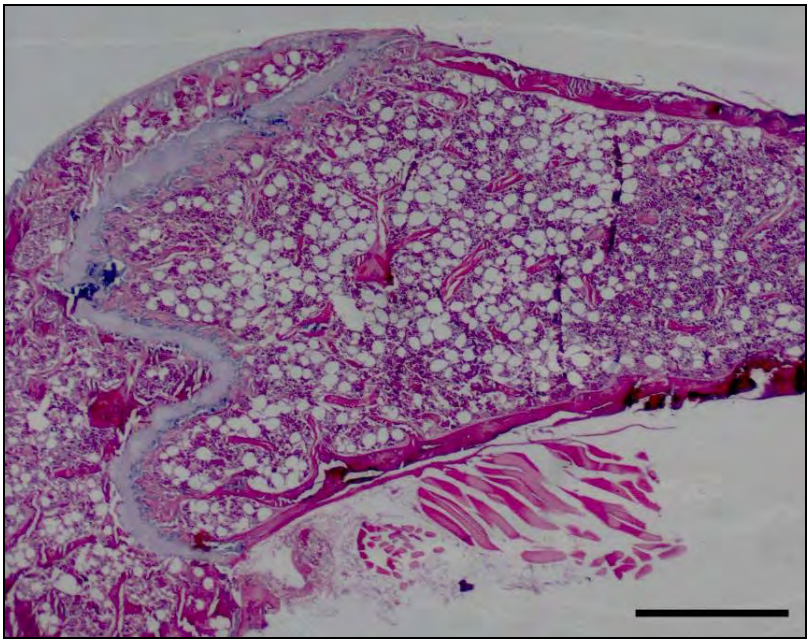

Figure 2.3 Sagittal section of a mouse distal femur stained with haematoxylin and eosin Unstained circular voids represent marrow adipocytes removed during processing of femur (Original magnification 5X). Bar represents 0.5mm.

2.3.8 Histomorphometry

2.3.8.1 Trabecular bone indices

For analysis of trabecular bone volume in the distal femur, all trabecular bone proximal to the epiphyseal growth plate extending 4.5mm was sampled as previously described (Baldock et al., 2002). Trabecular bone indices were estimated using a high resolution camera (Leica DFC 320 with a 0.63x c-mount, Leica Microsystems, Heerbrugg, Switzerland), connected to a microscope (Leica DM-RB; Leica Microsystems, Heerbrugg, Switzerland) and interfaced with Leica QWin analysis software (Leica Microsystems, Heerbrugg, Switzerland). The final image at 25x magnification was adjusted to an appropriate threshold for staining intensity and manually edited for artefacts of sectioning or staining. Total sample area (TA), bone area (BA), and bone perimeter (Peri) were recorded, and used to calculate trabecular bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), and trabecular number (Tb.N, /mm) (Parfitt et al., 1983).

2.3.8.2 Formation indices

Bone formation was estimated using flurochrome labelled sections. Following removal of MMA with acetone, sections were dehydrated 2x in 100% ethanol, cleared in xylene (2x 100%), then mounted unstained in xylene based mountant Eukitt (Kinder GmbH and Co., Freiburg, Germany). Demeclocycline and calcein chelate to calcium and are incorporated into bone as it is laid down during the mineralisation process. When viewed under UV light with a 420nm filter (Leica Microsystems, Heerbrugg, Switzerland), with a fluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland), the tetracycline labels were visible as fluorescent bands marking sites of active bone formation at the time of their administration (section 2.3.3). Demeclocycline appeared as a faint yellow/orange band, while calcein appeared as a bright green band (Figure 2.4).

Interlabel distance was measured at multiple sites along the labelled surfaces within the sample region at 400x magnification using the Leica QWin imaging software, and used to calculate the mineral apposition rate. The distance was measured from the internal origin of each fluorescent band (Figure 2.4). Total mineralising surface was determined manually at

100x magnification using the Leica QWin imaging software, from the length of double and single labelled trabecular bone surfaces. Bone formation rate was calculated from mineralising surface and the mineral apposition rate.

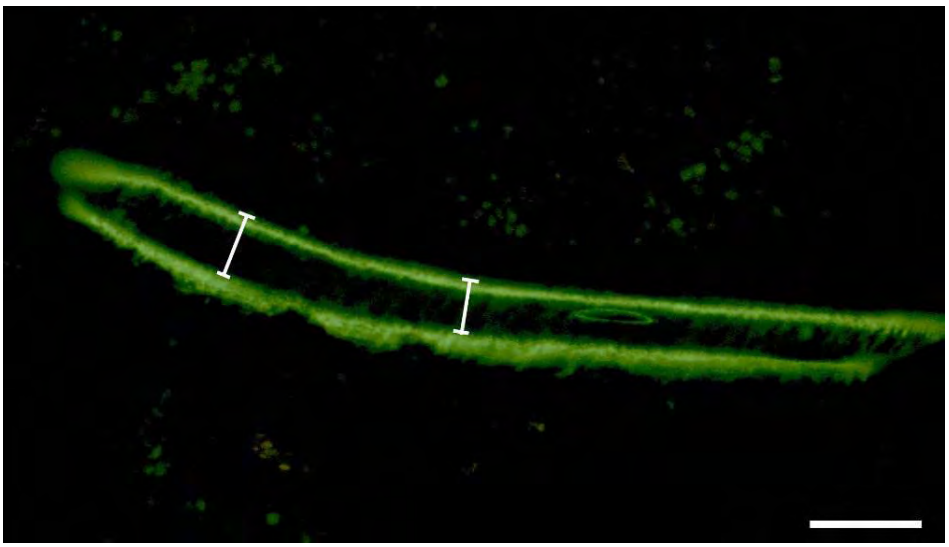

Figure 2.4 Flurochrome (calcein) labelled sites of active bone mineralisation
Fluorescent calcein labels appear green under UV light. The distance between the inner margin of each band perpendicular to the bone surface (white bars) represents 7 days of continuous bone formation and was used to estimate mineral apposition rate (original magnification 40X). Bar represents 10µm.

2.3.8.3 Resorption indices

Bone resorption was estimated following the enzymatic identification of bone resorbing osteoclastic cells (section 2.3.7.2). Osteoclast cells were identified using a microscope (Leica DM-RB; Leica Microsystems, Heerbrugg, Switzerland) at 200x magnification as multinucleated cells with a red stained cytoplasm lying adjacent to the bone surface. Osteoclast surface was calculated from the percentage of total trabecular bone surface covered by multinuclear TRAP stained osteoclasts using Leica QWin imaging software.

2.3.8.4 Indices of marrow adiposity

Marrow adiposity was assessed using haematoxylin and eosin stained sections (section 2.3.7.3) and calculated based on the identification of circular voids in the bone marrow

made by adipocytes which were subsequently removed during processing. These were distinguished from sinusoids and blood vessels by lack of haematoxylin and eosin staining using a microscope (Leica DM-RB; Leica Microsystems, Heerbrugg, Switzerland) at 100x magnification. Adipocyte number and mean area were calculated using Leica QWin imaging software, and expressed as total adipocyte volume as a percentage of total marrow volume.

2.3.9 Calculations

2.3.9.1 Static trabecular indices

The total area, bone area, and bone perimeter data obtained from the image analysis system were used to estimate trabecular morphology using the following equations;

Trabecular bone volume (%)	BV/TV = (BA/TA) x 100
Trabecular number (/mm)	Tb.N = BV/TV/Tb.Th x 10
Trabecular thickness (μm)	Tb.Th = (BA/TA)/(0.5 x Peri/TA)

[total sample area (TA), bone area (BA), and bone perimeter (Peri)]

2.3.9.2 Cellular indices

Interlabel distance, double and single labelled surface, osteoclast surface, and bone perimeter data obtained from the image analysis system were used to estimate indices of cellular activity using the following equations;

Mineral apposition rate (μm/day)	MAR = interlabel distance/interlabel period
Mineralising surface (%)	MS = ((dLS + 0.5 x sLS)/Peri) x 100
Bone formation rate ($\mu m^2/\mu m$/day)	BFR = MS/100 x MAR
Osteoclast surface (%)	= Oc.S/Peri x 100

[double labeled surface (dLS), single labeled surface (sLS), and bone perimeter (Peri)]

2.3.9.3 Indices of adiposity

Adipocyte number, adipocyte area, and total area obtained from the image analysis system were used to estimate trabecular morphology using the following equations;

Mean adipocyte size	= total adipocyte area/number of adipocytes
Marrow adipocyte volume	AdV/TV = (total adipocyte area/TA) x 100

[total sample area (TA)]

2.4 SURGICAL PROCEDURES

2.4.1 Anaesthesia for gonadectomy and DXA measurements

Anaesthesia for gonadectomy or DXA measurements was performed by i.p injection 50mg/kg ketamine and 10mg/kg xylazine. Additional anaesthesia for gonadectomy was provided by inhalation of 2% methoxyfluorane as required. Completeness of anaesthesia was assessed prior to any operative procedure by applying acute pressure with non-toothed forceps to the skin between the toes of the hind foot. Any retraction of the limb was evidence of incomplete anaesthesia.

2.4.2 Gonadectomy

2.4.2.1 Orchidectomy

Following anaesthesia, animals were placed dorsal recumbent, and the area between the genitals and anus swabbed with ethanol. A small incision was made in-between the anus and the testes which was then torn open to approximately 0.8cm by extension of scissor blades in order to facilitate postoperative healing. The testes were identified and externalised. A small cut was made in the fibrous tunic to expose the testes. The testis was then ligated at the join between the spermatic cord and the base of the testis using absorbable 5-0 coated vicryl suture (Johnson and Johnson, Sydney, Australia). The testis was then dissected, and any exposed fat pad was replaced back into the cavity. The process was then repeated for the second testis. Sham operations were performed by exposing the testis for the same period of time taken to complete orchidectomy. The wound was closed using 5-0 silk suture (Johnson and Johnson, Sydney, Australia), and swabbed with povidone-iodine antiseptic. 0.01mg/kg of the analgesic buprenorphine was injected i.p following the completion of the procedure.

2.4.2.2 Ovariectomy

Following anaesthesia, animals were placed ventral recumbent, the lower half of the flank was shaved of hair and swabbed with ethanol. A small incision was made in the skin along

the long axis of the spine which was then torn open to approximately 1.5cm by extension of scissor blades in order to facilitate postoperative healing. Blunt dissection was used to expose the ventral left flank and a second incision was made in the superficial muscle distal to the kidney, overshadowing the fat pad of the lower flank. The ovary was easily located by its rich red colour and was externalised. The uterine horn was clamped below the fallopian tube then ligated with absorbable 5-0 coated vicryl suture (Johnson and Johnson, Sydney, Australia). The ovary was then dissected from the uterine horn above the ligation. The clamp was then removed slowly to check for excessive bleeding, and the uterine horn replaced. The internal wound was closed using absorbable 5-0 coated vicryl suture. The process was then repeated for the second ovary. Sham operations were performed by externalisation of the ovary for the same period of time taken to complete ovariectomy. The external wound was closed using 5-0 silk suture (Johnson and Johnson, Sydney, Australia), and swabbed with povidone-iodine antiseptic. 0.01mg/kg of the analgesic buprenorphine was injected i.p following the completion of the procedure.

2.4.3 Anaesthesia for adeno-associated virus injection

Anaesthesia for adeno-associated virus (AAV) injection of gonadectomised or aged mice was achieved by i.p injection of 100mg/kg ketamine and 20mg/kg xylazine. Completeness of anaesthesia was assessed prior to any operative procedure by applying acute pressure with non-toothed forceps to the skin between the toes of the hind foot. Any retraction of the limb was evidence of incomplete anaesthesia.

2.4.4 Adeno-associated virus injection

Injection of recombinant AAV vectors containing either the cre-recombinase gene or an empty cassette was performed using a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Anaesthetised $Y2^{lox/lox}$ mice were shaved of hair from the calvarial region and were placed securely within the stereotaxic frame (Figure 2.5 A). The shaved area was swabbed with ethanol and a 1.5cm opening was made using a #11 scalpel blade (Swann-Morton, Sheffield, England) to expose the skull. The membranes on top of the skull were then

cleaned away using a cotton bud (Johnson and Johnson, Sydney, Australia) to clearly visualise the sutures of the skull. The apparatus was adjusted so that the dorsal-ventral co-ordinates for bregma and lambda were equal, indicating that the skull surface was flat. AAV was injected using brain co-ordinates relative to bregma; posterior 2.3mm, lateral ± 0.4mm, ventral 5.6mm, corresponding to the arcuate nucleus (Franklin and Paxinos, 1997). One microliter of virus ($1x10^{14}$ genomic copies/ml) was injected bilaterally over 10 minutes using a 26-gauge guide cannula and a 33-gauge injector (Plastics One, Roanoke, VA, USA) connected to a Hamilton Syringe and a syringe infusion pump (World Precision Instruments, Waltham, MA, USA) (Figure 2.5 B). Validation of the injection co-ordinates was performed by injecting a blue dye followed by immediate collection of the brain. Sectioning of the frozen brain revealed the appearance of the needle tracks and the presence of blue dye within the arcuate nucleus (Figure 2.5 C). Following injection of AAV, the skin was sutured using 5-0 silk suture (Johnson and Johnson, Sydney, Australia), and swabbed with povidone-iodine antiseptic.

Figure 2.5 Deletion of hypothalamic Y2 receptors by injection of adeno-associated viral vector-expressing cre-recombinase.
Mouse is secured within stereotaxic apparatus (A), and virus is injected bilaterally into the hypothalamus (B). Injection of dye followed by immediate collection of brain shows needle tracks and evidence of blue dye within hypothalamus (C).

2.4.5 Recovery following surgery

Mice recovered from gonadectomy and hypothalamic injection of AAV in clean cages placed half on heating pads to aid thermoregulation until conscious and mobile. Mice were

checked every day for the following week to inspect wound healing, sutures and to monitor body weight. Weekly health checks were carried out on all animals to monitor body weight, general condition and well being.

2.5 ASSESSMENT OF BONE DENSITY AND BODY COMPOSITION

2.5.1 Bone densitometry and body composition analysis

Whole body lean mass, fat mass, bone mineral content (BMC) and bone mineral density (BMD) was measured in anesthetised mice using a dedicated mouse dual X-ray absorptiometry (DXA) (Lunar Piximus II, GE Medical Systems, Madison WI, USA). BMD and BMC was also measured for femur and tibia, with the sample region selected from the flexion joint at the hip to the ankle, and for the lumbar vertebrae, with a sample area 75 pixels in length proximal from the lower lumbar (Figure 2.6).

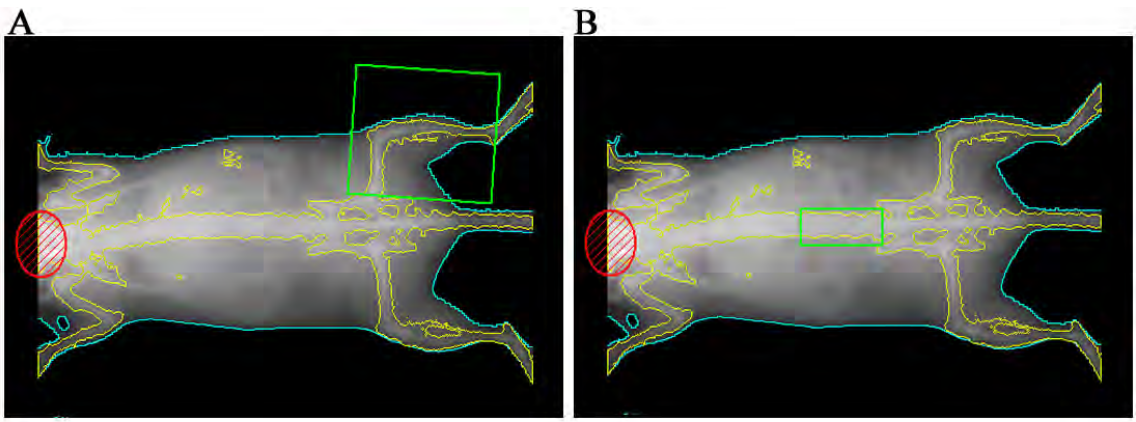

Figure 2.6 Measurement of BMD and BMC of leg and lumbar vertebrae
Sample area (green box) for leg (A), and lumbar vertebrae (B) for measurement of BMD and BMC by DXA.

In addition, excised individual femora were scanned following collection and fixation in 4% PFA for 16 hours, with tibiae attached and the knee joint in flexion to ninety degrees, in order to ensure consistent placement and scanning of the sagittal profile. In addition to

scanning the entire femur, femoral scans were analysed in linear thirds, including all mineralised tissue within the distal, shaft and proximal thirds.

2.5.2 Peripheral Quantitative Computed Tomography

Excised femurs were assessed by peripheral quantitative computed tomography (pQCT) with a Stratec XCT 960M (Norland Medical Systems, Fort Atkinson, WI, USA) with software version 5.40 to determine distal metaphyseal bone mass. A scan of a 1mm thick cross-section was made in the distal femur metaphysis 2.5mm proximal from the distal end, and a second scan of a 1mm thick cross-section was made in the femoral diaphysis, 8mm proximal from the distal end. A low density threshold of 1300 was set to distinguish mouse bone from soft tissue and an upper threshold of 2000 to differentiate cortical bone from bone of a lower density. A voxel size of 0.150mm was used. Volumetric mineral content, density and area were determined for total, trabecular, and cortical bone. Trabecular parameters were excluded for analysis of the diaphyseal region due to the very low trabecular content within this region. In addition, cortical thickness, periosteal and endocortical circumferences were determined at both sites.

2.5.3 Micro Computed Tomography

Femurs were scanned using micro-CT (Micro-CT40, Scanco Medical, Auenring 6-8, Bassersdorf, Switzerland) with software version 3.1. A cross-section of the distal femur metaphysis of 50 consecutive 16μm thick slices was taken at 2.3 to 3.1mm proximal to the distal end to determine parameters of trabecular bone morphology.

2.6 ISOLATION AND CULTURE OF BONE MARROW STROMAL CELLS

2.6.1 Isolation of bone marrow stromal cells

To isolate plastic-adherent bone marrow stromal cells (BMSCs), 5 to 9 week old male wild type and germline $Y2^{-/-}$ mice were sacrificed by cervical dislocation. Femurs and tibias were dissected and cleaned of muscle and connective tissue. The ends of the bones were removed, and the marrow was flushed using a 1ml syringe (Becton Dickinson, Singapore), and a 25-gauge needle (Terumo Corporation, Tokyo, Japan) with α-MEM control media containing 10% FBS, 2mM L-Glutamine, 2.2g/L sodium bicarbonate, 0.1M HEPES, 100IU/ml, 100μg/ml penicillin/streptomycin, and 34000IU/ml, 34 mg/ml gentamycin. Cells were plated at a density of 1.9 x 10^6 cells/cm^2 in 50cm^2 plastic tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA), and maintained at 37^0C with 5% humidified CO_2. The non-adherent cell population was removed after 72h by a medium change. The cells were then continuously cultured for a further 4 days, before passaging.

2.6.2 Passaging bone marrow stromal cells

Stromal cells were passaged once before inducing differentiation. On day 7, when the cells had reached approximately 70% confluence, cells were washed with PBS, and adherent cells were dissociated by addition of 0.25% trypsin containing 0.53mM EDTA for 5 minutes at 37^0C, after which the reaction was stopped by addition of α-MEM control media, and cells were then gently detached by scraping with a plastic cell scraper (Corning Inc., NY, USA). Cells were then sub-cultured by plating at 6.6 x 10^4 cells/cm^2 in either 12-well or 24-well plastic plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in α-MEM control media as above. Cells were changed into differentiation media 2 days later (day 0 of experiment).

2.6.3 Assessment of bone marrow stromal cell proliferation

Proliferation of BMSCs was estimated by counting viable cell number at days -1, 0, 1, 2, 3, and 6 of the experiment of cells cultured in control α-MEM media (that is, starting from the day after subculture). Cells were counted from 4 wells per genotype at each time point. Cells were washed twice with warm PBS, and detached with 0.25% trypsin containing 0.53mM EDTA for 5 minutes at 37^0C. The trypsin reaction was stopped by addition of control media and a single cell suspension was prepared by gentle pipetting. The cell suspension was then diluted 1:1 with 0.4% trypan blue, and viable cells counted using a haemocytometer.

2.6.4 Differentiation of bone marrow stromal cells

Cells were changed into differentiation media 2 days after passaging (day 0 of experiment), when the cells had reached approximately 90% confluence. Osteoblast differentiation was induced by culturing cells in osteogenic media consisting of the control α-MEM media supplemented with 50mg/ml ascorbic acid and 10mM β-glycerol phosphate. Adipogenic media, to induce the differentiation of adipocytes consisted of the control α-MEM media supplemented with 5μg/ml insulin and 10nM dexamethasone. Media was changed 3 times weekly, for 21 days of culture. All differentiation experiments were performed using triplicate wells for each experimental group

2.6.5 Assessment of osteoblast and adipocyte differentiation

Cells from triplicate wells in osteogenic, adipogenic, or control media in 24-well plates were fixed and stained every third day to assess progression of differentiation. Osteoblast differentiation and mineralisation of extracellular matrix was visualised by staining with alkaline phosphatase (ALP) and von Kossa. Formation of adipocytes was visualised using Oil Red-O staining.

2.6.5.1 Alkaline phosphatase staining of cultures

Cells were washed twice in PBS then fixed in 4% PFA for 10 minutes at room temperature, then washed 3 times with PBS and once with freshly made 100mM Tris-HCl (pH7.5) containing 0.5% $MgCl_2$ (pH9.4). Cells were then stained in freshly made and filtered (0.2µm filter; Sartorius, Hannover, Germany) staining solution containing 100mM Tris-HCl (pH7.5) with 0.5% $MgCl_2$ (pH9.4), with 0.1% Fast-Blue RR salt and 0.1% Napthol AS-BI phosphate disodium salt (from 2% dilution in DMF) for 20 minutes at 37^0C. The reaction was terminated by 2 washes with PBS.

2.6.5.2 Von Kossa staining of cultures

Cells were washed twice in PBS then fixed in 4% PFA for 10 minutes at room temperature. Fixed cells were then washed with deionised water (dH_2O) then incubated with 2% silver nitrate under UV light for 30 minutes. Cells were then washed again with dH_2O and incubated with 2.5% sodium thiosulphate for 5 minutes followed by a wash with dH_2O.

2.6.5.3 Quantification of von Kossa staining

Extent of mineralisation was assessed from imported images of von Kossa stained wells using a Leica QWin imaging system. Total mineralised nodule area was measured and expressed as percentage mineralisation per $2cm^2$.

2.6.5.4 Oil red O staining of cultures

Cells were washed twice in PBS then fixed in 4% PFA for 10 minutes at room temperature. Following fixation, cells were rinsed once with 50% isopropanol, then washed 3 times with dH_2O. Cells were then stained with fresh filtered (0.2µm filter) Oil Red-O solution (1.2% Oil Red-O, 60% isopropanol) for 15 minutes at room temperature. Following staining, cells were rinsed quickly with 50% isopropanol and washed twice with dH_2O.

2.6.5.5 Quantification of adipocyte number

The extent of adipogenic differentiation was assessed by counting the number of cells containing well-stained oil droplets in 10 random low-power visual fields using the Leica QWin imaging system. Adipocyte number was expressed as number of adipocytes per mm^2.

2.6.6 Isolation of total RNA from cultured stromal cells

Total RNA was isolated from triplicate cultures of BMSCs in 12-well plates using Trizol® Reagent as per the manufacturers instructions. Adherent cells were washed with ice-cold PBS then incubated with Trizol® Reagent (1ml per $10cm^2$) for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. The lysates were sheared by passing the cell lysate through a 25-gauge needle four times, after which 0.2ml chloroform per 1ml Trizol® Reagent was added to separate the organic phase. Lysates were then vortexed for 15 seconds, and incubated 2 to 3 minutes at room temperature. Samples were then centrifuged 12,000 x g for 15 minutes at 4^0C. The colourless upper phase containing the RNA was carefully transferred to a fresh tube without disturbing the interphase or the lower phase. The RNA was then precipitated by addition of isopropyl alcohol (0.5ml of isopropyl alcohol per 1ml Trizol® Reagent used), and incubated for 10 minutes at room temperature, followed by centrifugation (12,000 x g, 10 min, 4^0C). The RNA pellet was then washed with 75% Ethanol, and air dried and resuspended in DEPC-treated water for quantification.

2.6.7 First strand cDNA synthesis

Following extraction, 1µg of total RNA, 5µM oligo$(dT)_{20}$, and 0.25mM each dNTP (dATP, dCTP, dGTP, dTTP) in a final volume of 10µl were incubated at 65^0C for 5 minutes, then cooled on ice. To this reaction was added 2µl 10X RT-buffer (25mM Tris HCl, (pH8.3), 37.5mM HCl, 1.5mM $MgCl_2$), 5mM $MgCl_2$, 10mM DTT, 40U RNaseOUT™ recombinant ribonuclease inhibitor, and 10U Superscript™ III reverse transcriptase in a final reaction

volume of 20µl, and incubated at 50^0C for 50 minutes, before inactivation by heating to 85^0C for 5 minutes. The reaction was then chilled on ice. cDNA was stored at -20^0C.

2.6.8 Polymerase Chain Reaction

PCR amplification was carried out in a 1x PCR buffer (20mM Tris-HCl pH 8.3, 50mM KCl), with 3mM $MgCl_2$, 200µM each dNTP, 0.5µM each primer, 1U of Taq DNA polymerase, and 1µl reverse transcribed template, in a final volume of 20µl using specific primers and annealing temperatures as shown in table 2.1. Cycling conditions for amplification were as follows:

Hold	94^0C	5 minutes
Cycling		
Denaturation	94^0C	35 seconds
Annealing	see table 2.1	35 seconds
Extension	72^0C	45 seconds
Extension	72^0C	6 minutes
Cooling	4^0C	∞

To exclude possible contamination of genomic DNA, PCR was also applied to reactions without reverse transcription. Cycling reactions were carried out using a RoboCycler® Gradient 40 Thermal Cycler (Stratagene, La Jolla, CA, USA). The amplified complementary DNA was electrophoresed through a 1% agarose gel, and photographed under UV light.

Gene		Sequence	Annealing Temp	Size (bp)
mY1 R	Forward	CTCGCTGGTTCTCATCGCTGTGGAACGG	55^0C	323
	Reverse	GCGAATGTATATCTTGAAGTAG		
mY2 R	Forward	TCCTGGATTCCTCATCTGAG	60^0C	520
	Reverse	GGTCCAGAGCAATGACTGTC		
mY4 R	Forward	TCTACAGACAGTAGACCAGG	56^0C	367
	Reverse	GTAGGTTGGTCACATTGGAC		
mY5 R	Forward	GGGCTCTATACATTTGTAAGTCTTCTGGG	60^0C	204
	Reverse	CATGGCTTTGCCGAACATCCACTGATCC		
mY6 R	Forward	GGAGGGATGGTTATTGTGAC	56^0C	347
	Reverse	GTTGTTGCTCTTGCCACTGG		
mGAPDH	Forward	ACTTTGTCAAGCTCATTTCC	57^0C	269
	Reverse	TGCAGCGAACTTTATTGATG		

Table 2-1 Sequences of mouse primers used in RT-PCR.
Sequence, annealing temperature, and product size in base pairs (bp) of primers used for amplification of Y1 receptor (mY1 R), Y2 receptor (mY2 R), Y4 receptor (mY4 R), Y5 receptor (mY5 R), Y6 receptor (mY6 R), and GAPDH.

2.6.9 Electrophoresis of DNA

DNA samples were prepared with 0.1 volume loading buffer [0.4% bromophenol blue and 50% glycerol (v/v)]. DNA fragments were separated by horizontal gel electrophoresis through 1.5 to 2% agarose (w/v) with 1μg/ml ethidium bromide, in 1x TAE buffer (4mM Tris acetate, 0.115% glacial acetic acid, 50mM EDTA) at 80V. Molecular weight markers SPP-1/*Eco*R1 and/or pUC19/*Hpa*II (500ng) were run alongside samples. Following electrophoresis the DNA fragments were visualised by UV transillumination and photographed with a Gel Doc 1000 gel documentation system (Biorad Laboratories, Hercules, CA, USA).

2.7 ISOLATION OF MESENCHYMAL STEM AND PROGENITOR CELLS

Mesenchymal stem cells (MSCs) and a more mature progenitor cell type were isolated from bone tissue of 8 to 15 week old male mice using a method developed in Paul Simmons' laboratory, involving extraction of bone cells, depletion of contaminating haematopoietic cells, and cell sorting. Both cell types have the potential to differentiate down the osteogenic, adipogenic, and chondrocytic lineages (Short et al., 2003),(Paul Simmons, personal communication), however, analysis of gene expression in the two populations has demonstrated an up-regulation of bone- and adipocyte- associated genes in the progenitor population, suggesting these are a more mature cell type in comparison to the MSCs.

2.7.1 Extraction of bone cells

Femurs, tibias, and iliac crests were dissected and thoroughly cleaned of muscle and connective tissue, after which the outer surface of the bones was scraped to remove the periosteal surface. Long bones and iliac crests were crushed separately using a mortar and pestle in washing buffer (2% FBS in PBS). Crushed long bones and iliac crests were then pooled together and fragmented further in 3mg/ml Worthington type 1 collagenase (ScimaR, Templestone, VIC, Australia) using a #22 scalpel blade. Bone fragments were then collagenase digested for 45 minutes on a shaker at 300rpm at 37^0C. Following digestion, bone fragments were thoroughly washed in washing buffer (2% FBS in PBS), as above), and large fragments were subsequently removed by filtering the cell suspension using a 70µm nylon mesh cell strainer (BD Biosciences, San Jose, CA, USA). Cells were collected by centrifugation at 170 x g for 4 minutes at room temperature, resuspended in 2ml washing buffer, and viable cell number was counted using 0.4% trypan blue.

2.7.2 Depletion of haematopoietic cells

To remove contaminating haematopoietic cells (B-lymphocytes, granulocytes, macrophages, T-cells, and erythrocytes) lineage depletion was performed using magnetic

activated cell sorting (MACS) microbeads. Cells were incubated with a cocktail consisting of the purified antibodies against the haematopoietic cell surface antigens B220, Gr-1, Mac-1, CD4, CD8, CD3, CD5 (all 1:500 dilution), and TER119 (1:1000 dilution) (Pharmingen, Franklin Lakes, NJ, USA) in washing buffer in a total volume of 2.5ml for 20 minutes at 4^0C. Excess antibody was removed by the addition of 8ml washing buffer, centrifugation at 240 x g for 4 minutes, and discarding the supernatant. The pellet was then resuspended in 80µl washing buffer per 10^7 cells. The cell suspension was then incubated with MACS® goat anti-rat IgG magnetic microbeads (20µl per 10^7 cells, Miltenyi Biotech, Bergisch-Gladbach, Germany) for 15 minutes at 4^0C. Following the incubation, the cells were washed by addition of 8ml washing buffer and centrifugation at 240 x g, for 5 minutes at 4^0C to remove unbound magnetic beads, and resuspended in 5.5ml washing buffer. The cell suspension containing the bound magnetic beads was then applied to a VarioMACS® separator with a LS MACS® Cell Separation Column (Miltenyi Biotech, Bergisch-Gladbach, Germany) and washed through with 5ml washing buffer. Eluted negatively selected cells devoid of haematopoietic cells were collected, centrifuged at 240 x g for 4 minutes, resuspended in 500µl washing buffer, and viable cells counted using 0.4% trypan blue.

2.7.3 Preparation of cells for sorting

To prepare cells for cell sorting to select the MSCs and progenitor populations, cells (approximately $3x10^6$) were incubated for 20 minutes at 4^0C with 2.5µl each of the fluorochrome- or biotin- conjugated antibodies against the surface markers Sca-1, CD45, CD31, and CD51. Appropriate controls (no antibody, isotype control using non-specific IgG, and fluorochrome controls using FITC, PE, and biotin-conjugated antibodies) were also set up by incubation of 0.5µl appropriate antibody with approximately 20,000 cells for each control group. After addition of 9ml of washing buffer and centrifugation (240 x g , 5min), the supernatant was discarded to remove unbound antibody and labelled cells were re-suspended in 2ml washing buffer. The secondary APC-conjugated streptavidin (1:500 dilution) was then added to all tubes containing the biotinylated-CD51 antibody (isotypes

control, CD51 control tube, and experimental samples), incubated for a further 15 minutes at 4^0C, then washed in washing buffer by centrifugation and again resuspended in 2ml washing buffer. To detect non-viable cells, 100µl of diluted 7AAD (1:300 dilution) was added to each experimental tube. Cells were then sorted using a FACS Vantage SE™ (BD Biosciences, San Jose, CA, USA) with FACSDiva SE™ software version 4.1.2 (BD Biosciences, San Jose, CA, USA) using a nozzle size of 70µm at 4^0C.

2.7.4 Cell sorting to isolate MSC and progenitor cells

Sorting was performed first by removing duplets, followed by removal of non-viable cells based on identification of 7AAD-labelled cells. Remaining contaminating haematopoietic cells were then removed by selecting $CD31^-$ and $CD45^-$ cells. The remaining cell population, now entirely devoid of haematopoietic cell contamination was then sorted based on Sca-1. Sca-1^+ cells were collected. Studies by Paul Simmons' laboratory have demonstrated this population to be a multipotential immature MSC cell type (Short et al., 2003). Sca-1^- cells were further sorted based on the marker CD51 to eliminate $CD51^-$ erythroid precursor cells, and Sca-1^-CD51^+ cells were collected. These cells have been shown to be a more mature progenitor cell type which retain the capability to differentiate into osteoblastic, adipocytic, and chondrogenic cell types (Paul Simmons, personal communication).

2.7.5 Culturing of sorted MSC and progenitor cells

Sorted cells were plated at a density of 1000 cells/well in 6-well plates (approximately 100cells/cm^2) and cultured in control media (section 2.6) containing 20% FBS, initially in 5%O_2, 10%CO_2, 85%N_2 (Air Liquide, Melbourne, Australia) at 37^0C for 3 days, then changed into regular culture conditions at 37^0C and 5% CO_2.

2.7.6 Colony Forming Assays of sorted MSC and progenitor cells

Formation of colonies by the MSC and mature progenitor cells was assessed by evaluation of colony forming units (CFUs) on day 7 of culture, when individual colonies were easily identifiable. Cells were washed twice with warm PBS, and fixed with 4% formaldehyde for 10 minutes at room temperature. Fixed cells were then washed again twice with PBS, and then stained with filtered (0.2μm filter) 1% toluidine blue for 15 minutes at room temperature. The number of positively-stained colonies containing >5 cells were counted for each experimental group.

2.7.7 Statistical analyses

Results were either assessed by factorial ANOVA, followed by Fisher's post hoc tests, or by 2-tailed t-tests unless otherwise indicated, using StatView version 4.5 (Abacus Concepts, San Francisco, CA, USA). Data are presented as means ± SEM. For all statistical analyses, $P<0.05$ was accepted as being statistically significant.

Chapter 3

Specificity of Y receptor action in the control of bone remodelling in the mouse

3.1 INTRODUCTION

A role for the neuropeptide Y receptor system in the regulation of bone formation was initially revealed following deletion of neuropeptide Y2 receptors in mice. Germline deletion of Y2 receptors ($Y2^{-/-}$) resulted in a two-fold increase in trabecular bone volume in the distal femur compared with wild type mice (Baldock et al., 2002). This difference in trabecular bone volume was associated with a significant elevation in the rate of mineral apposition and bone formation with no significant change in osteoclast or mineralising surface (Figure 3.1, and data not shown) (Baldock et al., 2002).

As noted in chapter 1 (section 1.10.6), while germline deletion of neuropeptide Y4 receptors did not result in a bone phenotype, double deletion of Y2 and Y4 receptors ($Y2^{-/-}$ $Y4^{-/-}$) resulted in a three-fold increase in trabecular bone volume associated with enhanced parameters of bone formation in the distal femur of male, but not female mice (Sainsbury et al., 2003). Bone volume and osteoblast surface were significantly greater in $Y2^{-/-}Y4^{-/-}$ bone compared with $Y2^{-/-}$, suggesting synergistic interaction between the Y2 and Y4 receptor pathways in the regulation of bone formation. Parameters of bone resorption in $Y2^{-/-}Y4^{-/-}$ mice were also elevated relative to wild type, despite no change in osteoclast surface in Y2 or Y4 single knockout mice (Baldock et al., 2002; Sainsbury et al., 2003).

These findings indicated that the specific actions of individual Y receptor subtypes and their interaction with each other vary with respect to the regulation of bone formation and resorption. The work in this chapter explored the role of another Y receptor subtype, the Y1 receptor, in the control of bone formation and resorption, and further investigated the extent to which the Y1 receptor might interact with Y2 and Y4 receptors in the regulation of bone remodelling. In order to evaluate the contributions of the different receptors in the regulation of bone remodelling, germline $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$ double knockout mice, and germline $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout mice were generated and assessed for changes in trabecular bone morphology and bone cell activity of the distal femur compared with wild type, and single $Y1^{-/-}$ and $Y2^{-/-}$ models using histomorphometry. Specifically, phenotypes

were analysed for additive effects, indicative of alternate or separate pathways, or lack of additive effects, indicative of a shared pathway or a regulatory feedback loop.

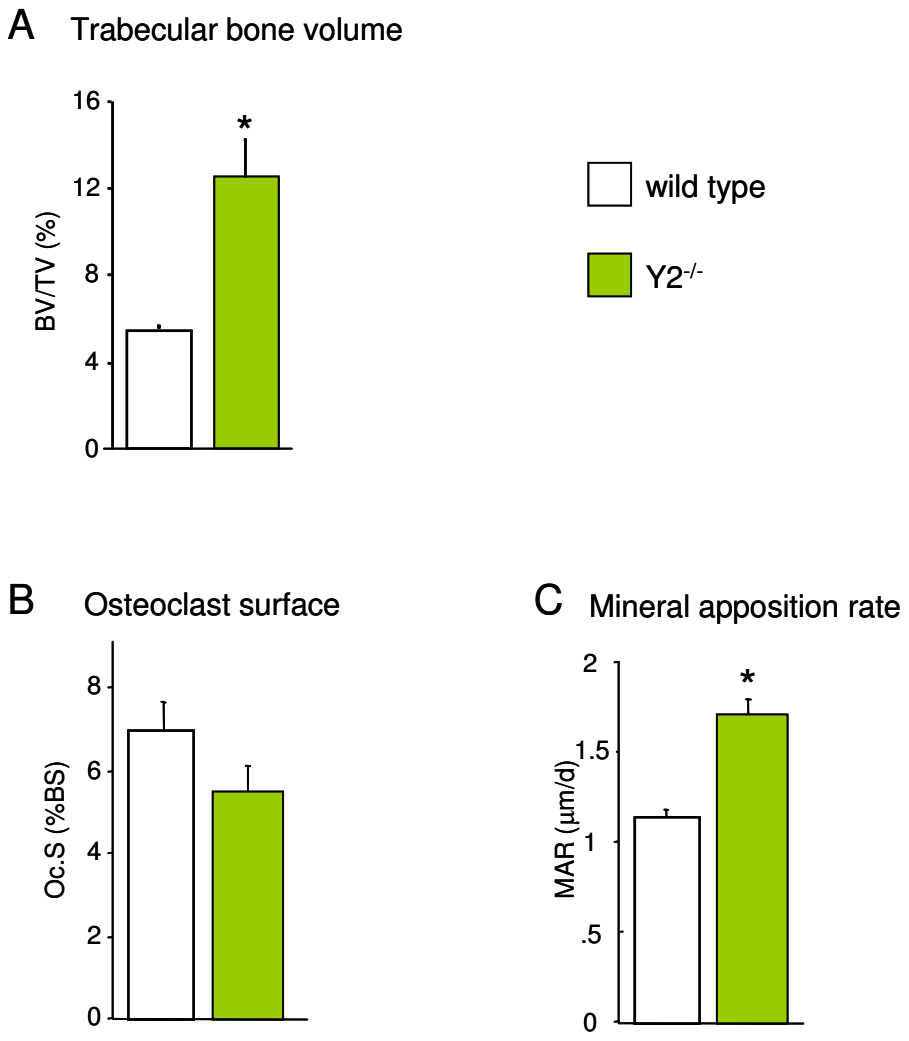

Figure 3.1 Germline deletion of Y2 receptors in mice results in greater trabecular bone volume associated with increased mineral apposition rate.
Trabecular bone volume (A), osteoclast surface (B), and mineral apposition rate (C). * $P < 0.05$ versus wild type. Adapted from; Baldock et al., 2002.

3.2 MATERIALS AND METHODS

3.2.1 Generation of germline $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$ double knockout and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout mice

Deletion of Y1, Y2, and Y4 receptor genes was performed as described in materials and methods (section 2.3.1). $Y2^{-/-}Y4^{-/-}$ double knockout mice were obtained as previously described (Sainsbury et al., 2003). $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ double knockout mice were obtained by crossing $Y1^{-/-}$ and $Y2^{-/-}$ or $Y4^{-/-}$ mice. $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout mice were generated in a three-step process performed by crossing the different combinations of double knockout mice, and the lack of genes confirmed by Southern Blot Analysis. Triple knockout mice were then maintained as homozygous breeding pairs.

3.2.2 Animals

Analyses were carried out in both male and female mice. Mice in this study were injected with the fluorescent acetomethoxy compound calcein 10 days prior to collection and the tetracycline related compound demeclocycline 3 days prior to collection as described in materials and methods (section 2.3.3). Mice were killed by cervical dislocation at 16 weeks of age. All analyses presented in this chapter were from the same cohort of animals, such that the same wild type, $Y1^{-/-}Y2^{-/-}$, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice were used in sections 3.3.2 and 3.3.3 and in sections 3.3.4 and 3.3.5.

3.2.3 Histomorphometry

Following fixation, the right femur was bisected transversely at the midpoint of the shaft, and the distal half embedded, undecalcified, in MMA as described in materials and methods (section 2.3.5). 5μm sagittal sections were analysed as described in materials and methods (section 2.3.6). BV/TV, Tb.N, and Tb.Th were calculated (section 2.3.9.1) from von Kossa stained sections (section 2.3.7.1). Oc.S was estimated (section 2.3.8.3) from TRAP stained sections (section 2.3.7.2). MAR was estimated from unstained sections (section 2.3.8.2). Unfortunately, the demeclocycline labels used in this study faded faster

than expected, and had nearly disappeared by the time single and double labelled surface was to be measured. As a consequence, calculations of mineralising surface (MS) and bone formation rate (BFR) could not be accurately assessed.

3.2.4 Statistical analyses

Statistical analyses were performed as described in materials and methods (section 2.7.7). Although the same wild type, $Y1^{-/-}Y2^{-/-}$, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ animals were used in sections 3.3.2 and 3.3.3 in comparison to $Y1^{-/-}$, and in sections 3.3.4 and 3.3.5 in comparison to $Y2^{-/-}$, mice were grouped for ANOVA and post hoc analyses as indicated by the graphs for each section. Analysis of $Y1^{-/-}$ versus $Y2^{-/-}$ measurements was performed using two-tailed unpaired t-tests. Numbers of mice used per group (n) were between 3 and 16, with specific "n" values specified in the figures.

3.3 RESULTS

3.3.1 Effect of Y1 receptor deletion on trabecular bone of the distal femur

Similar to the previously reported effect of Y2 receptor deletion on trabecular bone volume (Baldock et al., 2002), germline deletion of Y1 receptors also resulted in a significantly greater trabecular bone volume (BV/TV, %) in the distal femur of both male and female mice, with bone volume elevated approximately 2-fold relative to wild type (Figure 3.2, and data not shown). Analysis of trabecular bone cell activity revealed this was again associated with an increased rate of mineral apposition (Figure 3.2C), while later studies using double calcein labelling demonstrated that similar to the $Y2^{-/-}$ model, mineralising surface was also unchanged in the absence of Y1 receptor signalling (chapter 5). However, unlike the $Y2^{-/-}$ model, osteoclast surface was also elevated in $Y1^{-/-}$ mice (Figure 3.2B). These initial findings therefore not only revealed a new model in which trabecular bone volume was significantly elevated, but further demonstrated specific actions of the individual Y receptor subtypes in their regulation of different skeletal responses, leading to examination of the contributions and interactions of the Y1 receptor with other Y receptor

subtypes in the regulation of bone remodelling, achieved by the generation of double and triple Y receptor knockout mice.

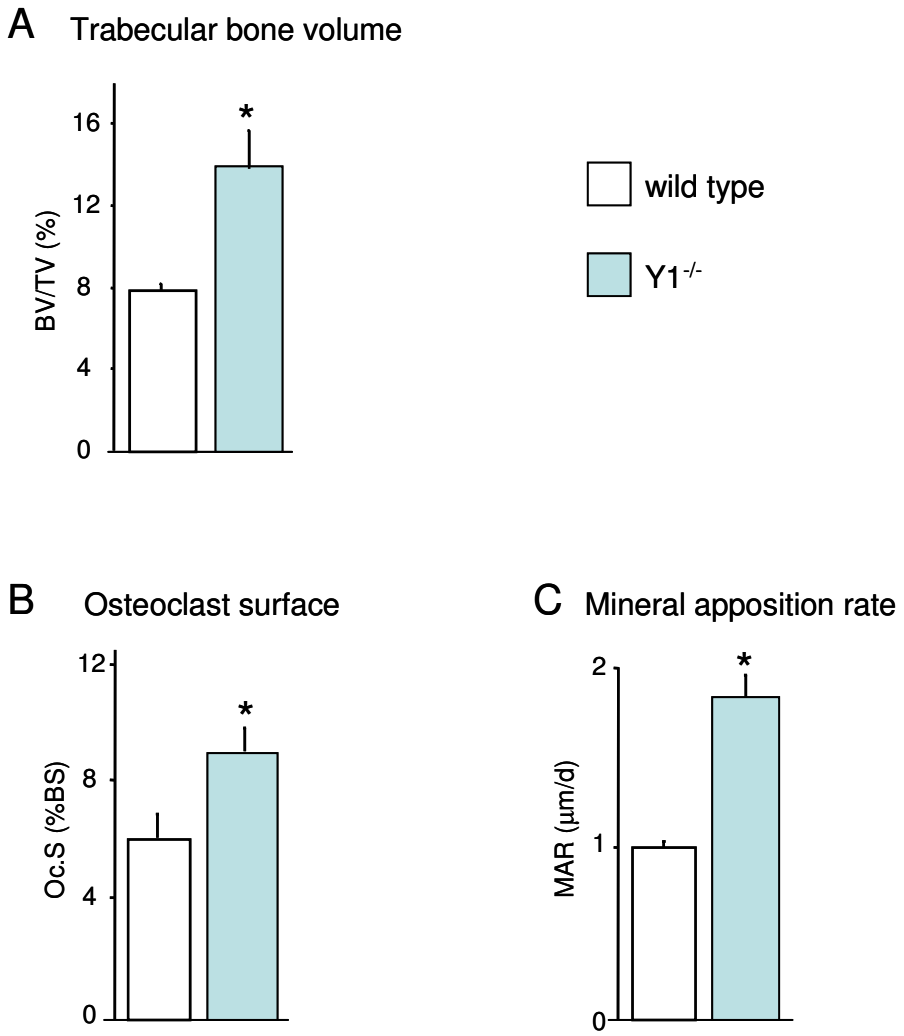

Figure 3.2 Germline deletion of Y1 receptors in mice results in greater trabecular bone volume associated with increased mineral apposition rate and osteoclast surface.
Trabecular bone volume (A), osteoclast surface (B), and mineral apposition rate (C). * $P < 0.05$ versus wild type. Numbers of mice per group (n); wild type: 5, $Y1^{-/-}$: 8.

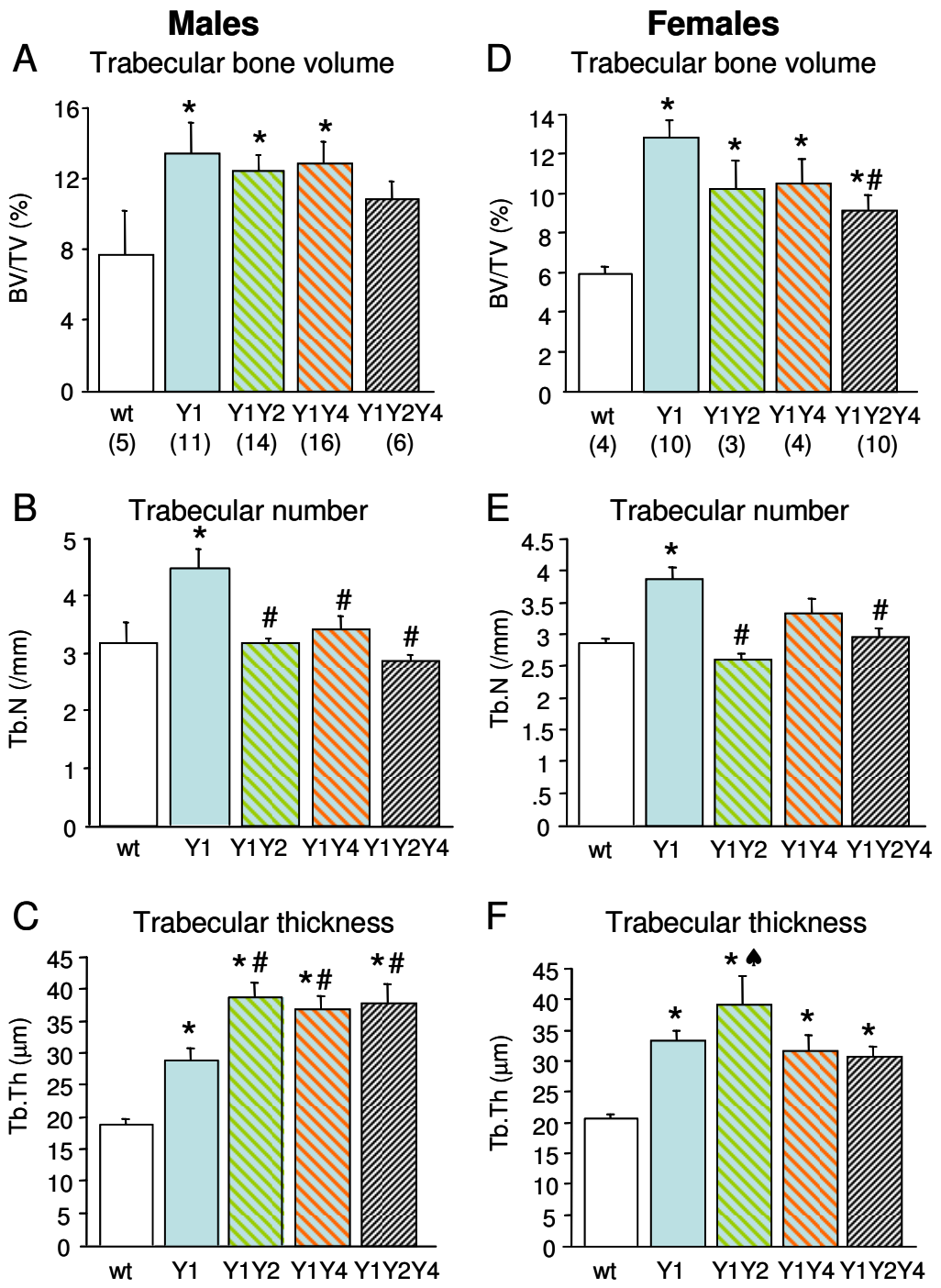

Figure 3.3 Effect of compound models lacking Y1 receptor on distal femur trabecular bone morphology. Male (A-C) and female (D-F) mice. Trabecular bone volume (A,D), trabecular number (B,E), and trabecular thickness (C,F). * $P < 0.05$ versus wild type (wt), # $P < 0.05$ versus $Y1^{-/-}$ (Y1), ♠ $P < 0.05$ versus $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ (Y1Y2Y4). Numbers of mice for each group indicated in parentheses.

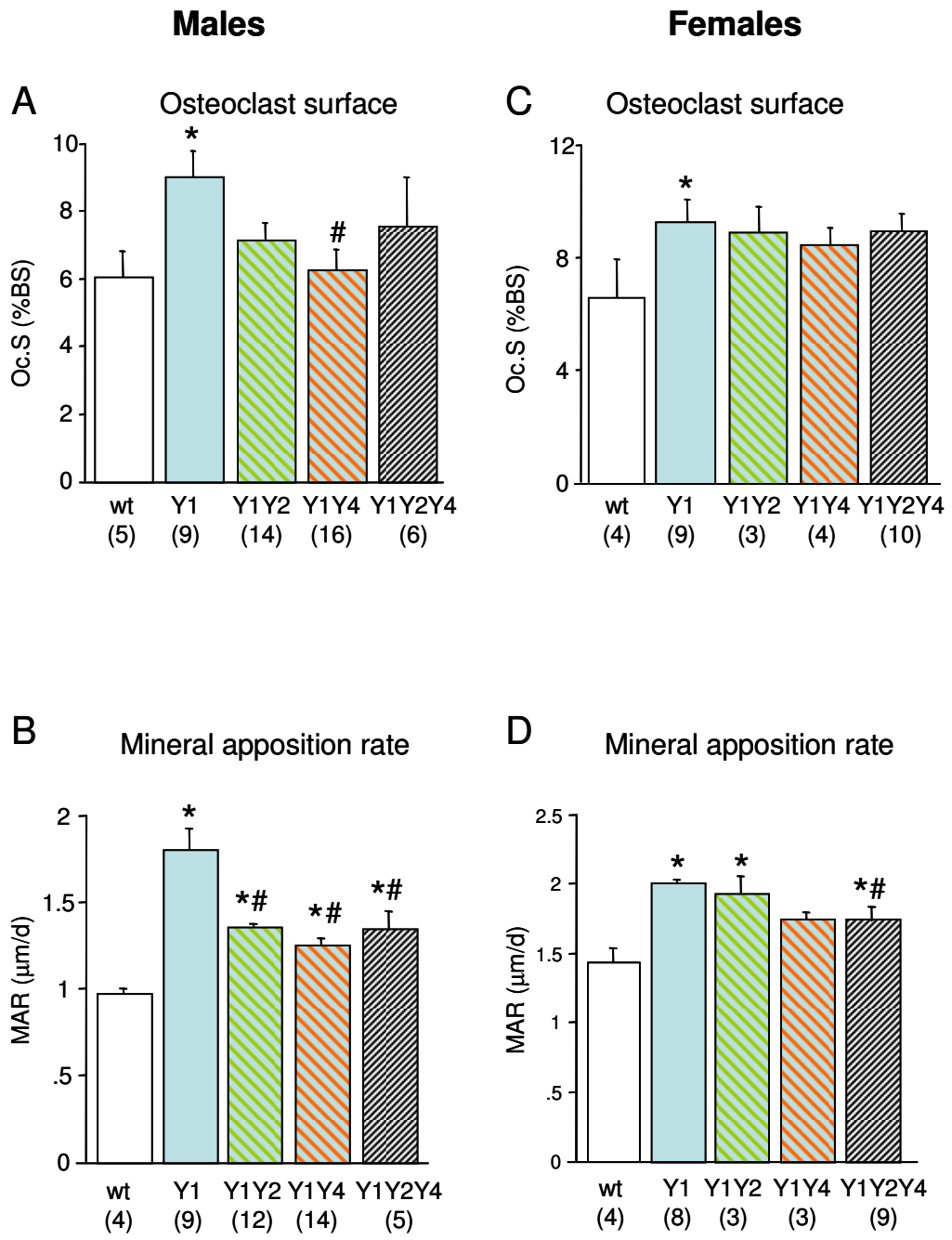

Figure 3.4 Effect of compound models lacking Y1 receptor on distal femur trabecular bone cell activity. Male (A-B) and female (C-D) mice. Osteoclast surface (A), and mineral apposition rate (B). * $P < 0.05$ versus wild type (wt), # $P < 0.05$ versus $Y1^{-/-}$ (Y1). Numbers of mice for each group indicated in parentheses.

3.3.2 Trabecular bone in distal femur of compound mutant mouse models lacking Y1 receptor

Trabecular bone volume of $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ double knockout mice were both elevated compared with wild type; however, in both sexes, levels were not further increased over those observed for $Y1^{-/-}$ mice (Figure 3.3 A,D). BV/TV was also increased in female $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout mice compared with wild type, although significantly lower than $Y1^{-/-}$ levels. A similar trend in male triple knockout mice did not reach statistical significance with either comparison (Figure 3.3 A,D).

The increase in bone volume observed in male and female $Y1^{-/-}$ mice resulted from significant elevations in both trabecular number (Tb.N, /mm) and thickness (Tb.Th, μm) compared with wild type (Figure 3.3 B,C,E,F). This is in contrast to trabecular bone microarchitecture in $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ double knockout, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout mice in which the greater BV/TV relative to wild type levels resulted from elevated Tb.Th (Figure 3.3 C,F), with no significant increases in Tb.N evident in either male or female mice (Figure 3.3 B,E). Tb.N in male $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice was in fact significantly reduced compared with $Y1^{-/-}$ levels. This lack of change in Tb.N in the compound models however, together with the significantly greater Tb.Th of the double and triple knockout models compared with $Y1^{-/-}$ male mice, likely explains the comparable elevations of BV/TV in these models (Figure 3.3). A similar trend was evident in females, with significantly reduced Tb.N in $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice compared with $Y1^{-/-}$ (Figure 3.3 E). However, in contrast to observations in males, Tb.Th in female double and triple knockout mice was not significantly greater than $Y1^{-/-}$ (Figure 3.3 F), consistent with a trend to slightly lower BV/TV values in the female compound mutants compared with $Y1^{-/-}$ levels, reaching statistical significance only in the $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ group (Figure 3.3). Importantly however, with the exception of elevated trabecular thickness in male compound mutants relative to $Y1^{-/-}$ which is an indirect calculation from histomorphometric measurements, these analyses of trabecular morphology in double and triple knockout mice gave no indication of additive or synergistic elevations in bone volume over $Y1^{-/-}$ levels, consistent with a role for shared pathways or a common

regulatory feedback loop in the determination of trabecular bone volume in these mutant models.

3.3.3 Bone cell activity in distal femur of $Y1^{-/-}$ and compound Y receptor mutant mice

Osteoclast surface (Oc.S, %BS) was elevated 50% and 40% in male and female germline $Y1^{-/-}$ mice compared with wild type, respectively (Figure 3.4 A,C). In contrast, Oc.S in $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice did not differ from wild type, indicating that the elevation in resorptive surface observed in $Y1^{-/-}$ mice is counteracted by the deletion of additional Y receptors. However it is important to note that Oc.S in female compound mutants was only marginally lower than levels measured for $Y1^{-/-}$ ($Y1^{-/-}$; 9.3 ± 0.8, $Y1^{-/-}Y2^{-/-}$; 8.9 ± 1.0, $Y1^{-/-}Y4^{-/-}$; 8.4 ± 0.7, $Y1^{-/-}Y2^{-/-}Y4^{-/-}$; 8.9 ± 0.7) (Table 3-2), and so while not statistically different from wild type, the relevance of these differences to resorption in bone tissue can not be ascertained from these studies. Interestingly however, this trend was stronger in the male bones. Of particular note was the significant 30% reduction in Oc.S in $Y1^{-/-}Y4^{-/-}$ male but not female mice compared with $Y1^{-/-}$ levels, suggesting that the Y4 receptor may be involved in a gender-specific control of bone resorption in these mice. However, it must also be noted that this gender-specific reduction in osteoclast surface in male but not female $Y1^{-/-}Y4^{-/-}$ mice may relate in part to the low number of female $Y1^{-/-}Y4^{-/-}$ mice available for this study (n = 4).

Mineral apposition rate (MAR, μm/d) was significantly elevated approximately 80% and 50% in male and female $Y1^{-/-}$ mice, respectively, compared with wild type (Figure 3.4 B,D), consistent with the greater BV/TV of this model. MAR was also significantly elevated in male and female $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice compared with wild type, and in male $Y1^{-/-}Y4^{-/-}$ mice with a similar but non-significant trend in females (P=0.07), likely due to low numbers of female $Y1^{-/-}Y4^{-/-}$ mice used in this comparison (Figure 3.4 B,D). Interestingly however, this indicator of osteoblast activity was between 20-30% lower in $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ male mice compared with $Y1^{-/-}$, although there was no

difference between these genotypes in females, which may again be due to low numbers of female compound mutants available for these analyses. By contrast, MAR was significantly lower than $Y1^{-/-}$ levels in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice of both genders. Therefore, consistent with the lack of an additive bone volume in the compound mutants over $Y1^{-/-}$ levels, bone formation in these mice was also not increased over $Y1^{-/-}$ levels. The lack of a synergistic increase in bone formation or bone volume in these mice is suggestive of a shared regulatory feedback loop or common pathways in their regulation of osteoblast activity. However, the indication that bone formation in the compound mutants was in some instances reduced compared with $Y1^{-/-}$ may also indicate interference with the Y1-anabolic pathway occurring in the absence of multiple Y receptors.

MALES:

	BV/TV (%)	Tb.N (/mm)	Tb.Th (µm)	Oc.S (%BS)	MAR (µm/d)
Wild type	5.3 ± 0.5	2.8 ± 0.1	18.6 ±1.1	6.1 ± 0.8	1.0 ± 0.03
$Y1^{-/-}$	13.4 ± 1.8 *	4.5 ± 0.3 *	28.7 ± 2 *	9.0 ± 0.8 *	1.8 ± 0.1 *
$Y1^{-/-}Y2^{-/-}$	12.4 ± 0.9 *	3.2 ± 0.08 #	38.8 ±2.2 *#	7.2 ± 0.5	1.3 ± 0.02 *#
$Y1^{-/-}Y4^{-/-}$	12.8 ± 1.3 *	3.4 ± 0.2 #	36.7 ± 2.2 *#	6.3 ± 0.6 #	1.2 ± 0.04 *#
$Y1^{-/-}Y2^{-/-}Y4^{-/-}$	10.8 ± 1	2.9 ± 0.1 #	37.8 ± 2.9 *#	7.6 ± 1.4	1.3 ± 0.1 *#

Table 3-1 Effect of compound Y receptor deletion in combination with $Y1^{-/-}$ in male mice.
* $P < 0.05$ versus wild type, # $P < 0.05$ versus $Y1^{-/-}$.

FEMALES:

	BV/TV (%)	Tb.N (/mm)	Tb.Th (µm)	Oc.S (%BS)	MAR (µm/d)
Wild type	5.9 ± 0.3	2.9 ± 0.07	20.7 ± 0.7	6.5 ± 1.4	1.4 ± 0.1
$Y1^{-/-}$	12.9 ± 0.9 *	3.9 ± 0.2 *	33.2 ± 1.7 *	9.3 ± 0.8 *	2.0 ± 0.03 *
$Y1^{-/-}Y2^{-/-}$	10.2 ± 1.4 *	2.6 ± 0.09 #	39.1 ± 4.7 *♠	8.9 ± 1.0	1.9 ± 0.1 *
$Y1^{-/-}Y4^{-/-}$	10.5 ± 1.2 *	3.3 ± 0.3	31.6 ± 2.6 *	8.4 ± 0.7	1.7 ± 0.06
$Y1^{-/-}Y2^{-/-}Y4^{-/-}$	9.1 ± 0.7 *#	2.9 ± 0.1 #	30.8 ± 1.5 *	8.9 ± 0.7	1.7 ± 0.09 *#

Table 3-2 Effect of compound Y receptor deletion in combination with $Y1^{-/-}$ in female mice.
* $P < 0.05$ versus wild type, # $P < 0.05$ versus $Y1^{-/-}$, ♠ $P < 0.05$ versus $Y1^{-/-}Y2^{-/-}Y4^{-/-}$.

3.3.4 Comparison of $Y2^{-/-}$ trabecular microarchitecture to other Y receptor mutant models

Bone histomorphometric parameters of the double and triple mutants were also compared to the $Y2^{-/-}$ phenotype. Consistent with the previous characterisation of the $Y2^{-/-}$ model (Baldock et al., 2002), BV/TV at the distal femur was significantly greater in male and female germline $Y2^{-/-}$ mice relative to wild type (Figure 3.5 A,D).

Comparison of $Y2^{-/-}$ mice to $Y1^{-/-}$ revealed no significant difference in BV/TV, Tb.N, or Tb.Th in either males or females (data not shown). In both genders, Oc.S was non-significantly increased in $Y1^{-/-}$ compared with $Y2^{-/-}$ mice (males; $Y1^{-/-}$: 9.0 ± 0.8 vs. $Y2^{-/-}$: 6.0 ± 1.5%, $p = 0.08$, females; $Y1^{-/-}$: 9.3 ± 0.8 vs. $Y2^{-/-}$: 6.9 ± 0.8%, $p = 0.07$) (Table 3-1 - Table 3-4). Interestingly, MAR in female $Y2^{-/-}$ mice was significantly greater relative to $Y1^{-/-}$ ($Y1^{-/-}$: 2.0 ± 0.03 vs. $Y2^{-/-}$: 2.2 ± 0.04 μm/d, $p<0.05$). This difference in MAR between $Y1^{-/-}$ and $Y2^{-/-}$ was not apparent in male mice, with no significant difference between the genotypes ($Y1^{-/-}$: 1.8 ± 0.1 vs. $Y2^{-/-}$: 1.7 ± 0.09 μm/d).

BV/TV of $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice were elevated compared with wild type (section 3.3.2 and repeated in Figure 3.5 A,D), but were not elevated above $Y2^{-/-}$ levels, similar to observations in $Y1^{-/-}$ mice.

These findings contrast with the synergistic 3-fold elevation of bone volume in male $Y2^{-/-}$ $Y4^{-/-}$ mice relative to wild type, with BV/TV significantly greater than both wild type and $Y2^{-/-}$ levels (Figure 3.5 A), and compared to germline $Y4^{-/-}$ mice in which BV/TV was comparable to wild type levels in our previous study (Sainsbury et al., 2003). The synergistic increase in BV/TV observed in $Y2^{-/-}Y4^{-/-}$ double knockout mice was gender specific and absent in female mice, with BV/TV levels similar to those of $Y2^{-/-}$ mice (Figure 3.5 D), also consistent with prior observations (Baldock et al., 2005; Sainsbury et al., 2003). Interestingly, trabecular bone volume in $Y2^{-/-}Y4^{-/-}$ males was elevated compared with $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockouts (Figure 3.5 A), demonstrating that the synergistic increase in bone volume of $Y2^{-/-}Y4^{-/-}$ mice requires the presence of Y1 receptor signalling.

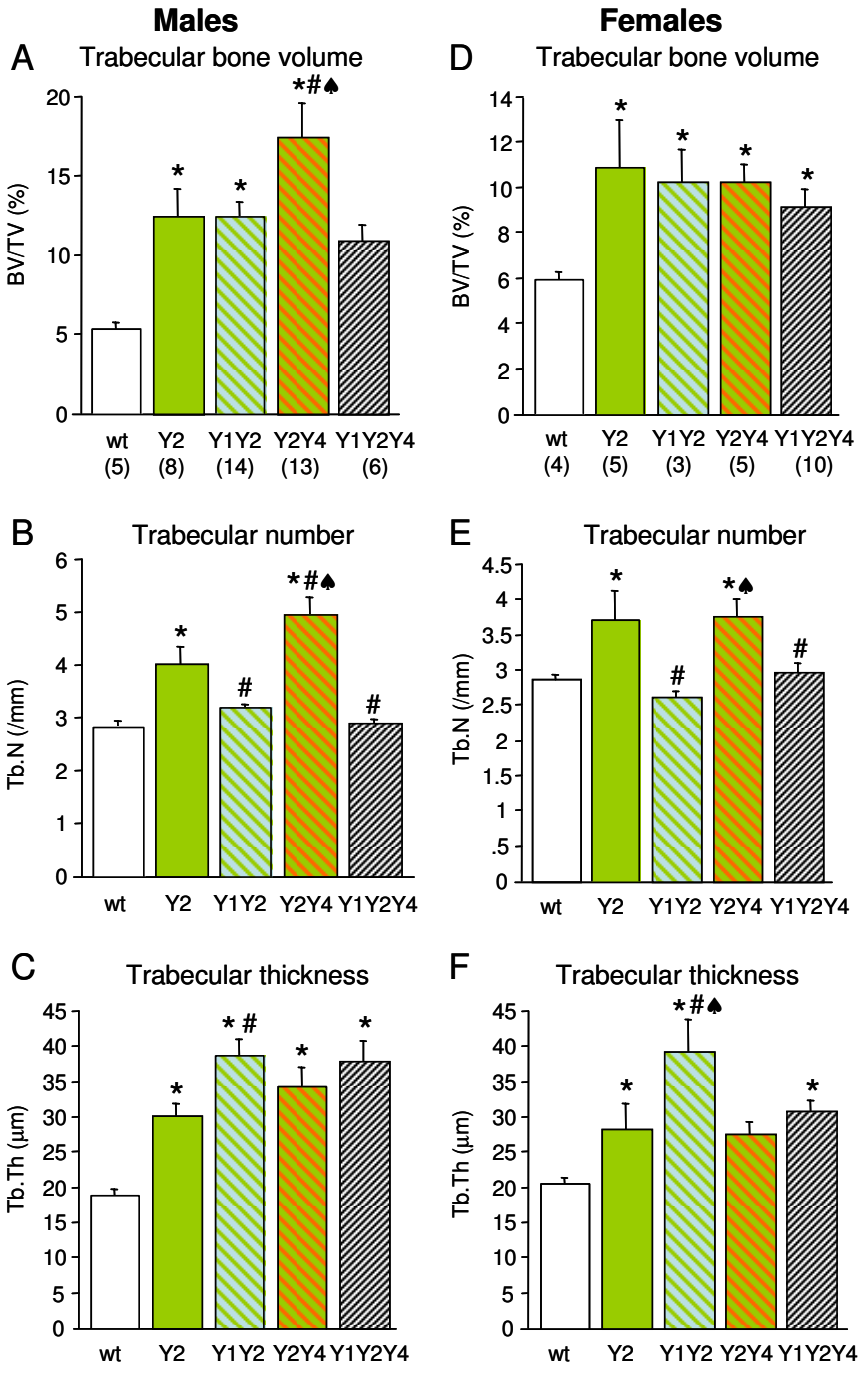

Figure 3.5 Effect of compound models lacking Y2 receptor on distal femur trabecular bone morphology. Male (A-C) and female (D-F) mice. Trabecular bone volume (A,D), trabecular number (B,E), and trabecular thickness (C,F). * $P < 0.05$ versus wild type (wt), # $P < 0.05$ versus $Y2^{-/-}$ (Y2), ♠ $P < 0.05$ versus $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ (Y1Y2Y4). Numbers of mice for each group indicated in parentheses.

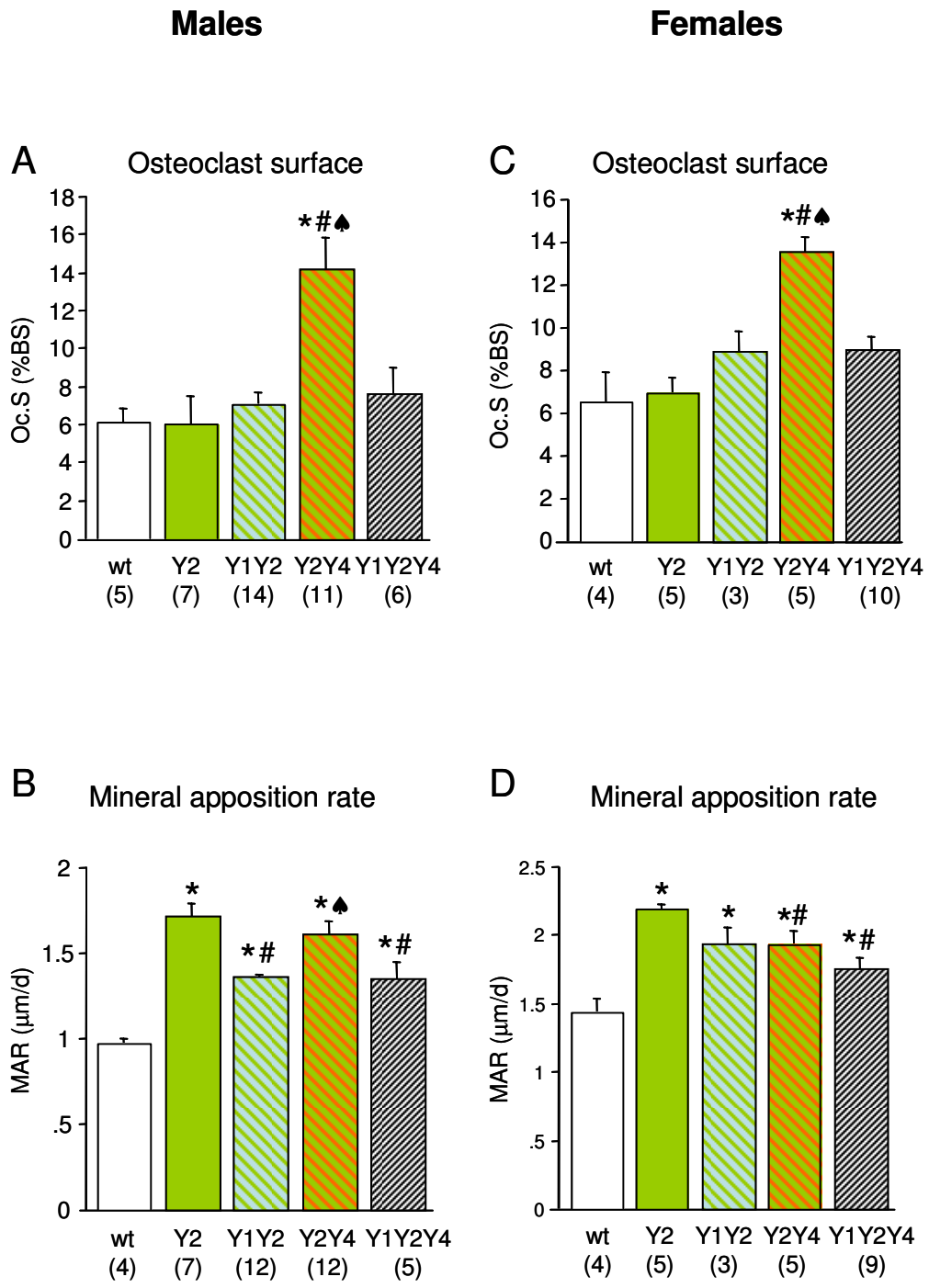

Figure 3.6 Effect of compound models lacking Y2 receptor on distal femur trabecular bone cell activity. Male (A-B) and female (C-D) mice. Osteoclast surface (A), and mineral apposition rate (B). * $P < 0.05$ versus wild type (wt), # $P < 0.05$ versus $Y2^{-/-}$ (Y2), ♠ $P < 0.05$ versus $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ (Y1Y2Y4). Number of mice for each group indicated in parenthesis.

Trabecular number and thickness were significantly elevated in both male and female $Y2^{-/-}$ mice compared with wild type (Figure 3.5 B,C,E,F), consistent with the previous characterisation of the $Y2^{-/-}$ model (Baldock et al., 2002). In contrast, Tb.N values in $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice were not different from wild type (section 3.3.2), and were significantly lower than $Y2^{-/-}$ levels in both male and female mice (Figure 3.5 B,E). However, Tb.Th was significantly greater in male and female $Y1^{-/-}Y2^{-/-}$ mice compared with $Y2^{-/-}$ levels, accounting for the similar BV/TV of the $Y2^{-/-}$ and the $Y1^{-/-}Y2^{-/-}$ models (Figure 3.5).

This pattern contrasts with that of male $Y2^{-/-}Y4^{-/-}$ double knockout mice, in which the synergistic increase in bone volume was associated with significantly greater Tb.N compared with wild type, $Y2^{-/-}$, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice (Figure 3.5 B) and greater Tb.Th compared with wild type (Figure 3.5 C). Similarly, Tb.N in female $Y2^{-/-}Y4^{-/-}$ mice was elevated compared with wild type and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice, although not different from $Y2^{-/-}$ levels (Figure 3.5 E), while Tb.Th was not different from wild type (Figure 3.5 F). These gender-specific differences in trabecular morphology in $Y2^{-/-}Y4^{-/-}$ double knockout mice are consistent with the gender-specific differences observed in BV/TV. Thus, similar to the findings from the previous section in which deletion of multiple Y receptors did not elevate BV/TV above $Y1^{-/-}$ levels, deletion of multiple Y receptor subtypes also did not elevate bone volume above $Y2^{-/-}$ levels, with the exception of a gender-specific synergistic elevation in bone volume in male $Y2^{-/-}Y4^{-/-}$ mice.

3.3.5 Histomorphometric comparison of $Y2^{-/-}$ bone cell activities to other Y receptor mutant models

Osteoclast surface was approximately 2-fold greater in $Y2^{-/-}Y4^{-/-}$ knockout mice of both genders compared with wild type and $Y2^{-/-}$ mice (Figure 3.6 A,C), consistent with earlier studies (Sainsbury et al., 2003). The reduced Oc.S in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ male and female mice compared with $Y2^{-/-}Y4^{-/-}$ counterparts, suggests complex interactions between the different Y receptors in the regulation of bone resorption, with the elevated Oc.S of the $Y2^{-/-}Y4^{-/-}$ model reduced in the absence of all three Y receptors.

MAR was significantly elevated in both male and female $Y2^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice compared with wild type (Figure 3.6 B,D), consistent with previous findings (Baldock et al., 2002; Sainsbury et al., 2003). While MAR in $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice was elevated relative to wild type, levels were lower compared with $Y2^{-/-}$ levels in male and female $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice, and in male $Y1^{-/-}Y2^{-/-}$ mice. MAR in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout mice was also significantly reduced compared with $Y2^{-/-}Y4^{-/-}$ levels in male, but not female mice, consistent with the gender-specific synergistic elevation of bone volume observed in male $Y2^{-/-}Y4^{-/-}$ mice (Figure 3.5 A, Figure 3.6).

MALES:

	BV/TV (%)	Tb.N (/mm)	Tb.Th (μm)	Oc.S (%BS)	MAR (μm/d)
Wild type	5.3 ± 0.5	2.8 ± 0.1	18.6 ±1.1	6.1 ± 0.8	1.0 ± 0.03
$Y2^{-/-}$	12.4 ± 1.7 *	4.0 ± 0.3 *	29.9 ± 2.0 *	6.0 ± 1.5	1.7 ± 0.09 *
$Y1^{-/-}Y2^{-/-}$	12.4 ± 0.9 *	3.2 ± 0.08 #	38.8 ±2.2 *#	7.2 ± 0.5	1.3 ± 0.02 *#
$Y2^{-/-}Y4^{-/-}$	17.4 ± 2.1 *#♠	4.9 ± 0.4 *#♠	34.4 ± 2.6 *	14.1 ± 1.8 *#♠	1.6 ± 0.08 *♠
$Y1^{-/-}Y2^{-/-}Y4^{-/-}$	10.8 ± 1	2.9 ± 0.1 #	37.8 ± 2.9 *	7.6 ± 1.4	1.3 ± 0.1 *#

Table 3-3 Effect of compound Y receptor deletion in combination with $Y2^{-/-}$ in male mice.
* P < 0.05 versus wild type, # P < 0.05 versus $Y1^{-/-}$.,♠ P < 0.05 versus $Y1^{-/-}Y2^{-/-}Y4^{-/-}$.

FEMALES:

	BV/TV (%)	Tb.N (/mm)	Tb.Th (μm)	Oc.S (%BS)	MAR (μm/d)
Wild type	5.9 ± 0.3	2.9 ± 0.07	20.7 ± 0.7	6.5 ± 1.4	1.4 ± 0.1
$Y2^{-/-}$	10.8 ± 2.1 *	3.7 ± 0.4 *	28.2 ± 3.6 *	6.9 ± 0.8	2.2 ± 0.04 *
$Y1^{-/-}Y2^{-/-}$	10.2 ± 1.4 *	2.6 ± 0.09 #	39.1 ± 4.7 *#♠	8.9 ± 1.0	1.9 ± 0.1 *
$Y2^{-/-}Y4^{-/-}$	10.2 ± 0.8 *	3.7 ± 0.3 *♠	27.5 ± 1.8	13.5 ± 0.8 *#♠	1.9 ± 0.1 *#
$Y1^{-/-}Y2^{-/-}Y4^{-/-}$	9.1 ± 0.7 *	2.9 ± 0.1 #	30.8 ± 1.5 *	8.9 ± 0.7	1.7 ± 0.09 *#

Table 3-4 Effect of compound Y receptor deletion in combination with $Y2^{-/-}$ in female mice.
* P < 0.05 versus wild type, # P < 0.05 versus $Y1^{-/-}$.,♠ P < 0.05 versus $Y1^{-/-}Y2^{-/-}Y4^{-/-}$.

3.4 DISCUSSION

These studies examined the involvement of Y1 receptor signalling in the regulation of trabecular bone morphology and bone cell activity in the mouse distal femur. The effect of Y1 and Y2 receptor deletion alone and in combination with each other, and/or Y4 receptor deletion was assessed to determine the extent of involvement of the different Y receptors in the regulation of bone remodelling, and to assess possible interaction between the different Y receptor pathways. Male and female mice were investigated in order to assess possible gender differences between the different Y receptors in the control of bone remodelling. With the exception of the synergistic elevation in bone volume previously reported in male $Y2^{-/-}Y4^{-/-}$ mice, the findings presented here demonstrate that deletion of multiple Y receptor subtypes does not further elevate bone volume or bone formation over levels observed in $Y1^{-/-}$ or $Y2^{-/-}$ mice, providing evidence for a shared regulatory feedback loop or commonality in the mechanism of action of the Y1 receptor- and Y2 receptor- mediated anabolic pathways. However, the elevated parameters of bone resorption in $Y1^{-/-}$ and $Y2^{-/-}$ $Y4^{-/-}$ mice which were not observed in other compound knockout models, suggests complex interactions in the regulation of osteoclast activity by the specific Y receptors. Interestingly, while there was no evidence for synergistic increases in the rate of mineral apposition in the compound mutants compared with single Y1- or Y2-receptor deletion, there were several instances in which MAR was reduced relative to the single knockout models, suggesting interactions between Y receptor subtypes in the control of bone formation may be more complex than these initial analyses reveal.

3.4.1 Trabecular bone phenotypes of Y1 receptor and compound Y receptor knockout models

Germline deletion of the Y1 receptor gene resulted in increases in trabecular bone volume, number, and thickness similar to the $Y2^{-/-}$ model, revealing the involvement of a novel Y receptor subtype in the regulation of bone cell activity. However, while double deletion of Y1 in combination with the other Y receptors ($Y1^{-/-}Y2^{-/-}$ or $Y1^{-/-}Y4^{-/-}$) resulted in elevated bone volume compared with wild type mice, no further synergistic increase in bone volume

was apparent. Interestingly, in both male and female mice, deletion of all three receptor subtypes ($Y1^{-/-}Y2^{-/-}Y4^{-/-}$) did not elevate BV/TV above levels measured in $Y1^{-/-}$ or $Y2^{-/-}$ mice or in $Y1^{-/-}Y2^{-/-}$ or $Y1^{-/-}Y4^{-/-}$ double knockout lines. In fact, in female $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice, bone volume was reduced compared with $Y1^{-/-}$ alone.

The greater trabecular bone volume of $Y1^{-/-}$ and $Y2^{-/-}$ mice was associated with elevations in both trabecular number and thickness, while the greater bone volume of $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ knockout mice was associated only with elevated trabecular thickness, with no change in number, regardless of gender. However, despite the lack of elevated Tb.N in these mice, bone volume was remarkably similar between the genotypes apparently due to a greater elevation in Tb.Th compensating for the lack of change in Tb.N in these double and triple Y receptor knockout mice.

These findings differ from the male-specific synergistic three-fold increase in trabecular bone volume previously observed in $Y2^{-/-}Y4^{-/-}$ mice, which was significantly higher than either $Y2^{-/-}$ or $Y4^{-/-}$ levels, and was also associated with greater trabecular number and thickness (Baldock et al., 2005; Sainsbury et al., 2003). The lack of a similar synergistic elevation in trabecular bone volume in the triple knockout mice was perhaps surprising. However, BV/TV and Tb.N in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice was in fact significantly reduced compared with $Y2^{-/-}Y4^{-/-}$. These findings suggest that the synergistic elevation in bone volume observed in male $Y2^{-/-}Y4^{-/-}$ double knockout mice is abolished when signalling through the Y1 receptor is eliminated, implicating a potential requirement for the Y1 receptor in the greater bone volume of $Y2^{-/-}Y4^{-/-}$ double knockout male mice. It has been proposed that the synergistic anabolic response of the $Y2^{-/-}Y4^{-/-}$ model is attributable to changes in leptin signalling (Baldock et al., 2005; Sainsbury et al., 2003). The finding that the synergistic response is absent in the triple knockout model supports this hypothesis, as is discussed in further detail below.

MAR was elevated in all knockout models compared with wild type mice, although this difference did not reach significance in female $Y1^{-/-}Y4^{-/-}$ mice, most likely due to the low numbers available for this study. However, MAR was actually lower in $Y1^{-/-}Y2^{-/-}$ males

compared with $Y1^{-/-}$ or $Y2^{-/-}$, and in $Y1^{-/-}Y4^{-/-}$ males compared with $Y1^{-/-}$, while in both males and females, MAR was lower in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice compared with $Y1^{-/-}$ and $Y2^{-/-}$ mice. These modest differences in the rate of mineral apposition could contribute to the differences in trabecular morphology discussed above, with the less marked elevations in MAR in $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$, and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice sufficient to increase trabecular thickness but not number, whereas the greater elevation of MAR in $Y1^{-/-}$, $Y2^{-/-}$, and male $Y2^{-/-}Y4^{-/-}$ mice compared to wild type appears to be sufficient to elevate both trabecular thickness and number.

It is somewhat surprising however, that bone volume was remarkably similar between the compound mutants and the $Y1^{-/-}$ or $Y2^{-/-}$ models, particularly in male mice, with the noted exception of the $Y2^{-/-}Y4^{-/-}$ model, despite a 20-30% reduction in the rate of mineral apposition in the compound mutants compared with $Y1^{-/-}$. It is possible that the modest reductions in Oc.S relative to $Y1^{-/-}$ might counteract the reduced MAR in these models. Alternatively, it is possible that these models exhibit differences in the extent of mineralising surface, with increased mineralising surface overcoming the reduction in MAR. While preliminary analyses suggested mineralising surface was not changed in the compound mutants relative to wild type (data not shown), marked fading of the demeclocycline labels used in this study rendered these findings inconclusive.

3.4.2 Lack of synergism in bone formation in compound mutants lacking Y1 receptor

Analysis of distal femurs from $Y1^{-/-}Y4^{-/-}$ mice revealed a similar trabecular bone phenotype to $Y1^{-/-}$ in both males and females, with similarly elevated trabecular bone volume. MAR was also elevated, albeit to a lesser extent. Importantly however, there was no evidence of additive effects suggesting that, unlike the synergistic elevation in bone volume activated in the absence of Y2 and Y4 receptor signalling, deletion of Y4 receptor signalling does not significantly alter the bone formation response to Y1 receptor deletion. Similarly, double deletion of both Y1 and Y2 receptors did not significantly alter BV/TV beyond the elevated levels observed with single Y1 or Y2 gene deletion in males or females. This lack of

additive effect on bone volume was consistent with a lack of further elevation in the rate of mineral apposition in $Y1^{-/-}Y2^{-/-}$ mice. Despite these similarities between the $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ bone phenotypes suggesting common signalling pathways in their regulation of osteoblast activity, it remains a possibility that the Y1 and Y2 receptors may regulate bone formation via distinct mechanisms, as serum insulin which is known to affect bone formation (Cornish et al., 1996), is elevated in both $Y1^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ male mice but not in $Y2^{-/-}$ or $Y1^{-/-}Y2^{-/-}$ animals (Sainsbury et al., 2006). By contrast, changes in serum leptin appear not to contribute to the bone phenotypes of $Y1^{-/-}$, $Y2^{-/-}$, $Y1^{-/-}Y2^{-/-}$, or $Y1^{-/-}Y4^{-/-}$ models, as these lines exhibit wild type levels of this adipocytic hormone (Sainsbury et al., 2006). As discussed in chapter 1 (section 1.10.6) however, the male-specific synergistic increase in BV/TV in $Y2^{-/-}Y4^{-/-}$ mice has been proposed to result from additive Y2-mediated and leptin antiosteogenic responses attributable to a gender-specific reduction in serum-leptin levels correlating with the reduced white adipose tissue (WAT) mass of this model (Baldock et al., 2005; Sainsbury et al., 2003). The findings presented here support this hypothesis, as the synergistic elevation in BV/TV of male $Y2^{-/-}Y4^{-/-}$ mice was not observed in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice. Indeed, serum leptin levels in 16-week old male $Y1^{-/-}Y2^{-/-}$ $Y4^{-/-}$ mice fed a standard chow diet were similar to wild type levels and significantly greater than levels of $Y2^{-/-}Y4^{-/-}$ mice, corresponding to the greater WAT mass in the triple knockout model (Sainsbury et al., 2006). Therefore, it is plausible that the synergistic elevation in bone volume in $Y2^{-/-}Y4^{-/-}$ and not $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice, could result from differences in leptin signalling, possibly secondary to changes in energy homeostasis induced by deletion of Y1 receptor signalling in the absence of Y2 and Y4.

Gender-specific differences in these studies other than those observed in the $Y2^{-/-}Y4^{-/-}$ model were not as obvious. Osteoclast surface in the compound mutants was reduced to a greater extent compared with $Y1^{-/-}$ in male mice, however the significant reduction in Oc.S in male $Y1^{-/-}Y4^{-/-}$ mice which was not observed in females may be solely due to low numbers of female $Y1^{-/-}Y4^{-/-}$ mice used in this study. Alternatively, the gender-specific difference in the osteoclast response in these mice may reflect a real physiological response, with the very slight reduction in Oc.S in the female compound mutants contributing to the lack of elevated trabecular thickness and the slightly reduced bone

volume of the females compared with males, relative to $Y1^{-/-}$, and may indicate an important role for the sex hormones estrogen and testosterone in the regulation of the resorption and formation response of the $Y1^{-/-}$ and $Y2^{-/-}$ models. The role of sex hormones in the regulation of bone cell activity in the absence of Y1 or Y2 receptor signaling is addressed further in chapter 5.

3.4.3 Specificity in Y receptor regulation of bone resorption

The lack of an additive osteoblastic response or a synergistic elevation in trabecular bone volume in $Y1^{-/-}Y2^{-/-}$, $Y1^{-/-}Y4^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice compared with $Y1^{-/-}$ and $Y2^{-/-}$ therefore suggests a common pathway or feedback loop in their regulation of bone remodelling, or alternatively suggests an absolute maximum or biological limitation was reached, which was only exceeded in the male $Y2^{-/-}Y4^{-/-}$ mice with the added effects of reduced leptin signalling contributing to a further increase in bone volume. However, the response of osteoclastic parameters did not always correlate tightly with observed changes in osteoblast activity. Osteoclast responses were also not easily attributable to a specific Y receptor subtype, suggesting specificity but also interaction or redundancy between the different Y receptors in the regulation of bone resorption. Oc.S was statistically similar across all genotypes, except for the $Y1^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ models, in which Oc.S was significantly elevated compared with wild type levels. However, osteoclast surface was increased only 40% and 50% in female and male $Y1^{-/-}$ mice, respectively, compared with a 2-fold increase in $Y2^{-/-}Y4^{-/-}$ mice, which may reflect a mechanistic difference in the regulation of resorption in these two models. However, in both models, the greater Oc.S did not appear to have significantly deleterious effects on bone volume, with BV/TV in $Y1^{-/-}$ mice comparable to $Y2^{-/-}$ levels, and BV/TV of male $Y2^{-/-}Y4^{-/-}$ mice elevated above $Y2^{-/-}$ levels. Unexpectedly, the greater Oc.S of the $Y1^{-/-}$ model was not matched by significant increases in the compound Y receptor knockout models ($Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$). However, as Oc.S in female compound mutants was only marginally reduced compared with $Y1^{-/-}$, which may in part only be due to low numbers of $Y1^{-/-}Y2^{-/-}$ and $Y1^{-/-}Y4^{-/-}$ mice, the relevance of these apparent differences to cellular responses *in vivo* is not clear. These responses were however more evident in male compound mutants and may therefore still

suggest a possible physiological role for the Y2 and Y4 receptor subtypes in the regulation of bone resorption in the absence of Y1. Furthermore, the greater Oc.S of the $Y2^{-/-}Y4^{-/-}$ model was abolished when all three Y receptors were deleted, indicating a complex interaction such that while deletion of Y1 receptor signalling is itself associated with greater bone formation and resorption, deletion of Y1 receptors in the absence of Y2 and Y4 receptor signalling attenuates both the synergistic increase in bone formation, and the greater bone resorption of the $Y2^{-/-}Y4^{-/-}$ model.

The mechanisms for the observed alterations in bone formation and resorption are not clear. However several recent studies have however demonstrated the ability of several neural factors to also modulate bone cell activity. Like NPY, these factors are also involved in the regulation of energy homeostasis, suggesting they may be in part responsible for some of the changes observed in bone cell activity in the Y receptor knockout models.

3.4.4 Possible neural mechanisms for Y receptor specificity in the control of bone

A role for cocaine- and amphetamine-regulated transcript (CART) as an inhibitor of osteoclast differentiation has been described, with increased bone resorption in $Cart^{-/-}$ mice (Elefteriou et al., 2005). CART acts as both a neurotransmitter in the nervous system and as a neuro-hormone in peripheral organs, but it is not yet known exactly how it regulates bone resorption. Serum levels of CART were not measured in the mice used in this study, although expression of CART mRNA was significantly reduced in the hypothalamus of $Y1^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice compared with wild type in a different study (Karl et al., 2004; Sainsbury et al., 2003), indicating a possible role for CART in the greater Oc.S of these models. However, this seems unlikely as CART expression was similarly reduced in $Y2^{-/-}$ mice, in which Oc.S was unaltered (Sainsbury et al., 2003). $Y1^{-/-}$, $Y2^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice also have reduced hypothalamic levels of pro-opiomelanocortin (POMC) relative to wild type (Sainsbury et al., 2006; Sainsbury et al., 2002a), and would therefore be expected to have reduced levels of α-melanocyte-stimulating hormone (α-MSH), a POMC-derived peptide. α-MSH has been shown to stimulate osteoclastogenesis in bone marrow cultures and reduce trabecular bone volume when administered to mice (Cornish et al., 2003). Mice

and humans lacking the α-MSH melanocortin-4 receptor have increased bone volume due to reduced parameters of bone resorption through a pathway which has recently been demonstrated to be mediated by CART (Ahn et al., 2006). Levels of POMC are not available for all the compound Y receptor knockout models, but while it is possible that decreased α-MSH levels in $Y1^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice could contribute to the greater bone resorption of these models, this is again unlikely due to the similar reduction in POMC levels observed in germline $Y2^{-/-}$ mice.

3.4.5 Conclusion

This study investigated the bone phenotype of single, double, and triple Y receptor knockout mice to assess possible interaction between these receptors in an attempt to elucidate specific roles for the different Y receptor subtypes in the control of bone remodelling. Part of the difficulty in determining the specific roles of individual Y receptor subtypes in the control of bone remodelling is due to their complex and sometimes synergistic interactions. Furthermore, deletion of individual Y receptors has been found to alter the ligand binding pattern of remaining Y receptors (Lin et al., 2005), demonstrating a degree of redundancy in this system.

Nevertheless, the findings of the present study indicate specific actions of individual Y receptors in the regulation of bone formation and bone resorption. Bone volume was greater in $Y1^{-/-}$ and $Y2^{-/-}$ but not in $Y4^{-/-}$ mice relative to wild type (Sainsbury et al., 2003). No further elevation in BV/TV was observed with deletion of the Y4 receptor in the absence of Y1 receptor signalling, contrasting with the synergistic elevation in trabecular bone volume of male $Y2^{-/-}Y4^{-/-}$ mice, and demonstrating gender and receptor-subtype specificity in the control of bone formation. The lack of an additive bone formation response in $Y1^{-/-}Y2^{-/-}$ mice relative to single Y1 or Y2 receptor deletion alone suggests interaction or a common pathway by which these two receptors regulate osteoblast activity. However, the possibility of distinct pathways perhaps secondary to changes in insulin levels has not yet been excluded.

The trabecular bone morphology and bone formation phenotype of the $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ triple knockout model supported the hypothesis that the synergistic elevation in bone volume in male $Y2^{-/-}Y4^{-/-}$ mice resulted from additive effects of the leptin- and Y2-mediated anabolic pathways, with leptin levels in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice restored to wild type levels. The degree of interaction between Y receptor signalling and the leptin antiosteogenic response is further addressed in the following chapter.

These findings also indicate specific actions of different Y receptors in the regulation of bone resorption. Although the precise contribution of the different receptor subtypes to the regulation of osteoclast activity is not entirely clear, there appears to be interaction between specific subtypes in their control of bone resorption with the greater resorption of the $Y1^{-/-}$ and the $Y2^{-/-}Y4^{-/-}$ models abolished in the absence of all three receptors. It remains possible that the regulation of bone resorption by the Y receptor subtypes is mediated by downstream alterations in levels of other neuropeptides and related hormones, with secondary effects on bone.

Two other Y receptors are known to exist in mouse, Y5 and y6, and it is therefore possible that these receptors could also be involved in the regulation of bone formation. The generation of a Y5 receptor knockout model has been reported by one group (Marsh et al., 1998), but the effect of this mutation on bone physiology is yet to be addressed. A targeting vector for the deletion of the Y5 receptor is also currently being developed by our laboratory; however, while it is possible that this receptor will also interact with other Y receptors in the regulation of bone remodelling, the generation of double Y1 and Y5 deletion will not be possible as these two genes are localised only 20kb apart on the same chromosome. Nevertheless, the generation of the remaining Y receptor knockout combinations will be necessary to assess the contribution of these Y receptor subtypes to the regulation of bone formation.

Chapter 4

Interaction between Y receptor subtypes and leptin in the regulation of bone formation and marrow adiposity

4.1 INTRODUCTION

An important regulatory role for the neuropeptide Y receptor system in the control of bone formation has been demonstrated, with deletion of Y1 or Y2 receptors, alone or in combination with each other, resulting in a significantly greater trabecular bone mass in the mouse distal femur (section 3.3.1) (Baldock et al., 2002). Specificity in the actions of the Y receptors in regulating bone formation has also been demonstrated. $Y4^{-/-}$ mice do not have a bone phenotype, and deletion of Y4 receptors in the absence of other Y receptors does not provide additional stimulation of bone formation, with the exception of a synergistic increase in bone volume in male $Y2^{-/-}Y4^{-/-}$ double knockout mice (section 3.3) (Sainsbury et al., 2003). However, the data suggest that the synergistic elevation in bone volume in these mice may involve additional stimulation of bone formation related to a reduction in serum leptin levels, as opposed to direct effects induced by Y4 receptor deficiency.

Interestingly, the regulation of osteoblast activity by both leptin and Y2 receptors has been experimentally demonstrated to involve alterations in hypothalamic activity. A role for central Y2 receptors in the Y2-mediated anabolic response was demonstrated by conditional deletion of hypothalamus-specific Y2 receptors, which produced a rapid and potent increase in bone formation and bone volume (Baldock et al., 2002). Similarly, central administration of leptin normalised bone mass of leptin deficient ob/ob mice and reduced bone volume in wild type mice, revealing that leptin too, regulates bone formation via a hypothalamic relay (Ducy et al., 2000).

Thus it has been demonstrated that both the Y2 and leptin antiosteogenic pathways involve mediation through the hypothalamus, and ablation of either central Y2 receptor or leptin signalling results in a significant elevation in the rate of bone formation, and a comparably elevated trabecular bone mass (Baldock et al., 2002; Ducy et al., 2000). Similarly, deletion of Y1 receptor signalling has now also been shown to result in increased mineral apposition, and an equivalent increase in trabecular bone volume compared with the Y2 receptor- and leptin-deficient models (Figure 3.2).

It is well established that leptin and NPY interact in the regulation of energy homeostasis with evidence that NPY acts as a neuromodulator of leptin's actions to regulate food intake, energy expenditure and neuroendocrine signalling (section 1.8.2) (Ahima et al., 1996; Erickson et al., 1996b; Schwartz et al., 1996a; Schwartz et al., 1996b; Stephens et al., 1995). Recent studies using Y receptor knockout mice have begun to reveal roles for specific Y receptor subtypes in particular aspects of this process. For example, Y1 receptor deletion in ob/ob mice caused a reduction in food intake, body weight and white adipose tissue mass (Figure 4.1) (Pralong et al., 2002), implicating a role for Y1 receptor signalling in the hyperphagic and adipose response to leptin deficiency. On the other hand, deletion of Y2 receptor signalling has been shown to reduce body weight, adiposity, and the diabetic syndrome of ob/ob mice, without altering food intake (Naveilhan et al., 2002; Sainsbury et al., 2002b), demonstrating specificity in the actions of the different Y receptors. In addition, Y1 and Y4 receptors have also been implicated in the control of the gonadotropic axis by leptin, with deletion of Y4 receptors rescuing the fertility of ob/ob mice (Sainsbury et al., 2002c), and an enhanced efficacy of leptin to accelerate puberty reported in the absence of Y1 receptor signalling (Pralong et al., 2002).

The similarity in the trabecular bone mass and bone formation phenotypes of the $Y1^{-/-}$, $Y2^{-/-}$ and leptin-deficient models, and the known interaction of these pathways in the regulation of energy homeostasis and neuroendocrine function suggests a common mechanism may also exist in their control of bone formation. As leptin and Y2 receptors are co-expressed on neurons within the arcuate nucleus of the hypothalamus (Baskin et al., 1999; Broberger et al., 1997), the proposal that the leptin and NPY receptor systems may interact in the regulation of multiple functions including the regulation of bone formation, is not unreasonable. Furthermore, as levels of NPY are significantly elevated in both ob/ob and $Y2^{-/-}$ mice (Sainsbury et al., 2003; Sainsbury et al., 2002b; Wilding et al., 1993), it is possible that increased NPY signalling could be the common mechanism by which ablation of Y2 receptor or leptin signalling increases bone formation. However, continuous intracerebroventricular (icv) injection of NPY into wild type mice has been shown to actually decrease bone volume (Baldock et al., 2005; Ducy et al., 2000), albeit most likely also resulting in hyperleptinemia which would also be expected to reduce bone mass. These

studies, together with chemical lesion experiments in which ablation of NPY-synthesising neurons in the brain attenuated the ability of leptin to reduce body weight but not bone mass (section 1.10.7) (Takeda et al., 2002), has lead to the suggestion that leptin and NPY might actually use different pathways to control bone mass and body weight, thereby supporting a model in which the leptin and Y receptor antiosteogenic pathways are separate.

Thus while it at first seemed reasonable that the leptin and Y receptor mediated anabolic responses might share a common pathway to regulate bone formation, recent studies have suggested the regulation of osteoblast activity by the Y receptors and leptin may occur by distinct pathways. To further investigate whether Y receptor signalling plays a role in the anabolic response induced by leptin deficiency, $Y1^{-/-}$/ob, $Y2^{-/-}$/ob and $Y4^{-/-}$/ob double knockout mice were generated to determine the extent to which lack of specific Y receptor signalling could alter the leptin-deficient bone anabolic response, in order to assess the relatedness of these signalling pathways in their control of bone formation. As these pathways have been demonstrated previously to be involved in the regulation of peripheral adipose, adiposity within the bone marrow was also investigated to further characterise the role of these pathways on cellular activity within the bone microenvironment.

4.2 MATERIALS AND METHODS

4.2.1 Generation of germline $Y1^{-/-}$/ob, $Y2^{-/-}$/ob, and $Y4^{-/-}$/ob double knockout mice

$Y2^{-/-}$/ob and $Y4^{-/-}$/ob double knockout mice were generated as previously described (Sainsbury et al., 2002b; Sainsbury et al., 2002c). $Y1^{-/-}$/ob mice were generated using the same methods. Male and female heterozygous (Ob/ob) mice on a mixed C57/BL6-129/SvJ background were crossed with $Y1^{-/-}$, $Y2^{-/-}$, or $Y4^{-/-}$ mice on the same mixed background. Double heterozygous $Y1^{+/-}$Ob/ob, $Y2^{+/-}$Ob/ob, and $Y4^{+/-}$Ob/ob mice were crossed again to obtain $Y1^{-/-}$/ob, $Y2^{-/-}$/ob or $Y4^{-/-}$/ob homozygous double knockout mice. All mice generated were maintained on a mixed C57/BL6-129/SvJ background.

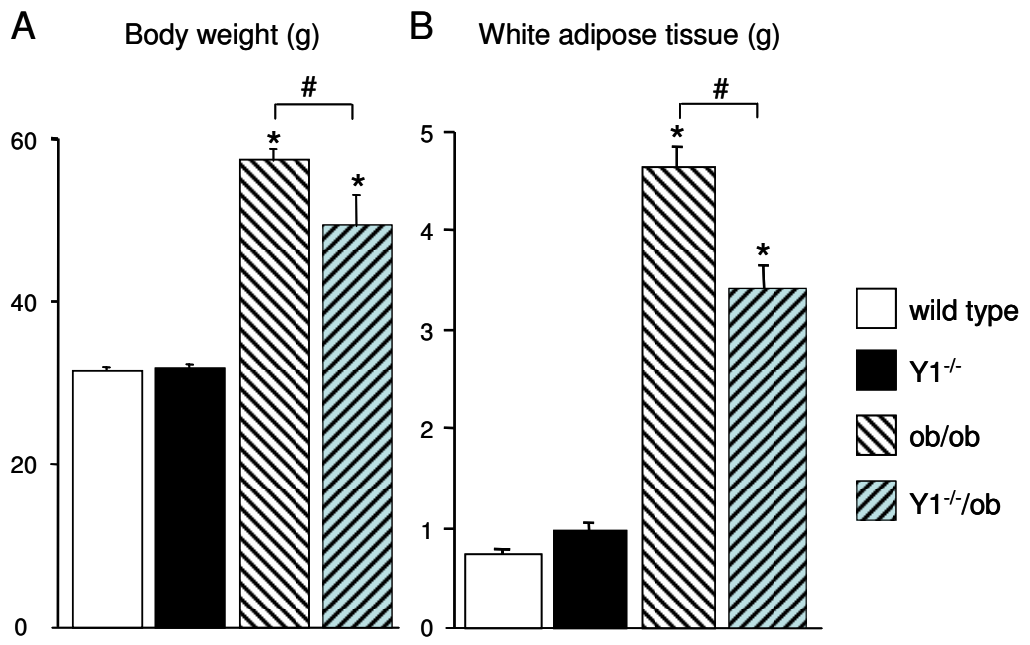

Figure 4.1 Effect of Y1 receptor deletion on body weight and white adipose tissue mass of ob/ob mice. Body weight (A), and white adipose tissue mass (B). * $P < 0.05$ versus wild type, # $P < 0.05$ as indicated. Unpublished data from A. Sainsbury.

4.2.2 Animals

Analyses were carried out in both male and female mice. Mice were injected with calcein and demeclocycline 10 days and 3 days prior to collection, respectively, as described in materials and methods (section 2.3.3). Mice were killed by cervical dislocation at 16 weeks of age. White adipose tissue mass was determined from the combined weights of specific depots (right inguinal, right epididymal or ovarian, right retroperitoneal, and mesenteric).

4.2.3 Histomorphometry

Following fixation, the right femur was bisected transversely at the midpoint of the shaft, and the distal half embedded, undecalcified, in MMA as described in materials and methods (section 2.3.5). 5µm sagittal sections were analysed as described in materials and methods (section 2.3.6). BV/TV, Tb.N, and Tb.Th were calculated (section 2.3.9.1) from

von Kossa stained sections (section 2.3.7.1). Oc.S was estimated (section 2.3.8.3) from TRAP stained sections (section 2.3.7.2). MAR was estimated from unstained sections (section 2.3.8.2). Marrow adipocyte number, average adipocyte size and calculations of AdV/TV were made from haematoxylin and eosin stained sections (section 2.3.8.4).

4.2.4 Statistical analyses

Statistical analyses were performed as described in materials and methods (section 2.7.7). Although the same wild type and ob/ob mice were used in sections 4.3.1 and 4.3.4 in comparison to $Y1^{-/-}$, in sections 4.3.2 and 4.3.5 in comparison to $Y2^{-/-}$, and in sections 4.3.3 and 4.3.6 in comparison to $Y4^{-/-}$, mice were grouped for ANOVA and post hoc analyses as indicated by the graphs for each section.

4.3 RESULTS

4.3.1 Role of Y1 receptor signalling in the regulation of marrow adiposity by leptin

Haematoxylin and eosin staining was used to identify circular holes within the marrow cavity which were occupied by adipocytes prior to processing (section 2.3.7.3). Initial comparison of stained sections from ob/ob and wild type mice revealed a striking difference in the extent of marrow adiposity between the two genotypes, with a distinctly greater number of circular voids made by adipocytes present in marrow from ob/ob bones, which in contrast were nearly absent in bones from wild type mice (Figure 4.2).

These observations were confirmed by quantification of the number of circular voids within the marrow cavity, with a substantially greater number of adipocytes present within the marrow of both male and female ob/ob mice compared with wild type (Figure 4.3 A,D). Furthermore, the average size of these adipocytes was larger compared with those from wild type mice (Figure 4.3 B,E), together accounting for the significantly greater total adipocyte volume within the marrow space of the ob/ob model (Figure 4.3 C,F).

In contrast to ob/ob mice, the number of adipocytes present within the marrow cavity of germline $Y1^{-/-}$ mice was similar to wild type, regardless of gender (Figure 4.3 A,D). Marrow adipocyte size and total adipocyte volume in $Y1^{-/-}$ mice were also comparable to wild type levels (Figure 4.3 B,C,E,F).

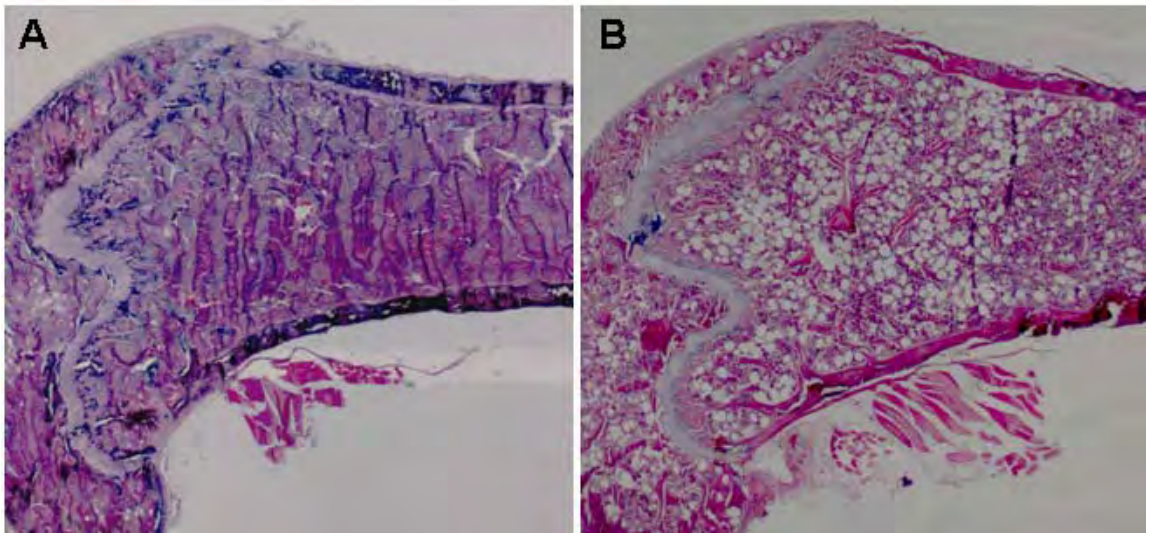

Figure 4.2 Haematoxylin and eosin stained distal femur sections.
Sections of distal femur from a wild type (A), and an ob/ob (B) mouse stained for haematoxylin and eosin.

Consistent with changes in body weight and white adipose tissue mass (Figure 4.1), marrow adipocyte number in $Y1^{-/-}$/ob double knockout mice was significantly reduced from ob/ob, by about 2-fold in male mice (Figure 4.3 A), and by nearly 3-fold in female mice (Figure 4.3 D). The reduction in adipocyte number in female $Y1^{-/-}$/ob double knockout mice was sufficient to produce a significant decrease in total marrow adipocyte volume (AdV.TV) compared with ob/ob, with adipocyte volume reduced to levels not significant from wild type (Figure 4.3 F). A similar but non-significant trend was also evident in male $Y1^{-/-}$/ob double knockout mice (P=0.07, Figure 4.3 C). Attenuation of Y1 receptor signalling had no effect on the average size of marrow adipocytes of ob/ob mice, regardless of gender.

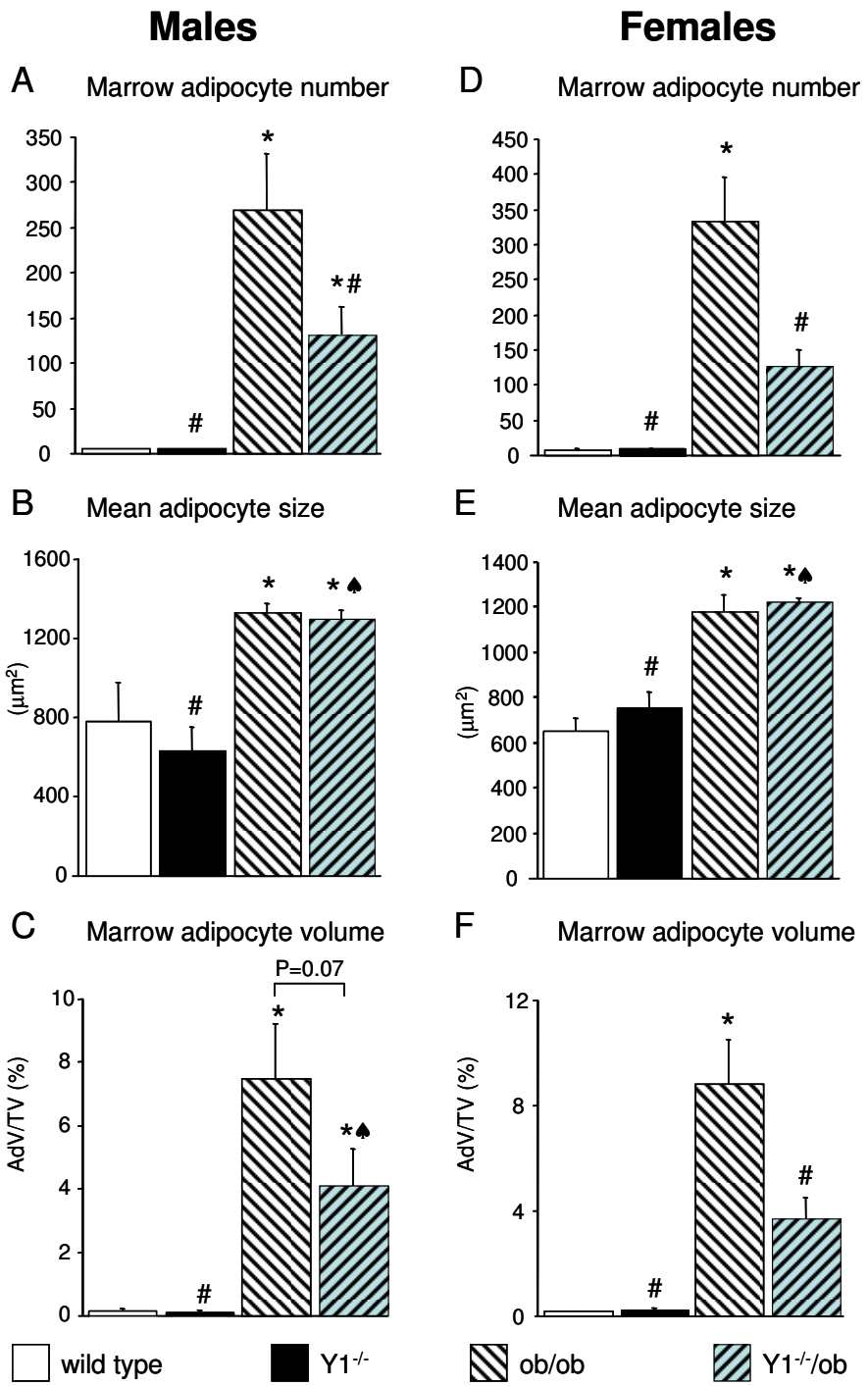

Figure 4.3 Effect of Y1 receptor deletion on marrow adiposity of ob/ob mice.
Male (A-C), and female (D-F) mice. Marrow adipocyte number (A,D), average adipocyte size (B,E), and marrow adipocyte volume (C,F). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y1^{-/-}$

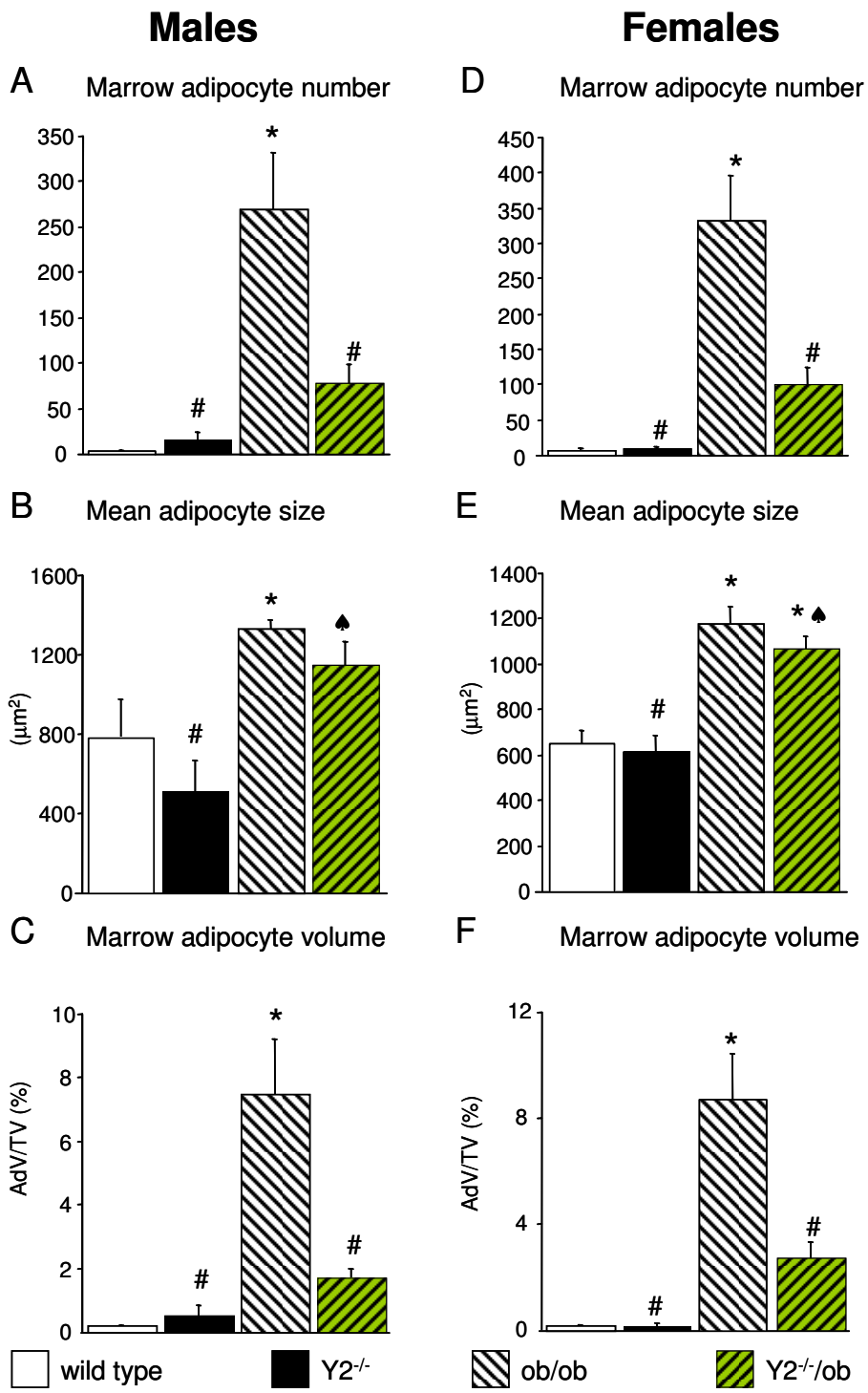

Figure 4.4 Effect of Y2 receptor deletion on marrow adiposity of ob/ob mice.
Male (A-C), and female (D-F) mice. Marrow adipocyte number (A,D), average adipocyte size (B,E), and marrow adipocyte volume (C,F). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y2^{-/-}$

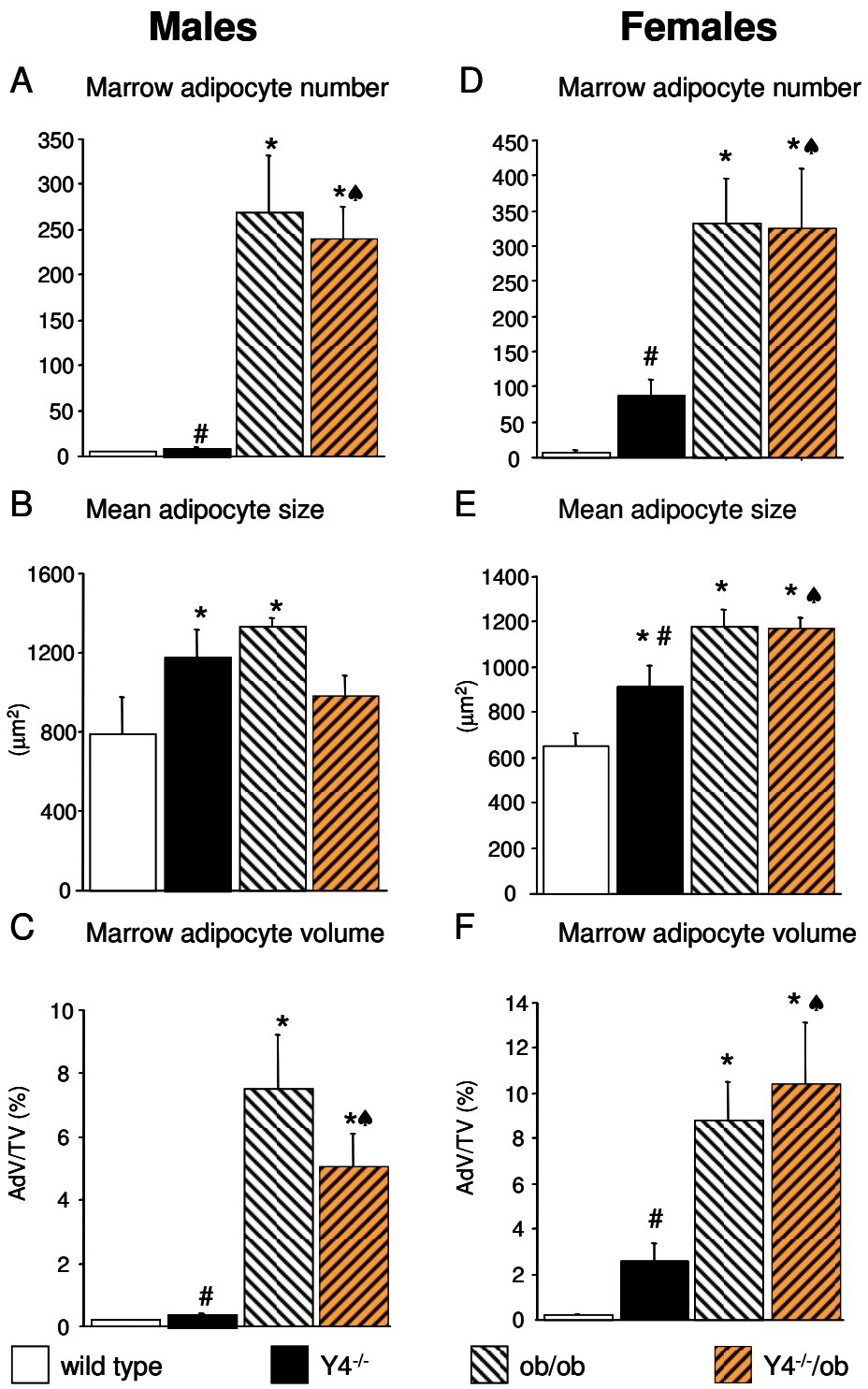

Figure 4.5 Effect of Y4 receptor deletion on marrow adiposity of ob/ob mice.
Male (A-C), and female (D-F) mice. Marrow adipocyte number (A,D), average adipocyte size (B,E), and marrow adipocyte volume (C,F). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y4^{-/-}$

4.3.2 Role of Y2 receptor signalling in the regulation of marrow adiposity by leptin

Similar to the effects of Y1 receptor deficiency, deletion of Y2 receptor signalling alone did not alter the number or size of marrow adipocytes from wild type mice of either gender (Figure 4.4 A,B,D,E). Again, similar to the effects of Y1 receptor deletion on the marrow adiposity of leptin deficient ob/ob mice, deletion of Y2 receptors in ob/ob mice significantly reduced the number of marrow adipocytes from ob/ob (Figure 4.4 A,D). This reduction in adipocyte number was greater than the effect seen in $Y1^{-/-}$/ob mice, with numbers reduced by over 3-fold in both male and female $Y2^{-/-}$/ob double knockout mice, to levels not significant from wild type in either gender (Figure 4.4 A,D).

Again, adipocyte size was not altered in the double knockouts from ob/ob measurements (Figure 4.4 B,E). However, the reduction in adipocyte number was sufficient to cause a marked reduction in AdV/TV from ob/ob, with adipocyte volume of both male and female $Y2^{-/-}$/ob mice not significant from wild type (Figure 4.4 C,F).

4.3.3 Role of Y4 receptor signalling in the regulation of marrow adiposity by leptin

In contrast to the effects of Y1 or Y2 receptor deletion on marrow adiposity, while the number of marrow adipocytes in $Y4^{-/-}$ mice was equivalent to wild type (Figure 4.5 A,D), the average size of the adipocytes in $Y4^{-/-}$ bone marrow were significantly larger than those in wild type marrow (Figure 4.5 B,E). In male $Y4^{-/-}$ mice, average adipocyte size was not significantly different from the size of adipocytes in ob/ob mice (Figure 4.5 B).

Furthermore, unlike the ability of Y1 or Y2 receptor deletion to reduce the adiposity of ob/ob marrow, deletion of Y4 receptor signalling did not reduce the number of marrow adipocytes, with numbers equivalent to those of ob/ob mice (Figure 4.5 A,D). Adipocyte size was also unaffected in $Y4^{-/-}$/ob double knockout mice (Figure 4.5 B,E).

The lack of change in the number or size of marrow adipocytes from ob/ob in $Y4^{-/-}$/ob double knockout mice, corresponded to a lack of change in Ad.V/TV between the two

genotypes, with adipocyte volume in $Y4^{-/-}$/ob double knockout mice remaining significantly greater than both wild type and $Y4^{-/-}$ levels (Figure 4.5 C,F).

4.3.4 Role of Y1 receptor signalling in the regulation of trabecular bone formation by leptin

Consistent with previous observations (Figure 3.2) (Ducy et al., 2000), BV/TV was significantly and similarly elevated in male and female $Y1^{-/-}$ and ob/ob mice compared with wild type (Figure 4.6 A,D), with greater Tb.Th in both models, regardless of gender (Figure 4.6 C,F). Tb.N was greater in $Y1^{-/-}$ mice and in female, but not male ob/ob mice compared with wild type (Figure 4.6 B,E).

BV/TV was significantly reduced in male $Y1^{-/-}$/ob double knockout mice compared with $Y1^{-/-}$, to levels not significant from wild type (Figure 4.6 A), with both Tb.N and Tb.Th reduced to wild type levels (Figure 4.6 B,C).

In contrast to the reduction in BV/TV and Tb.N observed in male $Y1^{-/-}$/ob mice, while it appeared that BV/TV and Tb.N in female $Y1^{-/-}$/ob mice were somewhat reduced compared with $Y1^{-/-}$, BV/TV remained significantly greater compared with wild type (Figure 4.6 D), despite a reduction in Tb.N to levels not significant to wild type (Figure 4.6 E). Thus the changes in trabecular morphology in female $Y1^{-/-}$/ob mice appeared similar to the pattern observed in males, although to a lesser extent.

Oc.S was significantly greater in male $Y1^{-/-}$ mice and in ob/ob mice of both genders compared with wild type, consistent with the previous characterisation of these models (Figure 3.2) (Ducy et al., 2000) (Figure 4.7 A,C). Oc.S was also significantly elevated in both male and female $Y1^{-/-}$/ob double knockout mice compared with wild type (Figure 4.7 A,C). However, Oc.S measurements in $Y1^{-/-}$/ob mice were comparable to $Y1^{-/-}$ and ob/ob levels, and would therefore not be expected to cause the reduction in BV/TV evident in male $Y1^{-/-}$/ob mice, although changes in resorption depth cannot be ruled out. In contrast, the greater MAR of the $Y1^{-/-}$ and ob/ob models, which is consistent with the greater

trabecular bone volume of these mice, was abolished in the double knockout model, with MAR in both male and female $Y1^{-/-}$/ob mice not significant from wild type (Figure 4.7 B,D), suggesting that the reduction in BV/TV seen in the $Y1^{-/-}$/ob double knockout mice is the result of a reduction in osteoblast activity.

4.3.5 Role of Y2 receptor signalling in the regulation of trabecular bone formation by leptin

Consistent with previous observations (Figure 3.1), BV/TV was significantly elevated in male and female $Y2^{-/-}$ mice compared with wild type (Figure 4.8 A,D), with greater Tb.Th in $Y2^{-/-}$ mice of both genders, and greater Tb.N in male $Y2^{-/-}$ mice (Figure 4.8 B,C,E,F).

BV/TV was significantly reduced in male $Y2^{-/-}$/ob double knockout mice compared with $Y2^{-/-}$, although levels remained significantly greater than wild type (Figure 4.8 A). However, the greater Tb.N of the $Y2^{-/-}$ model was reduced to wild type levels in $Y2^{-/-}$/ob double knockout mice (Figure 4.8 B).

In contrast to observations in male $Y2^{-/-}$/ob mice, BV/TV remained elevated in female $Y2^{-/-}$ /ob double knockout mice, similar to $Y2^{-/-}$ and ob/ob levels (Figure 4.8 D), with no significant effect on Tb.N or Tb.Th (Figure 4.8 E,F).

Interestingly, while osteoclast surface of both male and female $Y2^{-/-}$ mice was similar to wild type levels, Oc.S of $Y2^{-/-}$/ob double knockout mice was comparable to ob/ob levels, significantly greater than both wild type and $Y2^{-/-}$ (Figure 4.9 A,C). This significant elevation in Oc.S in $Y2^{-/-}$/ob mice compared with $Y2^{-/-}$, suggests increased bone resorption, which is consistent with the reduction in BV/TV observed in male $Y2^{-/-}$/ob mice. In contrast, MAR was similarly elevated in $Y2^{-/-}$, ob/ob and $Y2^{-/-}$/ob mice of both genders (Figure 4.9 B,D), demonstrating that deletion of Y2 receptor signalling did not alter the osteoblast activity of the ob/ob model, and therefore the reduction in bone volume in male $Y2^{-/-}$/ob mice is solely the response to altered osteoclastic activity.

4.3.6 Role of Y4 receptor signalling in the regulation of trabecular bone formation by leptin

As previously reported (Sainsbury et al., 2003), BV/TV, Tb.N, and Tb.Th in both male and female $Y4^{-/-}$ mice was similar to wild type (Figure 4.10). In contrast, BV/TV of $Y4^{-/-}$/ob double knockout mice was comparable to ob/ob levels, and significantly greater than both wild type and $Y4^{-/-}$ mice (Figure 4.10 A,C), with greater Tb.Th in both male and female $Y4^{-/-}$/ob mice, and greater Tb.N in male $Y4^{-/-}$/ob mice only relative to wild type levels (Figure 4.10 B,C,E,F).

Oc.S in male $Y4^{-/-}$/ob double knockout mice was also significantly greater than both wild type and $Y4^{-/-}$ mice, comparable with ob/ob levels (Figure 4.11 A). Similarly, while not significant from wild type, MAR in male $Y4^{-/-}$/ob mice approached ob/ob levels, with no significant difference between the two genotypes (Figure 4.11 B), suggesting that the similar trabecular bone phenotype of the $Y4^{-/-}$/ob and the ob/ob models was due to the similar bone cell activity of the two genotypes.

In contrast to the observations in male $Y4^{-/-}$/ob mice, Oc.S in female $Y4^{-/-}$/ob mice remained similar to wild type levels, with no apparent effect of leptin deficiency to increase bone resorption (Figure 4.11 C). Furthermore, there was no evidence for elevated MAR in female $Y4^{-/-}$/ob mice, with levels significantly reduced compared with ob/ob mice (Figure 4.11 D). Thus while leptin deficiency does not alter the bone cell activity of female $Y4^{-/-}$ mice, which remains similar to wild type levels, trabecular bone morphology in female $Y4^{-/-}$/ob mice appears similar to that of ob/ob mice, with elevated BV/TV and Tb.Th.

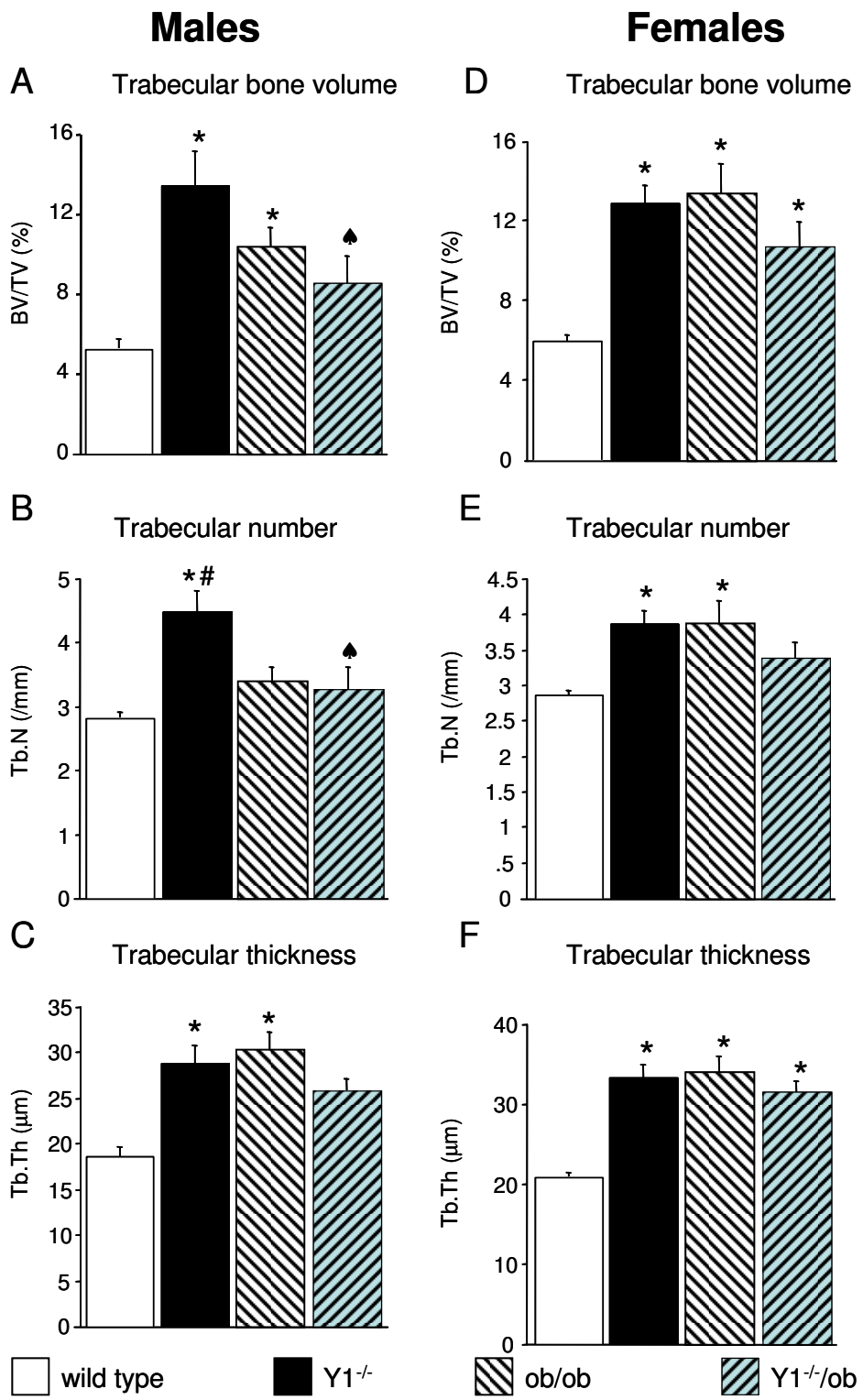

Figure 4.6 Effect of Y1 receptor deletion on trabecular bone morphology of ob/ob mice.
Male (A-C), and female (D-F) mice. Trabecular bone volume (A,D), trabecular number (B,E), and trabecular thickness (C,F). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y1^{-/-}$

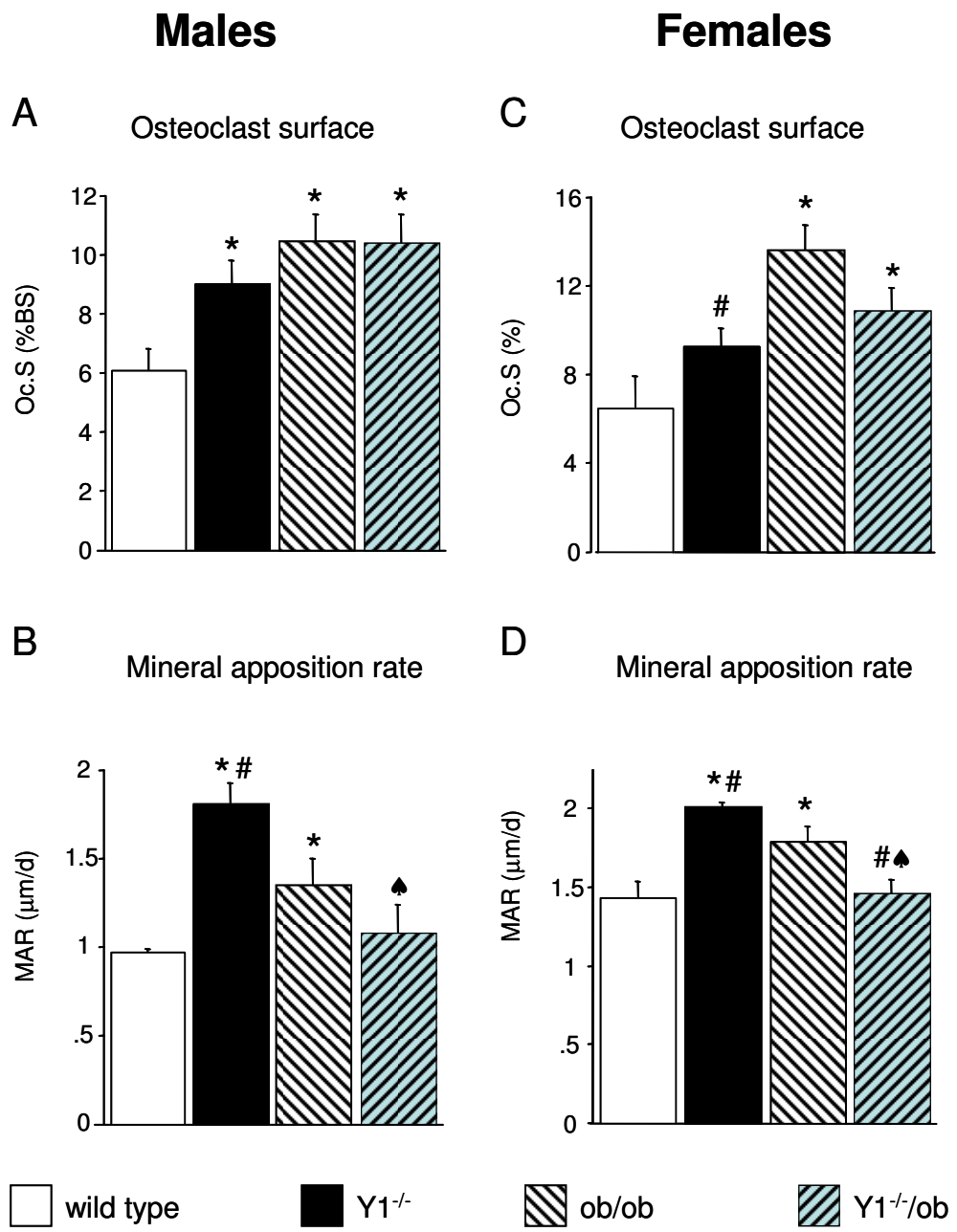

Figure 4.7 Effect of Y1 receptor deletion on parameters of bone resorption and formation of ob/ob mice. Male (A-B), female (C-D) mice. Osteoclast surface (A,C), and mineral apposition rate (B,D). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y1^{-/-}$

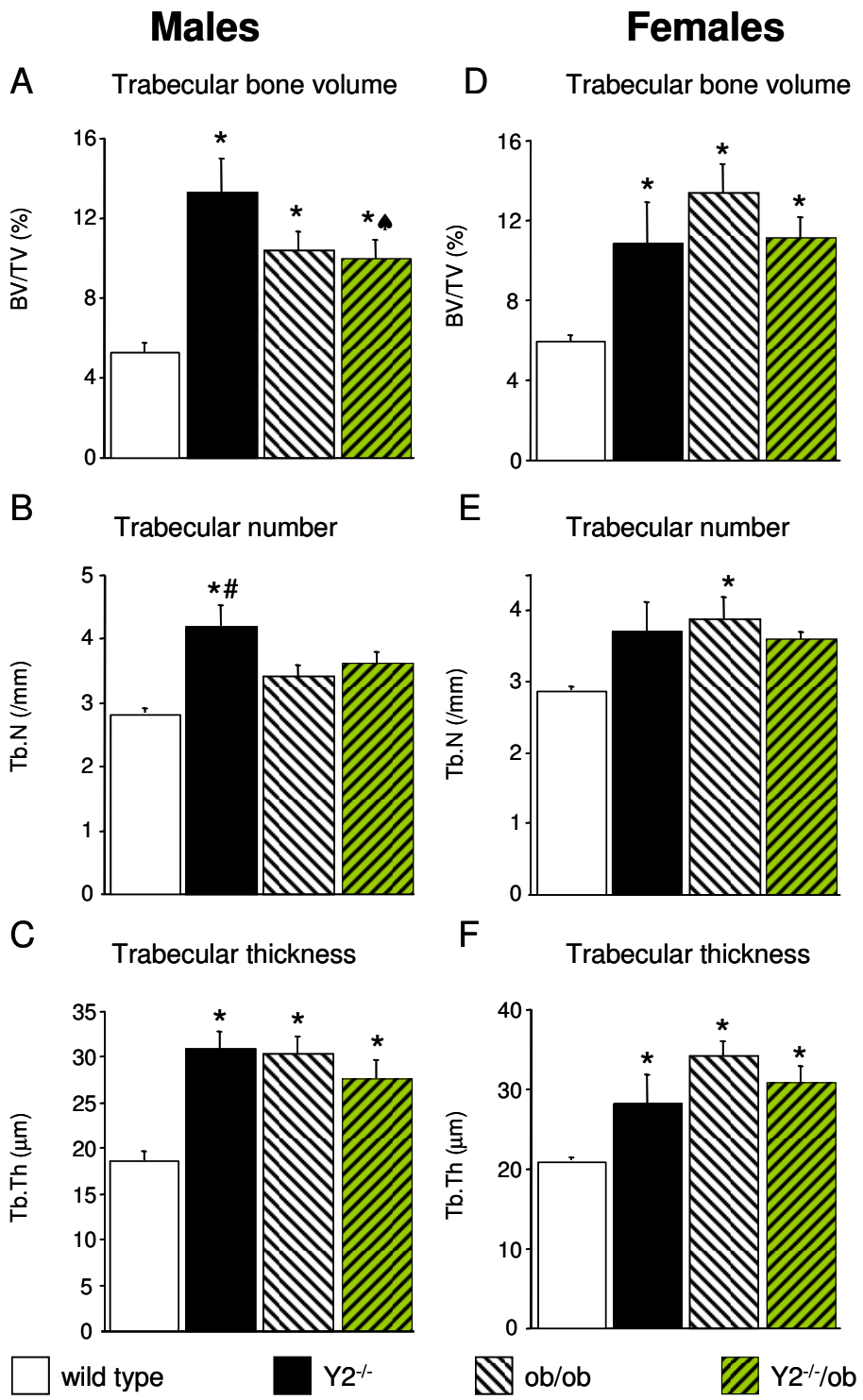

Figure 4.8 Effect of Y2 receptor deletion on trabecular bone morphology of ob/ob mice.
Male (A-C), and female (D-F) mice. Trabecular bone volume (A,D), trabecular number (B,E), and trabecular thickness (C,F). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y2^{-/-}$

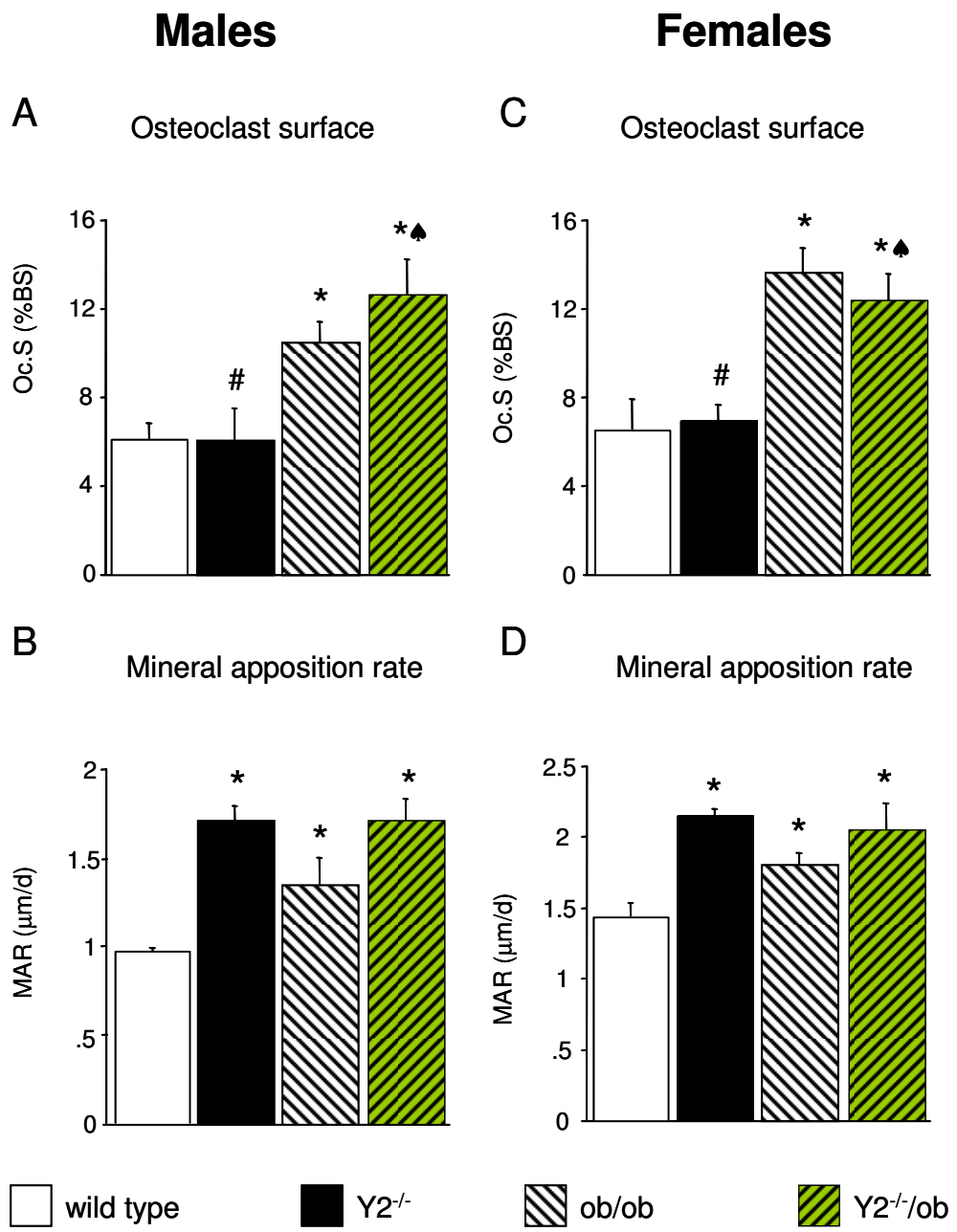

Figure 4.9 Effect of Y2 receptor deletion on parameters of bone resorption and formation of ob/ob mice. Male (A-B), female (C-D) mice. Osteoclast surface (A,C), and mineral apposition rate (B,D). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y2^{-/-}$

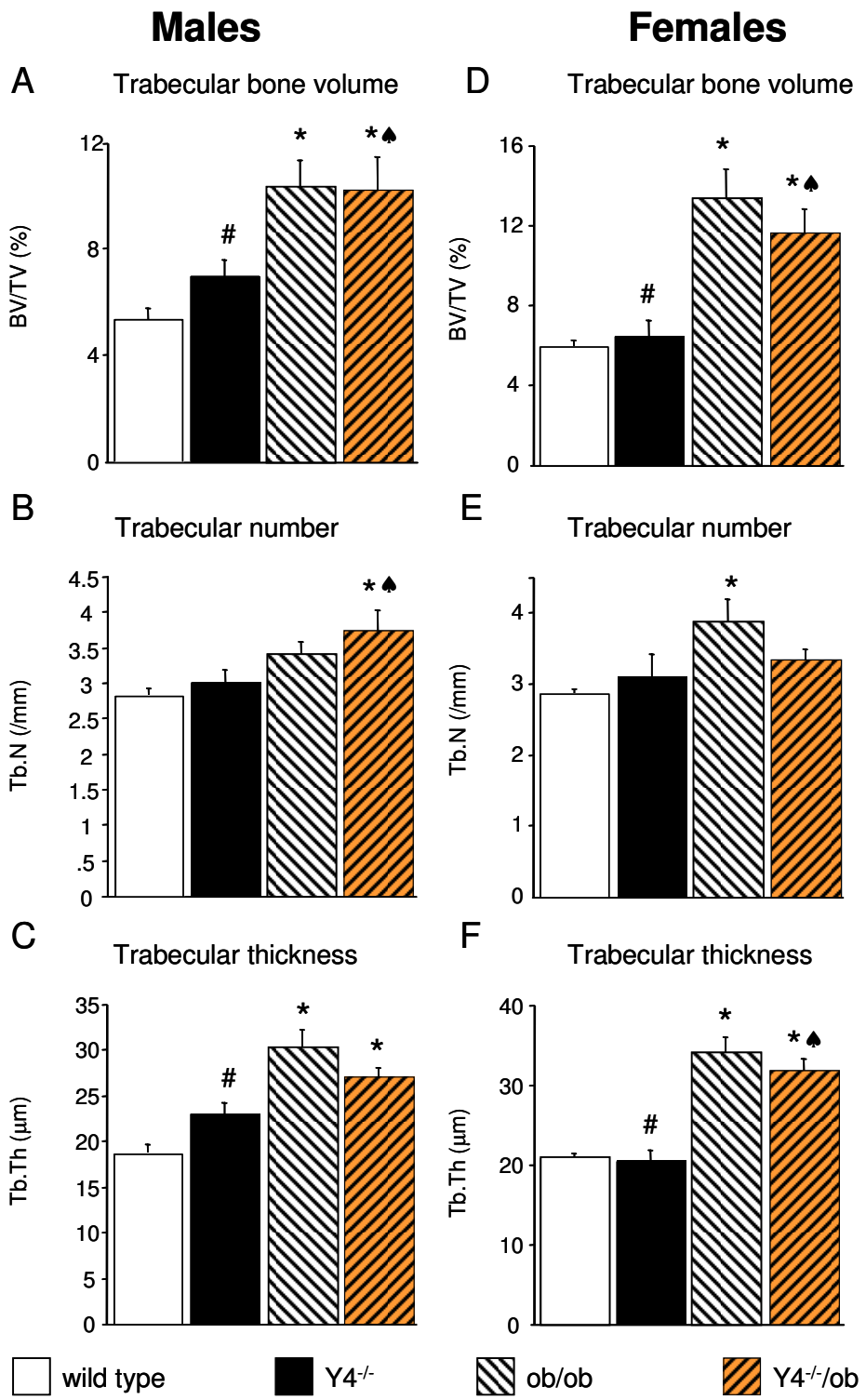

Figure 4.10 Effect of Y4 receptor deletion on trabecular bone morphology of ob/ob mice.
Male (A-C), and female (D-F) mice. Trabecular bone volume (A,D), trabecular number (B,E), and trabecular thickness (C,F). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y4^{-/-}$

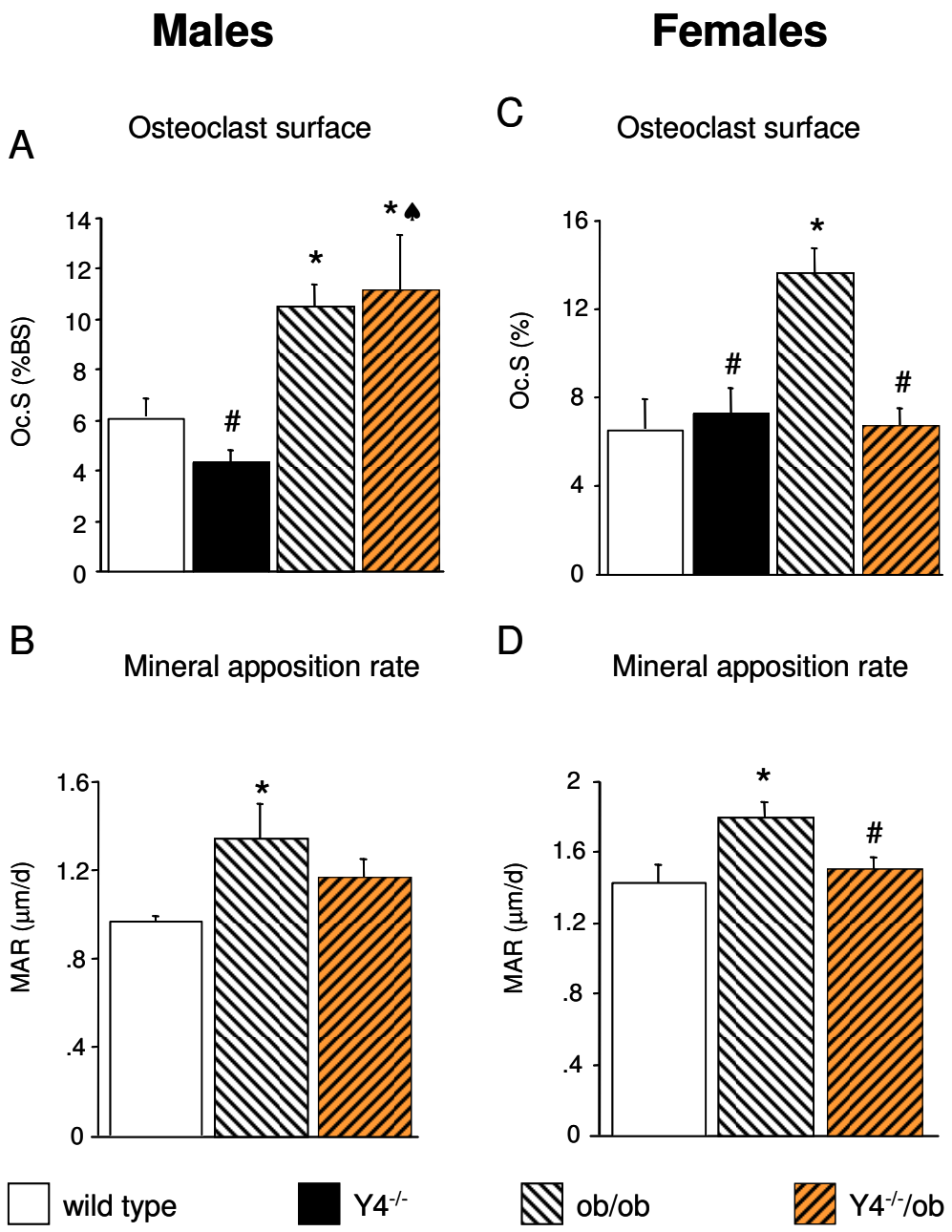

Figure 4.11 Effect of Y4 receptor deletion on parameters of bone resorption and formation of ob/ob mice. Male (A-B), female (C-D) mice. Osteoclast surface (A,C), and mineral apposition rate (B,D). *P < 0.05 versus wild type, #P < 0.05 versus ob/ob, ♠P < 0.05 versus $Y4^{-/-}$

4.4 DISCUSSION

4.4.1 Evidence for interaction between leptin and Y receptor pathways in the regulation of marrow adiposity

A number of studies have revealed roles for specific Y receptor subtypes in the regulation of the leptin deficient response in peripheral adipose and feeding (Naveilhan et al., 2002; Pralong et al., 2002; Sainsbury et al., 2002b). This study examined the role of Y receptor signalling in the leptin deficient response of adipose within the bone microenvironment, revealing for the first time the ability of specific Y receptor signalling to modify the adipose response to leptin deficiency within bone tissue. Deletion of Y1 or Y2 receptor signalling in leptin deficient ob/ob mice markedly reduced marrow adipocyte number and total adipocyte volume. This reduction was dramatic in both $Y1^{-/-}$/ob and $Y2^{-/-}$/ob models, with both adipocyte number and volume reduced to wild type levels in male and female $Y2^{-/-}$/ob mice. Interestingly, deletion of neither Y1 nor Y2 receptors had any significant effect on the size of marrow adipocytes from ob/ob mice, which were significantly larger compared with wild type, $Y1^{-/-}$, and $Y2^{-/-}$ models.

The reduction in marrow adipocyte number in the absence of leptin and either Y1 or Y2 receptor signalling suggests significant interaction between these two Y receptors and leptin in the regulation of marrow adiposity. The observation that only adipocyte number was affected, and not adipocyte size, suggests that Y1 or Y2 receptor signalling may be necessary for the appropriate proliferation or differentiation of adipocytes in the absence of leptin, but may not be involved in adipocyte hypertrophy.

Importantly, marrow adipocyte number of ob/ob was unaffected by deletion of Y4 receptor signalling, indicating specificity between the different Y receptors in their ability to modify the adipose response to leptin deficiency. It is also noteworthy that unlike the $Y1^{-/-}$ and $Y2^{-/-}$ models, in which marrow adipocyte number and size were similar to wild type, while adipocyte number in $Y4^{-/-}$ mice was also similar to wild type, $Y4^{-/-}$ marrow adipocytes were significantly larger than wild type, and in males were comparable in size to adipocytes from

ob/ob mice, suggesting differential regulation of marrow adiposity between the Y receptors in the presence of normal serum leptin levels.

The role of adipocytes within bone marrow is not well understood. It is not known whether adipocytes within the marrow play a similar role to extramedullary adipose in whole body lipid metabolism, by serving as an additional reservoir for the storage of excess lipid (Hussain et al., 1989). It has been proposed that marrow adipocytes may serve a role specifically as an energy source for processes occurring exclusively within the bone microenvironment (Gimble et al., 1996). Some evidence suggests differences between marrow and extramedullary adipocytes both in terms of their morphology, and in their response to various adipogenic stimuli, for example insulin (Gimble et al., 1996; Laharrague et al., 1998; Maurin et al., 2002; Nuttall et al., 1998). Alternatively, it has been proposed that marrow adipocytes may play a primarily passive role, by occupying space no longer needed for haematopoiesis (Meunier et al., 1971). However, with the identification of several hormones which are secreted by adipose cells, it has also been suggested that marrow adipocytes may in fact play an important physiological role within the bone microenvironment by regulating the function of stromal or haematopoietic cell types within the marrow through the release of locally acting endocrine factors. For example, the adipokines leptin and adiponectin are able to directly regulate bone cell activity (section 1.7), while the release of important soluble and cell surface factors by adipocyte stromal cells has been proposed to support the formation of osteoclasts *in vitro* (Kelly et al., 1998).

In this study, marrow adiposity was reduced in $Y1^{-/-}$/ob and $Y2^{-/-}$/ob double knockout mice compared with ob/ob. Interestingly, these two genotypes also exhibited a reduction in total adiposity compared with ob/ob (Figure 4.1) (Naveilhan et al., 2002; Pralong et al., 2002; Sainsbury et al., 2002b), suggesting the decrease in adipocyte number in the double knockout mice could simply be a refection of total body fat mass, supporting the proposal that marrow adipocytes may simply act as an additional storage compartment for excess lipid. However, while body weight and white adipose tissue mass are reduced in $Y4^{-/-}$ mice compared with control (Sainsbury et al., 2002c), marrow adipocytes in $Y4^{-/-}$ mice were significantly larger compared with control, and were comparable to the size of adipocytes

from ob/ob mice, demonstrating that in this instance, marrow adiposity did not directly reflect peripheral adipose tissue.

Both adipocytes and osteoblasts are derived from a common mesenchymal progenitor (Beresford et al., 1992; Nuttall et al., 1998; Pittenger et al., 1999), and the transcriptional mechanisms regulating the commitment of this progenitor to differentiate down either lineage is of considerable interest. Increasing evidence also suggests a high degree of plasticity between osteoblasts and adipocytes (Nuttall et al., 1998; Park et al., 1999; Wolf et al., 2003), suggesting one reason for the loss of bone seen with age could be due to a preferential differentiation of the progenitor towards that of the adipocyte. In support of this, osteoporotic bone loss is associated with an increase in marrow adipocyte content (Verma et al., 2002). Similarly, an increase in marrow adiposity is observed following ovariectomy (Wronski et al., 1986), with glucocorticoid treatment (Kawai et al., 1985; Wang et al., 1977), and with increasing age (Burkhardt et al., 1987; Meunier et al., 1971; Rozman et al., 1989), all conditions in which bone mass is concurrently decreased, suggesting a reciprocal relationship between bone and marrow fat. Interestingly however, ob/ob mice have a high trabecular bone mass phenotype concurrent with significant marrow adiposity. Furthermore, both bone volume and marrow adipose content were reduced in $Y1^{-/-}$/ob and $Y2^{-/-}$/ob mice, suggesting that, at least in the absence of leptin signalling, the formation of bone or fat does not always occur at the expense of the other. Whether this observation is exclusive to a situation in which leptin signalling is absent is yet to be determined.

As mentioned above, it has also been proposed that marrow adipocytes may function in an endocrine or paracrine manner to regulate the development and function of surrounding cell types. Human adipocytes including those within bone marrow express the enzyme aromatase, which converts circulating androgens into estrone or estradiol (Frisch et al., 1980), and it has been proposed that marrow adipocytes may act as a local source of estrogen following menopause in women (Gimble et al., 1996). Leptin deficient mice are hypogonadal; therefore it is possible that the substantial marrow adiposity seen in ob/ob mice might arise from a need for estrogen in order to maintain normal bone remodelling.

$Y1^{-/-}$/ob and $Y2^{-/-}$/ob mice are also hypogonadal and a need for estrogen equivalent to the ob/ob model would therefore be expected. However, marrow adiposity was actually reduced in these double knockout mice, which does not fit with the proposed role of marrow adipose cells to fulfil a need as a local source of estrogen production in these mice.

Bone marrow adipocytes also secrete other factors, for example leptin (Laharrague et al., 1998), which has been demonstrated to regulate bone cell activity *in vitro* (Cornish et al., 2002; Holloway et al., 2002; Reseland et al., 2001; Thomas et al., 1999), and *in vivo* (Burguera et al., 2001; Cornish et al., 2002; Ducy et al., 2000; Hamrick et al., 2005; Steppan et al., 2000). *In vitro* studies have demonstrated that leptin promotes osteogenesis and inhibits adipocyte differentiation in a human marrow stromal cell line (Thomas et al., 1999), and has stimulatory effects on proliferation, differentiation, and function in human and rat osteoblast cultures (Cornish et al., 2002; Gordeladze et al., 2002). Therefore in leptin deficient mice, the absence of leptin signalling might be expected to result in preferential adipogenesis over osteogenesis. While these findings are in line with our observations of marrow adiposity in leptin deficient ob/ob mice, the effects of leptin on osteogenesis *in vitro* are contradictory with our *in vivo* observations. There is increasing evidence that leptin can regulate bone metabolism by both locally and centrally mediated mechanisms as is discussed in chapter 1 (section 1.9). Therefore, it is important to appreciate that ob/ob and ob/ob-crossed mice lack production of leptin from both extramedullary and marrow adipose tissue, with effects on cellular activity within the bone microenvironment resulting not only from lack of leptin signalling via the hypothalamus, but also from a lack of direct effects, with leptin receptors or binding-sites reported for immortalised human stromal cells (Thomas et al., 1999), chondrocytes (Cornish et al., 2002; Maor et al., 2002; Steppan et al., 2000), and osteoblasts (Cornish et al., 2002; Enjuanes et al., 2002; Lee et al., 2002; Reseland et al., 2001; Steppan et al., 2000).

4.4.2 Interaction between leptin and Y receptor pathways in the regulation of bone formation

Deletion of Y1, Y2, or leptin signalling results in a similar elevation in osteoblast activity and trabecular bone volume (section 3.3.4) (Baldock et al., 2005; Baldock et al., 2002; Ducy et al., 2000). The data in this chapter demonstrate these pathways to interact in the regulation of adipose within the bone microenvironment, while the previous chapter demonstrated a lack of synergy between the Y1- and Y2-mediated anabolic pathways, suggesting interaction or a common feedback loop in their mechanism of action. This study investigated the relationship between leptin and the Y-receptor pathways in the regulation of bone formation. The effect of leptin deficiency on the control of bone formation by specific Y receptors was assessed by crossing $Y1^{-/-}$, $Y2^{-/-}$, and $Y4^{-/-}$ mice with leptin deficient ob/ob mice, followed by analysis of their trabecular bone phenotype.

Interestingly, trabecular bone volumes of male $Y1^{-/-}$/ob and $Y2^{-/-}$/ob mice were significantly reduced compared with $Y1^{-/-}$ or $Y2^{-/-}$, respectively, however, investigation of cellular activity revealed differences in the cellular mechanisms behind the decrease in bone volume between the two models. Germline $Y1^{-/-}$ mice and ob/ob mice have elevated parameters of bone resorption (Figure 3.2) (Ducy et al., 2000). Osteoclast surface was also elevated in $Y1^{-/-}$/ob double knockout mice compared with wild type, but was not elevated above levels of $Y1^{-/-}$ or ob/ob mice, suggesting the reduction in bone volume in this model was not the result of altered osteoclastic activity. However, the greater mineral apposition rate of the $Y1^{-/-}$ and ob/ob models was abolished in $Y1^{-/-}$/ob double knockout mice, suggesting that the reduced trabecular bone volume seen in $Y1^{-/-}$/ob mice was due to an attenuation of the bone formation activities of the $Y1^{-/-}$ and ob/ob models. In female $Y1^{-/-}$ /ob mice, while the changes in bone cell activity were equivalent to those observed in male mice, the extent of the changes in trabecular morphology were less marked, suggesting reduced sensitivity of these female mice to cellular changes in bone remodelling.

In contrast to $Y1^{-/-}$/ob mice in which trabecular bone volume was reduced from $Y1^{-/-}$ due to reduced bone formation, the rate of mineral apposition in $Y2^{-/-}$/ob mice was significantly

elevated compared with wild type, and comparable to both Y2$^{-/-}$ and ob/ob levels. Rather, the reduction in bone volume in Y2$^{-/-}$/ob double knockout mice was consistent with greater bone resorption in these mice compared with Y2$^{-/-}$ levels. This was observed in the measurements of osteoclast surface, which in Y2$^{-/-}$ mice were equivalent to wild type, but which were significantly elevated in Y2$^{-/-}$/ob mice to levels similar to ob/ob. Interestingly, in female Y2$^{-/-}$/ob mice, a similar pattern of bone cell activity was observed in the absence of any significant decrease in trabecular bone volume. Again, similar to the reduced extent of trabecular bone changes observed in female Y1$^{-/-}$/ob mice, this could relate to a reduced sensitivity of these female mice to cellular changes in bone remodelling, or a slower response to remodelling signals.

Male Y4$^{-/-}$/ob mice were found to have a trabecular bone phenotype markedly similar to ob/ob mice, with a similar elevation in trabecular bone volume, number and thickness, associated with similarly elevated osteoclast surface. Mineral apposition rate in Y4$^{-/-}$/ob mice was also not significantly different from ob/ob, together suggesting that deletion of Y4 receptor signalling does not in any way affect the leptin-deficient bone response in male mice. The trabecular bone phenotype of female Y4$^{-/-}$/ob mice was more unexpected. Similar to male Y4$^{-/-}$/ob mice, trabecular bone morphology in female Y4$^{-/-}$/ob mice was comparable to ob/ob, with elevated bone volume and trabecular thickness. Surprisingly however, the bone cell activity in female Y4$^{-/-}$/ob mice was not consistent with the changes observed in trabecular morphology, with both osteoclast surface and mineral apposition rate unchanged from wild type.

Together these results suggest specificity in interaction between Y receptor signalling and leptin in the regulation of bone formation. The reduction in bone volume in Y1$^{-/-}$/ob mice compared with Y1$^{-/-}$ was the result of reduced osteoblast activity, demonstrating that while bone formation is elevated in the absence of either Y1 or leptin signalling, in the absence of both Y1 and leptin, the increase in osteoblast activity is abolished. This suggests that the presence of leptin may be required for the anabolic activity of the Y1$^{-/-}$ model, and vice versa, the presence of an intact Y1 receptor is required for the anabolic activity of the ob/ob model. Thus, in the absence of both Y1 and leptin, the anabolic pathway remains inactive.

This response appears to be non-gender specific as similar cellular changes were evident in both male and female $Y1^{-/-}$/ob mice, however, the resultant extent of change in trabecular bone morphology in female mice appeared to be reduced or delayed compared with male mice.

In contrast to the $Y1^{-/-}$/ob model, the reduction in bone volume in $Y2^{-/-}$/ob mice compared with $Y2^{-/-}$ was the result of elevated bone resorption, evident in the increased osteoclast surface in $Y2^{-/-}$/ob compared with $Y2^{-/-}$ mice. This elevation in bone resorption is consistent with the hypogonadal status of the ob/ob and the $Y2^{-/-}$/ob models (Sainsbury et al., 2002b). The elevated bone resorption of the ob/ob model has also been attributed to reduced hypothalamic levels of CART (Elefteriou et al., 2005), a neuropeptide with inhibitory actions on bone resorption. Levels of CART within the arcuate nucleus of $Y2^{-/-}$/ob mice are reduced to levels similar to ob/ob (Sainsbury et al., 2002b), in fitting with the similar resorptive response of these models. CART levels are also significantly reduced in $Y2^{-/-}$ mice, albeit to a lesser extent (Sainsbury et al., 2002b), however no change in Oc.S was apparent in this model, suggesting perhaps that moderate reductions in levels of this neuropeptide may not affect resorptive activity. The lack of any additive stimulation of osteoblast activity in the absence of both leptin and Y2 receptor signalling demonstrates that in the absence of leptin, the Y2- and leptin-mediated bone formation pathways cannot be distinguished, and may therefore share a common mechanistic pathway, or regulatory feedback loop.

In addition to the comparable elevation in osteoblast activity in $Y2^{-/-}$, ob/ob, and $Y2^{-/-}$/ob mice, a significant elevation in hypothalamic NPY expression is present in all three models (Sainsbury et al., 2003; Sainsbury et al., 2002b; Wilding et al., 1993). Leptin is inversely proportional to NPY expression, with central leptin acting to inhibit NPY production in hypothalamic neurons (Stephens et al., 1995). Y2 receptors also inhibit the production of NPY (King et al., 2000). Thus, in the absence of leptin or Y2 receptors, hypothalamic NPY is consequentially increased, leading to the proposal that elevated NPY could be a common mechanism by which osteoblast activity is elevated in the $Y2^{-/-}$, ob/ob, and $Y2^{-/-}$/ob models. This question was recently addressed by our group by injecting NPY-expressing

recombinant virus into the hypothalamus of wild type and $Y2^{-/-}$ mice to experimentally elevate hypothalamic NPY (Baldock et al., 2005). Consistent with the known effects of NPY on energy homeostasis, body weight and adiposity of NPY-recipient mice rapidly increased, resulting in a concurrent elevation in serum leptin (Baldock et al., 2005). The elevation in NPY and leptin resulted in a decrease in osteoblast activity in both wild type and $Y2^{-/-}$ mice, consistent with a central anti-osteogenic action of leptin on trabecular bone (Elefteriou et al., 2004). However importantly, osteoblast activity remained significantly greater in $Y2^{-/-}$ mice compared with wild type (Baldock et al., 2005). These findings together with the above data suggest that elevated NPY does not regulate the leptin anti-osteogenic pathway, as NPY levels were elevated in both ob/ob and NPY-recipient mice, and were not consistent with the observed changes in osteoblast activity. However, elevated NPY cannot yet be ruled out as a mechanism for the greater osteoblast activity in $Y2^{-/-}$ mice, as elevated NPY expression has been a consistent feature of all $Y2^{-/-}$ models studied to date. The lack of any additive effect on osteoblast activity in $Y2^{-/-}$/ob mice suggests either that a permissive level of leptin may be necessary for the activation of the Y2-mediated anabolic pathway, or alternatively, a shared mechanistic pathway or shared feedback mechanism between the Y2- and leptin-mediated control of bone formation. However, the finding that osteoblast activity remained stimulated in $Y2^{-/-}$ mice in the presence of elevated leptin and NPY, indicates that the Y2-mediated stimulation of osteoblast activity is independent of leptin (Baldock et al., 2005). Recent studies of cortical bone have also provided evidence of distinct actions of the leptin and Y2-mediated pathways, with ablation of Y2 receptor signalling activating osteoblast activity to increase cortical bone mass, while in contrast leptin deficiency reduced cortical bone mass and density (Baldock et al., 2006b), revealing opposing activities of the leptin and Y2 pathways in cortical bone, and supporting a model in which the actions of these two pathways are distinct. The synergistic elevation in bone volume observed in lean male $Y2^{-/-}Y4^{-/-}$ double knockout mice suggests that further increases in bone volume are possible and argues against a shared pathway in the regulation of bone formation by leptin and Y2 receptors (Sainsbury et al., 2003), therefore supporting a model in which a permissive level of leptin is required for the activation of the Y2-anabolic pathway. Thus, while the findings from this study cannot distinguish between the leptin and the Y2-mediated pathways in the absence

of leptin, evidence from our complementary studies suggest distinct actions of the two pathways at least in the presence of normal or increasing concentrations of leptin.

While deletion of either Y1 or Y2 receptor signalling results in a two-fold greater trabecular bone volume in the mouse distal femur, deletion of Y4 receptor signalling does not result in a bone phenotype. Deletion of Y4 receptors in leptin deficient ob/ob male mice did not significantly alter the trabecular bone phenotype from ob/ob; with a similar trabecular bone volume and morphology, and similar changes in parameters of bone resorption and formation, indicating that Y4 receptors do not play a role in the leptin-mediated regulation of bone remodelling. However, while female $Y4^{-/-}$/ob mice also had a similar trabecular bone volume and morphology to ob/ob mice, osteoclast surface and mineral apposition rate were not elevated to ob/ob levels, instead remaining equivalent to wild type. Levels of CART within the hypothalamus of male $Y4^{-/-}$/ob mice are similar to those of ob/ob mice (Sainsbury et al., 2002c), consistent with the similar Oc.S of these models. Hypothalamic levels of CART for female $Y4^{-/-}$/ob mice however, have not yet been measured and therefore cannot yet be excluded as a possible mechanism for the gender-specific differences in osteoclast surface of this model. The similar trabecular bone volume of the $Y4^{-/-}$/ob and ob/ob models suggest a necessity for bone formation to be elevated above wild type levels. It is possible that this elevation was transient in female $Y4^{-/-}$/ob mice, and was therefore not detected by histology at 16 weeks of age. Interestingly, previous studies have demonstrated that crossing $Y4^{-/-}$ mice onto an ob/ob background improves the fertility of sterile ob/ob mice, with enhanced levels of testosterone in males, restoration of testis and seminal vesicle weight, and a complete rescue of fertility with 100% of male $Y4^{-/-}$/ob mice able to produce offspring (Sainsbury et al., 2002c). In female mice however, fertility was only partially restored with only 50% of female $Y4^{-/-}$/ob mice able to produce offspring accompanied by partial restoration of estrous cycling (Sainsbury et al., 2002c). Expression of gonadotropin releasing hormone (GnRH) was also increased within the forebrain of $Y4^{-/-}$/ob mice supporting a role for Y4 receptors in the control of the gonadotropic axis by leptin. Sex hormones are known to play an important protective role in bone, with loss of sex hormones leading to increased bone turnover, with excess bone resorption by osteoclastic cells (Manolagas et al., 2002). As deletion of Y4 receptors rescues the fertility

of ob/ob mice, it might be expected that $Y4^{-/-}$/ob mice would not display the greater osteoclast surface characteristic of the ob/ob model. Interestingly, this appeared to be the case for female, but not male $Y4^{-/-}$/ob mice, which was surprising, as the improvement in fertility in female $Y4^{-/-}$/ob mice was only partial (Sainsbury et al., 2002c). Nevertheless, it is highly likely that the restoration of fertility has a secondary influence on bone cell activity in these mice, and therefore it is difficult to separate the effects of leptin deficiency on bone, and the secondary effects resulting from improved gonadotropic activity.

4.4.3 Summary

These studies have revealed distinct differences between the Y receptors in their interactions with leptin in the control of adiposity and bone formation within the distal femur. The Y1 and Y2 receptors appear to be required for the full marrow adipose phenotype resulting from leptin deficiency, with deletion of either Y1 or Y2 receptor signalling significantly reducing the marrow adiposity of ob/ob mice. In contrast, Y4 receptors do not appear to be involved in this response, with no effect of Y4 deletion on the leptin-deficient response.

Crossing $Y1^{-/-}$ or $Y2^{-/-}$ onto the ob/ob background significantly reduced trabecular bone volume from $Y1^{-/-}$ or $Y2^{-/-}$, respectively; however, the mechanism behind the decrease in bone volume differs between the two models. The reduction in bone volume in $Y1^{-/-}$/ob double knockout mice results from an attenuation of the anabolic activity of the $Y1^{-/-}$ model, suggesting the presence of leptin or intact Y1 receptor signalling is required for the anabolic activity of either pathway. In contrast, the reduction in bone volume in $Y2^{-/-}$/ob double knockout mice results from an elevation in osteoclastic activity, possibly secondary to the hypogonadism of the leptin deficient model. While in this instance the leptin and Y2 receptor-mediated increase in bone formation are not distinguishable, other studies by our group have revealed distinct actions of the leptin and Y2 pathways in the control of bone formation. The differences in bone cell activity between the $Y1^{-/-}$ and $Y2^{-/-}$ pathways in the absence of functional leptin also provide evidence that the control of bone formation by these two Y receptor pathways may be distinct from one another. This finding could not be

determined from the study in the previous chapter investigating the bone phenotype of $Y1^{-/-}$ $Y2^{-/-}$ double knockout mice in the presence of normal levels of circulating leptin. It will therefore be of interest to assess the bone phenotype of the $Y1^{-/-}Y2^{-/-}$ model in the absence of leptin signalling. These studies have also provided evidence that the Y4 receptor is not involved in the leptin-deficient response in bone; however, alterations in sex hormone levels in the $Y4^{-/-}$/ob double knockout mice render it difficult to determine the exact role of the Y4 receptor in the control of bone remodelling by leptin.

The proposal that elevated levels of hypothalamic NPY may contribute to the greater trabecular bone volume of the ob/ob and $Y2^{-/-}$ models was investigated further using viral overexpression of NPY within the hypothalamus. While a role for elevated NPY in the leptin response in bone was ruled out, a role for NPY in the Y2-mediated anabolic response cannot yet be excluded (Baldock et al., 2005). The role of elevated NPY in the control of bone formation has also been questioned in other studies, with the finding that NPY knockout mice did not have a skeletal phenotype (Elefteriou et al., 2003). However, these mice also lack an obvious phenotype in terms of food intake and body weight (Erickson et al., 1996a), despite the known influence of NPY on energy homeostasis (Stanley and Leibowitz, 1985). It is likely therefore either that compensatory mechanisms exist and are sufficient to replace the requirement for NPY, or that elevated, but not reduced central NPY expression may regulate bone remodelling. It is noteworthy however, that levels of NPY in $Y1^{-/-}$ mice are actually non-significantly reduced within the arcuate nucleus (Karl et al., 2004), providing evidence that elevated NPY is not the mechanism for the greater bone volume in these mice, and again suggesting differences between the Y1- and Y2-mediated increases in bone formation may exist.

Chapter 5

Role of Y receptors in gonadectomy-induced changes in adipose and bone

5.1 INTRODUCTION

The musculoskeletal disease osteoporosis is a significant cause of disability affecting millions worldwide, characterised by the deterioration of bone density and microarchitecture resulting in weakened bones and increased risk of fragility fracture. The incidence of osteoporotic fracture is greater in women compared with men of a similar age, due to a rapid loss of bone that occurs with loss of the protective effects of estrogen on bone following menopause (Horowitz, 1993; Orwoll and Klein, 1995). In men, the rate of bone loss is slower and is concurrent with a more progressive decline in sex steroid production (Kaufman and Vermeulen, 2005; Melton et al., 1998; Riggs et al., 2000). Progressive increases in levels of circulating sex hormone-binding globulin also contribute to an age-related decrease in levels of unbound sex steroids (Riggs et al., 2002).

Sex hormones play a pivotal role in the regulation of bone turnover (section 1.5). In the absence of sex hormones, bone loss is characterised by a marked increase in the rate of bone resorption, with a coupled increase in formation. However, formation is unable to match resorption, resulting in a net loss of bone. Both estrogen and testosterone inhibit bone resorption (Falahati-Nini et al., 2000; Leder et al., 2003; Weinstein and Manolagas, 2000) by inducing osteoclast apoptosis (Chen et al., 2005) and by suppressing the activity of mature osteoclasts through direct receptor interactions (Oursler et al., 1994). Estrogen can also inhibit osteoclast formation by suppressing the production of receptor activator of NF-κB ligand (RANKL) and by increasing the production of the soluble decoy receptor osteoprotegerin (OPG) (Eghbali-Fatourechi et al., 2003; Hofbauer et al., 1999; Kawano et al., 2003), and also through the regulation of various bone-acting cytokines (Girasole et al., 1992; Horowitz, 1993; Jilka et al., 1992; Kimble et al., 1996; Kimble et al., 1994; Kitazawa et al., 1994; Manolagas and Jilka, 1995; Pacifici et al., 1991). Therefore the suppression of osteoclast formation and function by sex steroids occurs through both direct and indirect mechanisms, and loss of their protective effects consequentially increases resorptive activity.

Sex steroids also play an important role in regulating bone formation (Falahati-Nini et al., 2000), acting to increase osteoblast lifespan by inhibiting osteoblast apoptosis (Kousteni et al., 2001). Some *in vitro* studies have also shown an effect of estrogen on osteoblast proliferation and differentiation (Cao et al., 2003b; Dang et al., 2002; Monroe et al., 2003; Robinson et al., 1997; Waters et al., 2001). Thus, sex steroids are important for maintaining the balance between bone formation and resorption and loss of sex hormones due to mutation, gonadal failure, or surgery results in a rapid loss of bone mass in both males and females.

A number of treatments are currently available for osteoporosis. Hormone replacement therapy (HRT) decreases fracture risk (Cauley et al., 2003; Cauley et al., 1995; Delmas, 2002; Torgerson and Bell-Syer, 2001a; Torgerson and Bell-Syer, 2001b), however, concerns of increased breast cancer risk has resulted in controversy over the benefits versus the risks of HRT use (Beral and Collaborators., 2003; Chlebowski et al., 2003; Rossouw et al., 2002; Warren and Halpert, 2004). Antiresorptives such as bisphosphonates and calcitonin have been successfully used to inhibit osteoclastic resorption following menopause (Chesnut et al., 2000; Delmas, 2002). However, decreased responsiveness to calcitonin with time and the low oral bioavailability of bisphosphonates together with complications and side effects associated with bisphosphonate use, has led to the suggestion that their use for the long-term treatment of osteoporosis should be limited (Conte and Guarneri, 2004; Farrugia et al., 2006; Gennari and Agnusdei, 1994; Ott, 2005).

Most importantly, while antiresorptive treatments are effective at reducing further deterioration of bone microarchitecture, they are not able to stimulate bone formation to replace the already lost bone, leaving osteoporotic patients with significantly weakened bones and at risk of further fragility fracture. Once an osteoporotic fracture has occurred, the risk to an individual of subsequent fracture is increased up to 10-fold (Black et al., 1999; Haentjens et al., 2003; Klotzbuecher et al., 2000; Robinson et al., 2002; van Staa et al., 2002). The most effective antiresorptive therapy available today can reduce this risk to 5-fold (Cummings et al., 2002). There is therefore an urgent need to develop anabolic therapies, which can reverse the loss of bone by stimulating osteoblastic bone formation.

In addition to a reduction in bone mass, the decline in estrogen levels following menopause is also associated with an increase in body fat mass, associated with altered plasma lipoprotein metabolism (Godsland et al., 2004), and contributing to an increased risk of cardiovascular and metabolic diseases including diabetes (Godsland et al., 2004; Poehlman et al., 1995). This postmenopausal increase in adipose tissue deposition has been associated with a corresponding increase in hypothalamic levels of the orexigenic peptide NPY (Ainslie et al., 2001; Shimizu et al., 1996), suggesting increased NPY signalling may contribute to the mechanism by which adiposity is increased in the absence of sex hormones in females.

As our previous studies have demonstrated a role for the neuropeptide Y1 and Y2 receptors in the control of bone formation, and as Y1 and Y2 receptors are known to play an important role in the regulation of body weight by NPY and leptin (Naveilhan et al., 2002; Pralong et al., 2002; Sainsbury et al., 2002b), it is possible that Y1 and Y2 receptors may play an important role in two of the major endocrine consequences of sex hormone deficiency; bone loss and adipose accumulation.

Here we have investigated the effects of Y1 and Y2 receptor deletion on bone cell function using surgical gonadectomy (GX) to induce sex-steroid deficient bone loss in both male and female mice, to determine whether germline $Y1^{-/-}$ or $Y2^{-/-}$ mice are resistant to the effects of gonadectomy on bone. Secondly, conditional deletion of hypothalamic Y2 receptors was performed following the occurrence of gonadectomy-induced bone loss to investigate whether activation of the central Y2-mediated anabolic response can repair losses in bone mass. As the NPY system is also likely to be involved in the regulation of adipose in the absence of sex hormones, the effects of Y receptor deletion on gonadectomy-induced changes in WAT and BAT were also assessed to determine whether Y1 or Y2 receptor deletion can inhibit changes in fat accumulation following sex steroid deficiency.

5.2 MATERIALS AND METHODS

5.2.1 Study design

This study was performed in two parts. Firstly, to determine whether pre-existing deletion of Y1 or Y2 receptors provides resistance to gonadectomy-induced bone loss, male and female germline $Y1^{-/-}$, $Y2^{-/-}$, and wild type mice were either gonadectomised or sham-operated at 8 weeks of age. Mice were collected 8 weeks later at 16 weeks of age and adiposity and trabecular bone mass were assessed (Figure 5.1 A).

Secondly, to investigate whether conditional deletion of hypothalamic Y2 receptors can reverse or repair bone once gonadectomy induced bone loss has already been established, male and female $Y2^{lox/lox}$ mice were also either gonadectomised or sham-operated at 8 weeks of age. Over the following 8 weeks bone loss was allowed to occur. At 16 weeks of age, gonadectomised mice received either a hypothalamic injection of recombinant adeno-associated virus (AAV) vector containing an empty cassette (AAV-empty), thereby not affecting expression of the Y2 gene, or the AAV vector containing the cre-recombinase gene (AAV-cre) to delete the Y2 receptor gene within this area of the hypothalamus. Sham-operated mice only received AAV-cre. Mice were left for a further 6 weeks to allow the effects of Y2 receptor ablation to develop, and were collected at 22 weeks of age (Figure 5.1 B).

5.2.2 Surgery

5.2.2.1 Anaesthesia for gonadectomy

Anaesthesia of 8 week old male and female $Y1^{-/-}$, $Y2^{-/-}$, $Y2^{lox/lox}$, or wild type mice was performed as described in materials and methods, using a methoxyfluorane top up as required (section 2.4.1). Completeness of anaesthesia was assessed by applying acute pressure with non-toothed forceps to the skin between the toes of the hind foot. Any retraction of the limb was taken as evidence of incomplete anaesthesia.

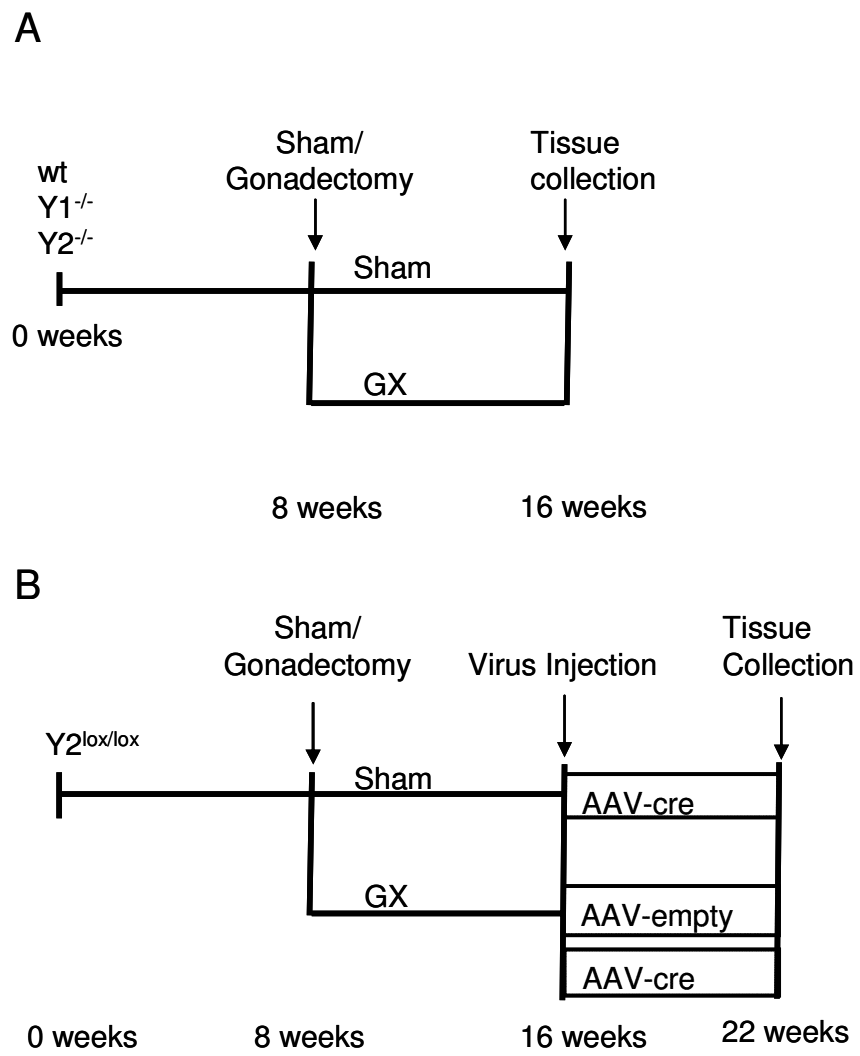

Figure 5.1 Experimental plan to determine effects of Y1 and Y2 receptor deficiency on gonadectomy-induced changes in adiposity and bone mass.
To determine the effect of pre-existing Y receptor deletion (A); wild type and germline $Y1^{-/-}$ or $Y2^{-/-}$ mice were gonadectomised at 8 weeks of age and collected at 16 weeks of age. To determine the response to activation of the Y2-mediated anabolic pathway following the onset of bone loss (B); $Y2^{lox/lox}$ mice were gonadectomised at 8 weeks of age. Conditional deletion of hypothalamic Y2 receptors was performed at 16 weeks, and mice were collected at 22 weeks of age.

5.2.2.2 Gonadectomy

Following anaesthesia, animals underwent ovariectomy, orchidectomy, or sham-operation as described in materials and methods (section 2.4.2). 0.01mg/kg of the analgesic buprenorphine was injected i.p. following the completion of the procedure.

5.2.2.3 Anaesthesia for adeno-associated virus injection

Anaesthesia of 16 week old $Y2^{lox/lox}$ mice for hypothalamic injection of adeno-associated viral vector containing either the cre-recombinase or empty-vector was performed as described in materials and methods (section 2.4.3). Completeness of anaesthesia was assessed by applying acute pressure with non-toothed forceps to the skin between the toes of the hind foot. Any retraction of the limb was taken as evidence of incomplete anaesthesia.

5.2.2.4 Adeno-associated virus injection

Deletion of hypothalamic Y2 receptors was performed in gonadectomised and sham-operated $Y2^{lox/lox}$ mice at 16 weeks of age as described in materials and methods (section 2.4.4). Control gonadectomised mice received a hypothalamic injection of recombinant AAV vector containing an empty cassette instead of the cre-recombinase.

5.2.2.5 Recovery following surgery

Mice recovered from gonadectomy and hypothalamic injection of AAV in clean cages placed half on heating pads as described in materials and methods (section 2.4.5). For the daily monitoring of food intake and body weight mice were housed individually. Mice in which food intake was not measured were group housed. Mice were injected with the fluorescent tetracycline compound calcein as described in materials and methods (section 2.3.3), 10 days and 3 days prior to collection. Germline $Y1^{-/-}$ and $Y2^{-/-}$ mice were killed by cervical dislocation at 16 weeks of age, while $Y2^{lox/lox}$ mice were collected 6 weeks following injection of either AAV-cre or AAV-empty, at 22 weeks of age.

5.2.2.6 Tissue Collection

Trunk blood was collected immediately following cervical dislocation. Completeness of orchidectomy or ovariectomy was confirmed at death in all ORX and OVX mice by the absence of testes or the absence of ovarian tissue and atrophied uterine horns, respectively.

White adipose tissue (WAT) depots (right inguinal, right retroperitoneal, and mesenteric) were collected and weighed. Weight of these tissues were summed and expressed as total WAT mass. Interscapular brown adipose tissue (BAT) was also excised and weighed. In addition to collecting femora, lumbar vertebrae were also collected for histomorphometric analysis.

5.2.3 Histomorphometry

Following fixation, the right femora was bisected transversely at the midpoint of the shaft, and the distal half embedded, undecalcified, in MMA as described in materials and methods (section 2.3.5). A lumbar vertebral body (L4) was identified by X-ray, and isolated for processing and analysis. 5µm sagittal sections were analysed as described in materials and methods (section 2.3.6). BV/TV, Tb.N, and Tb.Th were calculated (section 2.3.9.1) from von Kossa stained sections (section 2.3.7.1). Oc.S was estimated (section 2.3.8.3) from TRAP stained sections (section 2.3.7.2). MAR was estimated from unstained sections (section 2.3.8.2). MS and BFR were calculated as described in materials and methods (section 2.3.9.2).

5.2.4 Serum Biochemistry

Radioimmunoassay kits were used to determine serum concentrations of IGF-1 and corticosterone and were performed according to supplied protocols. Osteocalcin was measured using an ELISA kit specific for mouse osteocalcin, and was performed in supplied 96-well plates according to manufacturer's instructions.

5.2.5 Statistical analyses

Statistical analyses were performed as described in materials and methods (section 3.2.4). As the same wild type mice were used in sections 5.3.1 and 5.3.2 in comparison to $Y1^{-/-}$, and in sections 5.3.3 and 5.3.4 in comparison to $Y2^{-/-}$, mice were grouped for ANOVA and post hoc analyses as indicated by the graphs for each section.

5.3 RESULTS

5.3.1 Germline Y1 receptor deletion and gonadectomy-induced changes in body weight, adipose mass and biochemical parameters

In order to investigate whether deletion of Y1 receptors affects gonadectomy-induced changes in adipose tissue deposition, germline $Y1^{-/-}$ mice were assessed for changes in body weight and adiposity at 16 weeks of age, 8 weeks after gonadectomy.

In female mice, body weight increased following recovery from surgery in all groups, however body weight was increased to a greater extent in ovariectomised $Y1^{-/-}$ mice compared with all other groups (Figure 5.2 A). A similar trend was also noted for orchidectomised $Y1^{-/-}$ mice (Figure 5.2 B). At the study endpoint, body weight was significantly greater in both female and male $Y1^{-/-}$ mice compared with wild type, regardless of operation (Figure 5.3 A, 5.4 A). Body weight in ovariectomised wild type and $Y1^{-/-}$ mice was also elevated compared with sham-operated mice of the same genotype (Figure 5.3 A), associated with significant elevations in inguinal and retroperitoneal mass in wild type, and mesenteric mass in $Y1^{-/-}$ mice (Figure 5.3 B-D), and corresponding to a significant elevation in total WAT mass in ovariectomised wild type compared with sham-operated mice and a similar but non-significant trend in ovariectomised $Y1^{-/-}$ mice (Figure 5.3 E). Therefore, investigation of the specific adipose depots revealed differences between the genotypes in the response of specific fat depots to ovariectomy, with a 38% increase in mesenteric mass following ovariectomy of $Y1^{-/-}$ mice, which was not apparent in wild type, while in contrast, retroperitoneal mass was significantly increased in ovariectomised wild type but not $Y1^{-/-}$ mice (Figure 5.3). Thus while female $Y1^{-/-}$ mice had significantly greater fat mass compared with wild type in the sham-operated state, Y1 receptor deficiency did not alter the overall adipose response to ovariectomy with increased deposition of WAT in both genotypes in the absence of sex hormones. However, differences in the regional distribution of adipose mass in $Y1^{-/-}$ mice following ovariectomy, suggests differential responses of specific adipose depots to sex hormone deficiency.

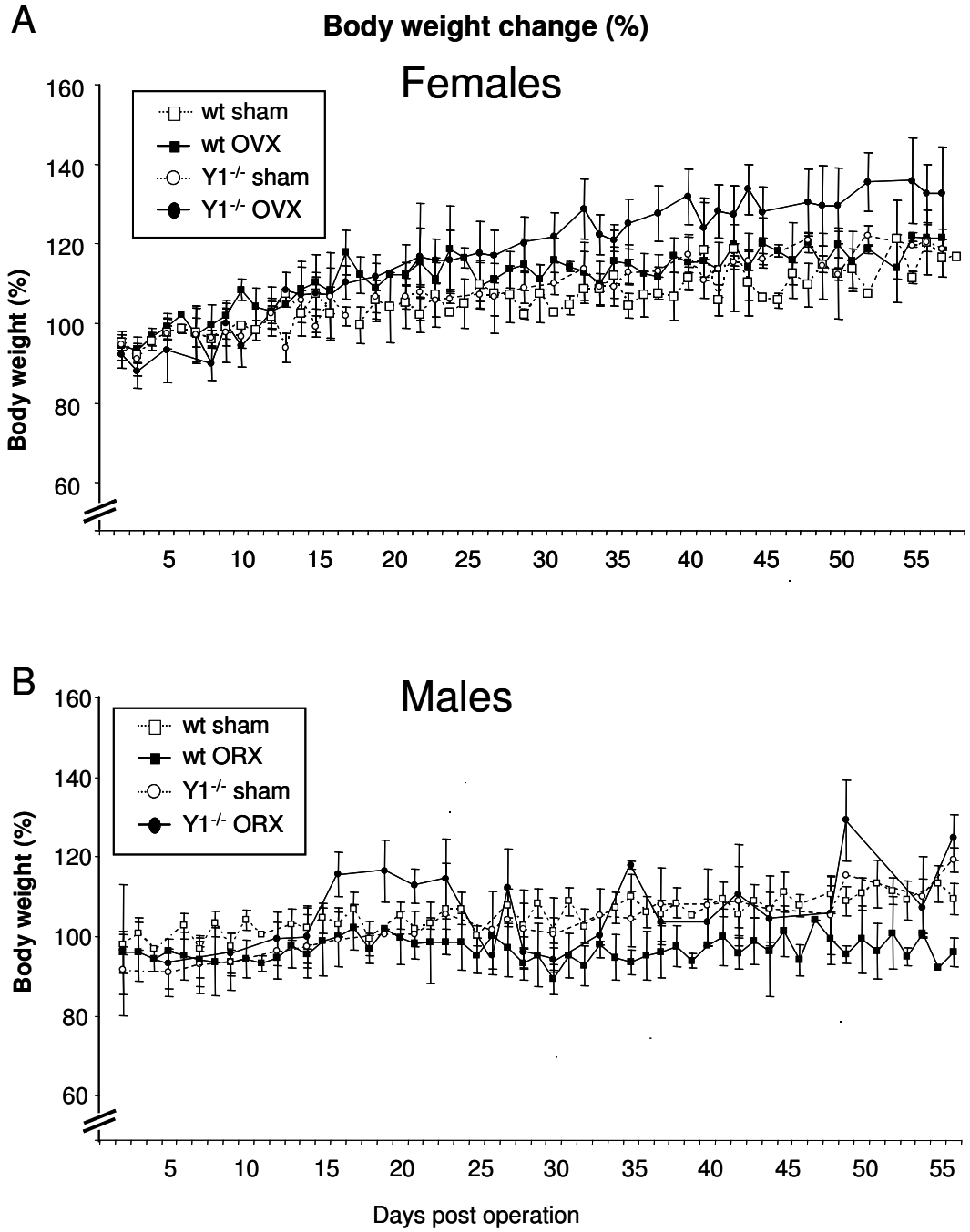

Figure 5.2 Effect of gonadectomy on body weight change in wild type and germline $Y1^{-/-}$ mice.
Female (A), and male (B), wild type and germline $Y1^{-/-}$ mice following gonadectomy (OVX and ORX) or sham-operation. Data are means ± std dev.

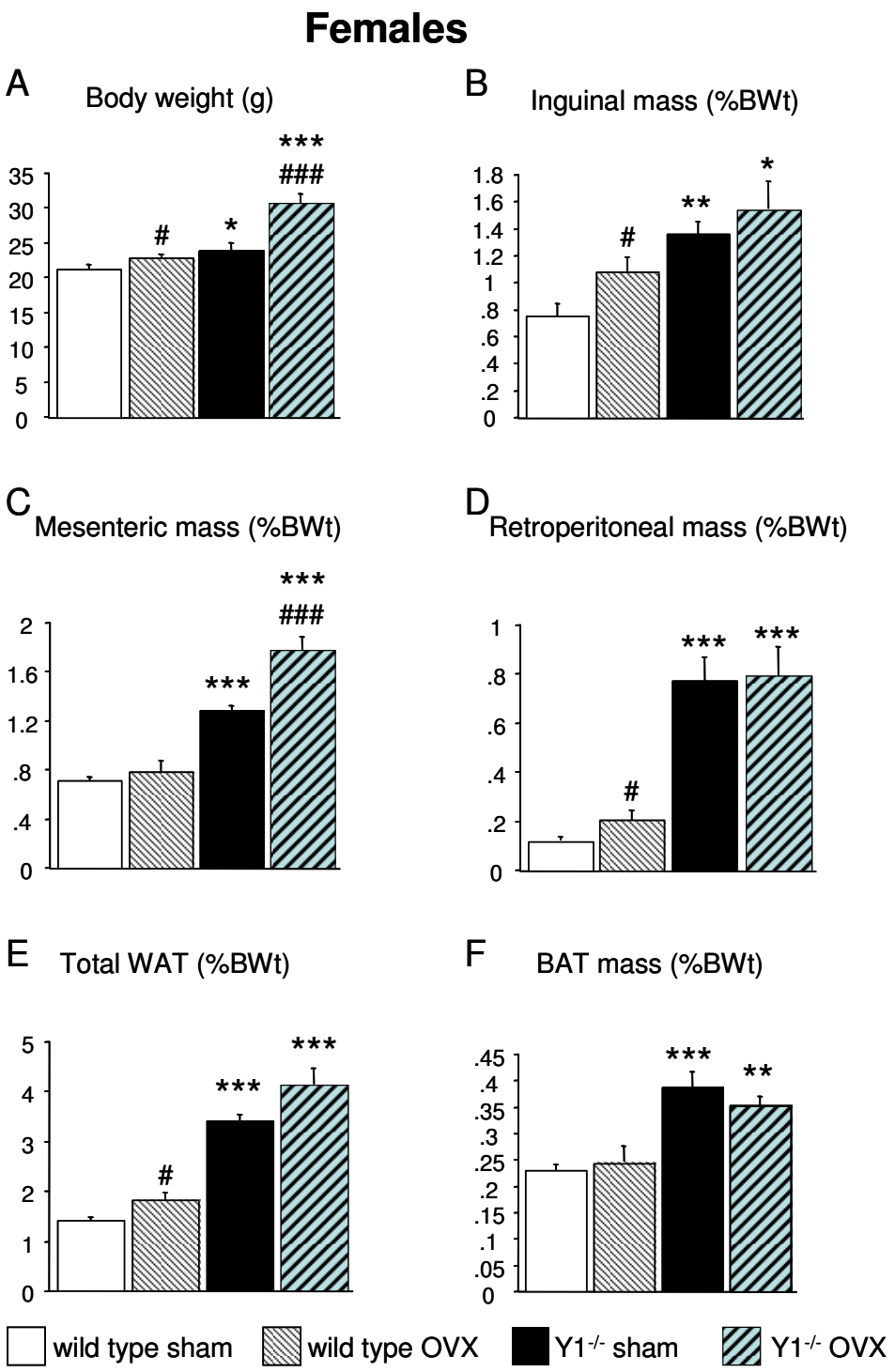

Figure 5.3 Effect of ovariectomy and germline Y1 receptor deletion on body composition of female mice. Body weight (BWt) (A), inguinal (B), mesenteric (C), retroperitoneal (D), total white adipose tissue (E), and brown adipose tissue (F). Asterisks indicate statistically significant differences versus wild-type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #<0.05, ###P<0.001.

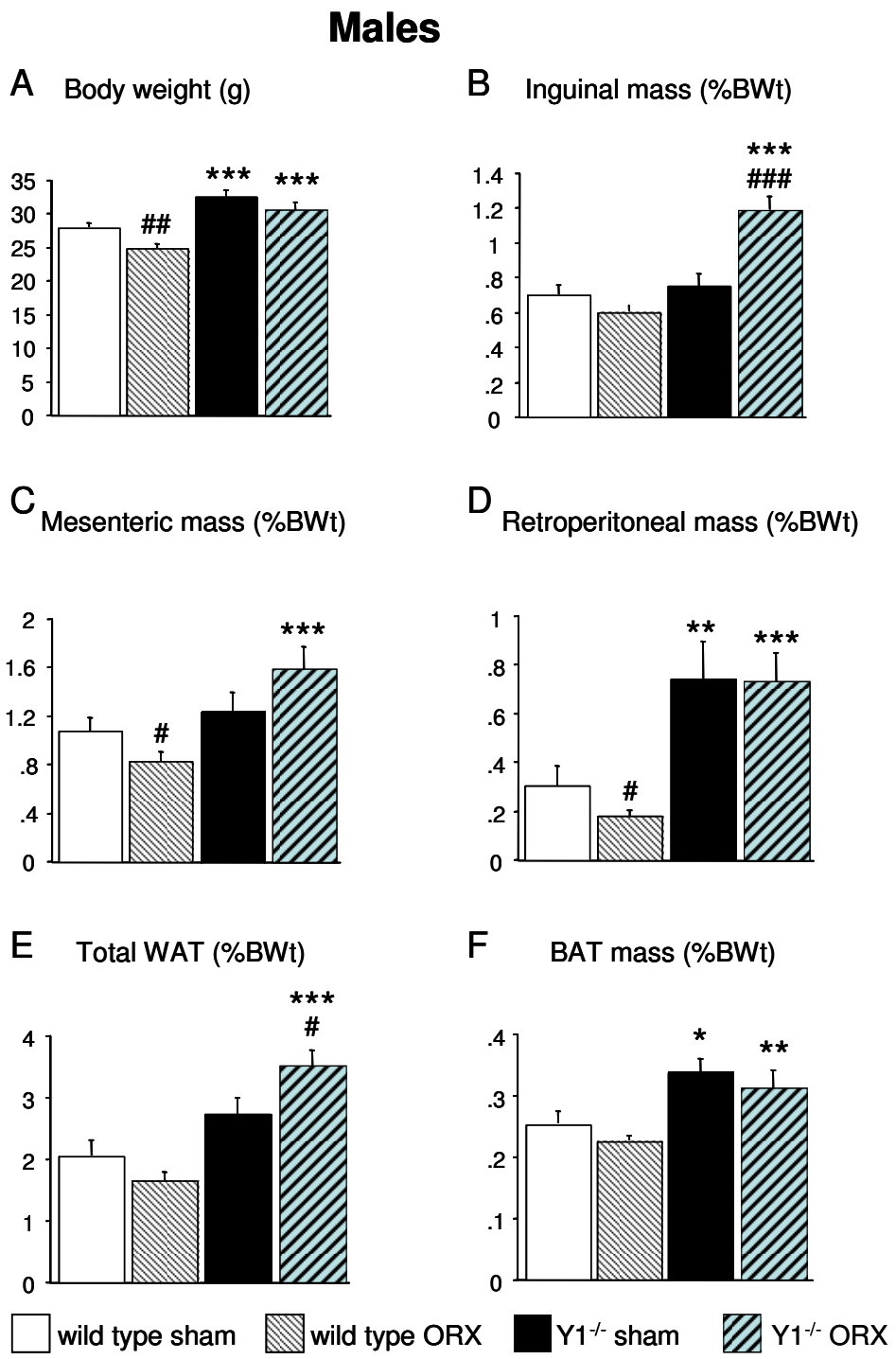

Figure 5.4 Effect of orchidectomy and germline Y1 receptor deletion on body composition of male mice. Body weight (BWt) (A), inguinal (B), mesenteric (C), retroperitoneal (D), total white adipose tissue (E), and brown adipose tissue (F). Asterisks indicate statistically significant differences versus wild-type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #<0.05, ##P<0.01, ###P<0.001.

In contrast to females, orchidectomy of male wild type mice decreased body weight, associated with decreased mesenteric, retroperitoneal adipose mass, and a trend for decreased total WAT (Figure 5.4 A,C,D,E). In contrast to wild type mice however, orchidectomy of $Y1^{-/-}$ mice did not change body weight from sham-operation, but resulted in a greater than 50% increase in inguinal mass and significantly greater total WAT mass with a trend for increased mesenteric mass compared with sham-operated $Y1^{-/-}$ and similarly operated wild type mice (Figure 5.4 A,B,C,E). Thus attenuation of Y1 receptor signalling in male mice modified the response of subcutaneous and central adipose deposition to orchidectomy suggesting a potential role for Y1 receptor signalling in the regulation of adipose in sex hormone deficiency in males.

BAT mass was significantly greater in both female and male $Y1^{-/-}$ mice compared with wild type, suggesting reduced thermogenesis in these mice, which would also likely contribute to the greater body weight and adiposity of these mice, however no change in BAT mass was noted with gonadectomy (Figure 5.3 A, 5.4 A).

Serum concentrations of IGF-1 were significantly greater in both male and female $Y1^{-/-}$ mice compared with wild type regardless of operation (Table 5-1, 5-2). Corticosterone levels were unchanged in $Y1^{-/-}$ mice compared with wild type in both genders, but were elevated in orchidectomised compared with sham-operated $Y1^{-/-}$ mice (Table 5-1, 5-2).

FEMALES:

	wild type sham	wild type OVX	$Y1^{-/-}$ sham	$Y1^{-/-}$ OVX
IGF-1 (ng/ml)	150 ± 11	151.8 ± 15	206.4 ± 7 **	233.8 ± 20 ***
Corticosterone (ng/ml)	100 ± 8	117.3 ± 23	93.2 ± 18	121.9 ± 13

Table 5-1 Effect of ovariectomy on serum levels of IGF-1 and corticosterone in female wild type and germline $Y1^{-/-}$ mice.
Asterisks indicate statistically significant differences versus wild type equivalent operation **P<0.01, ***P<0.001.

MALES:

	wild type sham	wild type ORX	$Y1^{-/-}$ sham	$Y1^{-/-}$ ORX
IGF-1 (ng/ml)	178.2 ± 18	145.6 ± 7	232 ± 11 *	228 ± 24 ***
Corticosterone (ng/ml)	78.8 ± 14	121.7 ± 16	53.1 ± 5	112.2 ± 17 #

Table 5-2 Effect of orchidectomy on serum levels of IGF-1 and corticosterone in male wild type and germline $Y1^{-/-}$ mice.
Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #<0.05.

5.3.2 Germline Y1 receptor deletion and gonadectomy-induced bone loss in the distal femur

Distal femur BV/TV was significantly greater in female and male sham-operated $Y1^{-/-}$ mice compared with wild type (Figure 5.5 A,B,E). Gonadectomy at 8 weeks of age significantly reduced BV/TV of both wild type and $Y1^{-/-}$ mice (Figure 5.5 A,B,E). In female $Y1^{-/-}$ mice, despite a similar percentage reduction in bone volume compared with wild type mice following ovariectomy, the remaining bone volume in ovariectomised $Y1^{-/-}$ mice was comparable to wild type sham levels, remaining significantly greater than bone volume of ovariectomised wild type mice (Figure 5.5 A,B). Similarly, Tb.N and Tb.Th both remained significantly greater in ovariectomised $Y1^{-/-}$ mice compared with similarly-operated wild type levels (Figure 5.5 C,D), suggesting that germline deletion of Y1 receptors provided some protection against ovariectomy-induced bone loss and deterioration of bone microarchitecture in the distal femur.

In contrast to observations in ovariectomised female mice, orchidectomy of wild type and $Y1^{-/-}$ male mice abolished the difference in BV/TV between the two genotypes with markedly reduced BV/TV in orchidectomised $Y1^{-/-}$ mice (Figure 5.5 E). The loss of BV/TV in orchidectomised $Y1^{-/-}$ mice was associated with a reduction in Tb.N of a similar magnitude (Figure 5.5 F).

The reduction in BV/TV in female and male wild type mice was associated with an elevation in Oc.S compared with sham-operated mice (Figure 5.6 B, 5.7 B). Oc.S was slightly but non-significantly greater in sham-operated female and male $Y1^{-/-}$ mice compared with similarly-operated wild type (Figure 5.6 B, 5.7 B), consistent with the previous characterisation of this model in non-operated mice (section 3.3.1). Similar to observations in wild type mice, Oc.S was further elevated following orchidectomy in male $Y1^{-/-}$ mice, with Oc.S significantly greater than both sham-operated $Y1^{-/-}$ and similarly-operated wild type mice (Figure 5.7 B). A similar but non-significant trend was also apparent in ovariectomised female $Y1^{-/-}$ mice (Figure 5.6 B).

Mineralising surface (MS), a measure of the extent of mineralisation, was similar between wild type and $Y1^{-/-}$ sham-operated male and female mice (Figure 5.6 C, 5.7 C), revealing for the first time that similar to the $Y2^{-/-}$ model, the greater trabecular bone volume of the $Y1^{-/-}$ model also results from a greater rate of mineral apposition, with no change in the extent of mineralisation. Following gonadectomy, MS was non-significantly elevated in both male and female wild type and $Y1^{-/-}$ mice, importantly with no difference between the genotypes.

MAR in female sham-operated $Y1^{-/-}$ mice was significantly elevated compared with similarly-operated wild type, consistent with the greater bone volume of this model (Figure 5.6 A,D). Bone formation rate (BFR) was also significantly elevated in sham-operated $Y1^{-/-}$ females compared with wild type (Figure 5.6 E). Importantly, MAR and BFR in $Y1^{-/-}$ mice were not reduced by ovariectomy, remaining significantly greater than similarly-operated wild type (Figure 5.6 A,D,E), suggesting that the loss of bone volume observed in ovariectomised $Y1^{-/-}$ mice was the result of elevated resorption which was somewhat elevated in ovariectomised compared with sham-operated $Y1^{-/-}$ mice.

MAR in male sham-operated $Y1^{-/-}$ mice was also significantly greater compared with similarly-operated wild type, but in contrast to observations in female $Y1^{-/-}$ mice, the elevated rate of mineral apposition of the $Y1^{-/-}$ model was abolished following orchidectomy, with MAR reduced to levels not significant from wild type (Figure 5.7 A,D). BFR in male $Y1^{-/-}$ mice was also not different from wild type levels, regardless of operation (Figure 5.7 E). The lack of change in BFR in sham-operated $Y1^{-/-}$ mice compared with wild type is the result of elevated MAR with no change in MS, while in orchidectomised, $Y1^{-/-}$ mice, the lack of elevated BFR was due to comparable MAR to wild type levels in the sex hormone deficient state (Figure 5.7 C-E). These findings suggest that the greater extent of bone loss observed in gonadectomised male compared with female mice is the result of both increased resorptive activity and loss of osteoblastic function, and indicates that the anabolic activity of the $Y1^{-/-}$ pathway in male mice may be dependent on the presence of sex hormones.

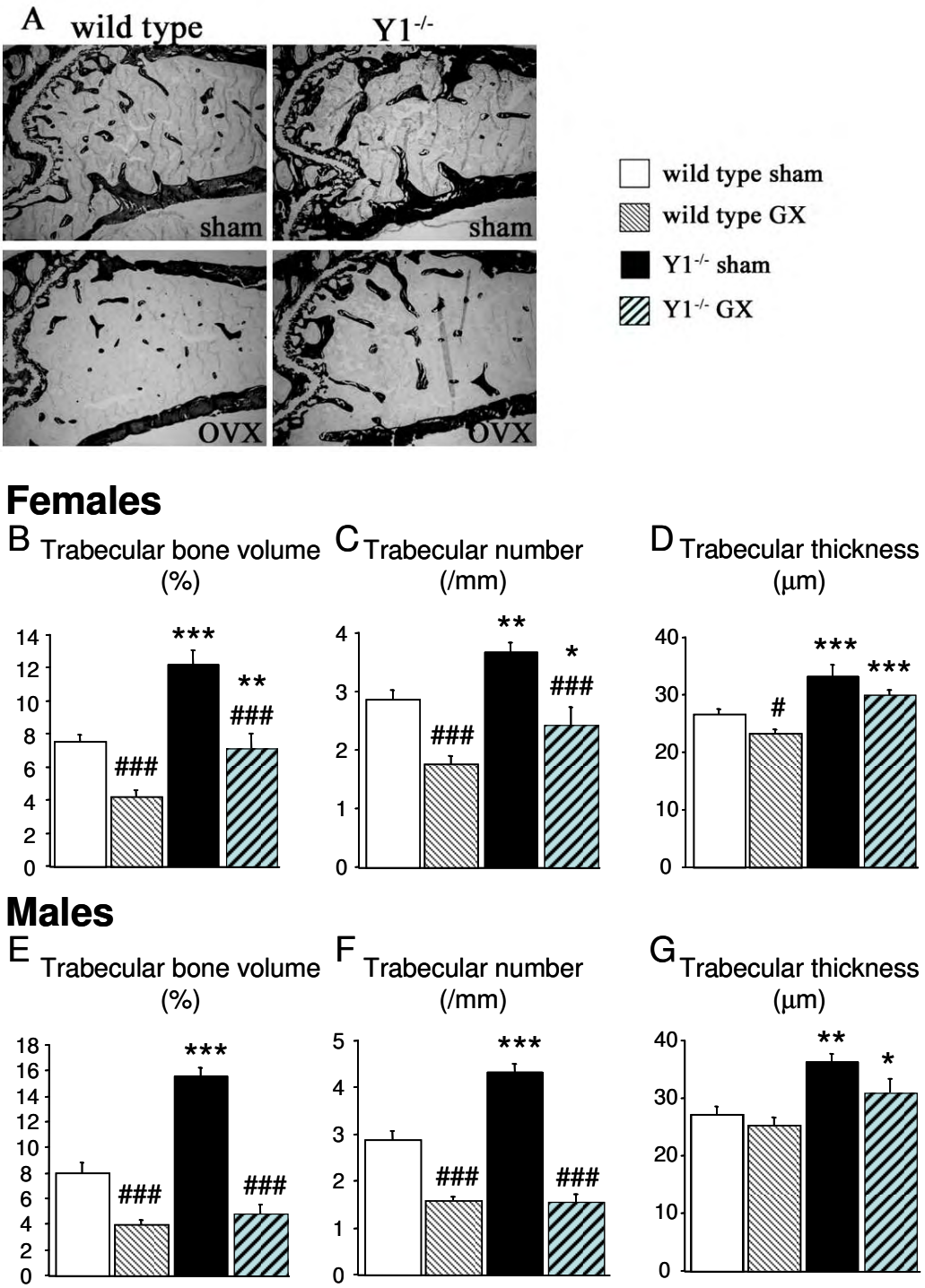

Figure 5.5 Effect of gonadectomy and germline Y1 receptor deletion on trabecular bone of distal femur. Female (A-D) and male (E-G) mice. Von Kossa stained sections of distal femur from ovariectomised wild type and Y1$^{-/-}$ mice (A), trabecular bone volume (B,E), trabecular number (C,F), and trabecular thickness (D,G). Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #<0.05, ###P<0.001.

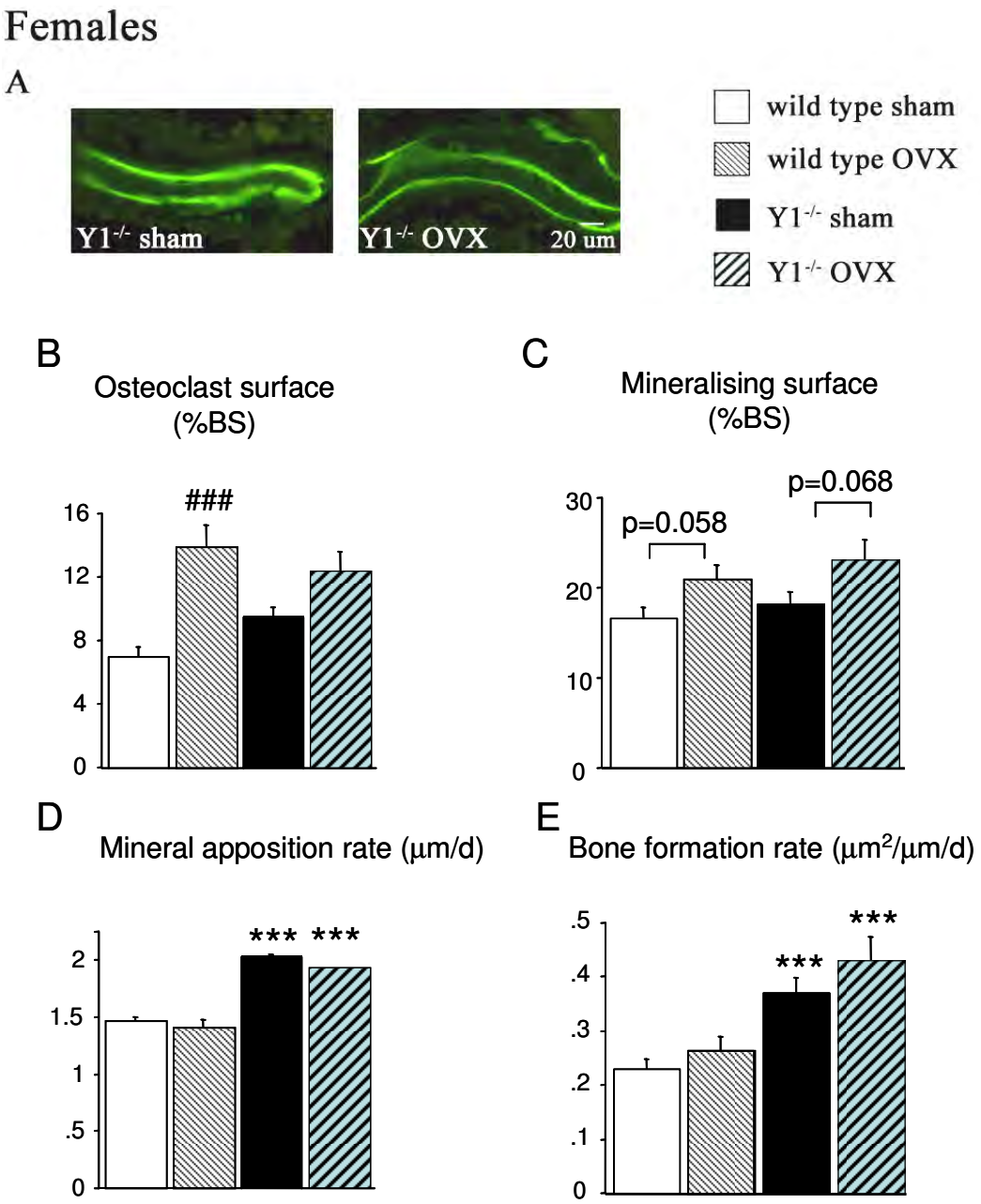

Figure 5.6 Effect of ovariectomy and Y1 receptor deletion on parameters of bone resorption and formation in the distal femur.
Double calcein labels (A), osteoclast surface (B), mineralising surface (C), mineral apposition rate (D), and bone formation rate (E). Asterisks indicate statistically significant differences versus wild-type equivalent operation ***P<0.001. Hashes indicate significant differences versus sham within the same genotype ###P<0.001.

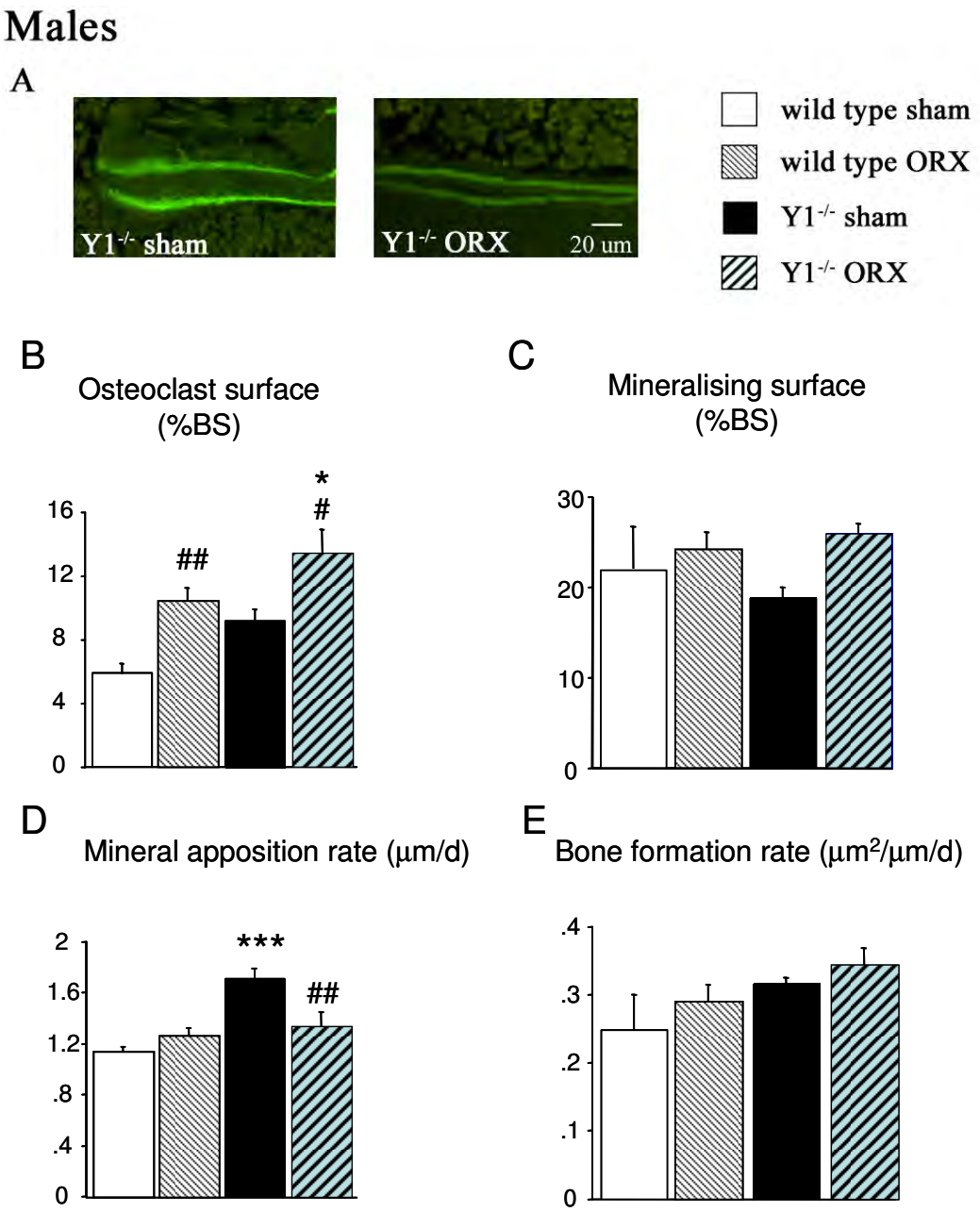

Figure 5.7 Effect of orchidectomy and Y1 receptor deletion on parameters of bone resorption and formation in the distal femur.
Double calcein labels (A), osteoclast surface (B), mineralising surface (C), mineral apposition rate (D), and bone formation rate (E). Asterisks indicate statistically significant differences versus wild-type equivalent operation *P<0.05, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #P<0.05, ##P<0.01.

5.3.3 Germline Y2 receptor deletion and gonadectomy-induced changes in body weight, adipose mass and biochemical parameters

Similar to the above study in $Y1^{-/-}$ mice, germline $Y2^{-/-}$ mice were also assessed for changes in body weight and adiposity at 16 weeks of age, 8 weeks following gonadectomy.

Body weight change following surgery did not differ between $Y2^{-/-}$ and wild type mice, with increasing body weight in all female mice (Figure 5.8 A), while in male mice, body weight gradually increased in sham-operated mice and decreased in orchidectomised mice regardless of genotype (Figure 5.8 B). At the study endpoint, body weights of female sham-operated wild-type and $Y2^{-/-}$ mice were similar, with elevated body weight in wild type but not $Y2^{-/-}$ mice following ovariectomy (Figure 5.9 A). The increase in body weight in ovariectomised wild type mice was associated with increased inguinal, retroperitoneal, and total WAT mass (Figure 5.9 B,D,E), which was also not increased following ovariectomy in $Y2^{-/-}$ mice. Total WAT mass was significantly lower in ovariectomised $Y2^{-/-}$ mice compared with wild-type (Figure 5.9 E), suggesting that germline Y2 receptor deletion provides resistance to the ovariectomy-associated increase in adipose mass. No difference in BAT was observed between genotypes in female mice regardless of operation (Figure 5.9 F).

Endpoint body weight of male germline $Y2^{-/-}$ mice was significantly lower than wild type mice (Figure 5.10 A), with lighter inguinal, mesenteric, retroperitoneal, and total WAT in $Y2^{-/-}$ males regardless of operation (Figure 5.10 B-E). BAT mass was also significantly lower in $Y2^{-/-}$ sham-operated males compared with wild-type (Figure 5.10 F), suggesting increased thermogenesis which would also likely contribute to reduced body weight. As expected from post-surgery monitoring (Figure 5.8 B), orchidectomised mice were lighter compared with sham-operated genotype-matched controls (Figure 5.10 A), demonstrating that although lighter than wild-type mice, germline deletion of Y2 receptors does not significantly alter changes in adipose mass that occur in response to orchidectomy.

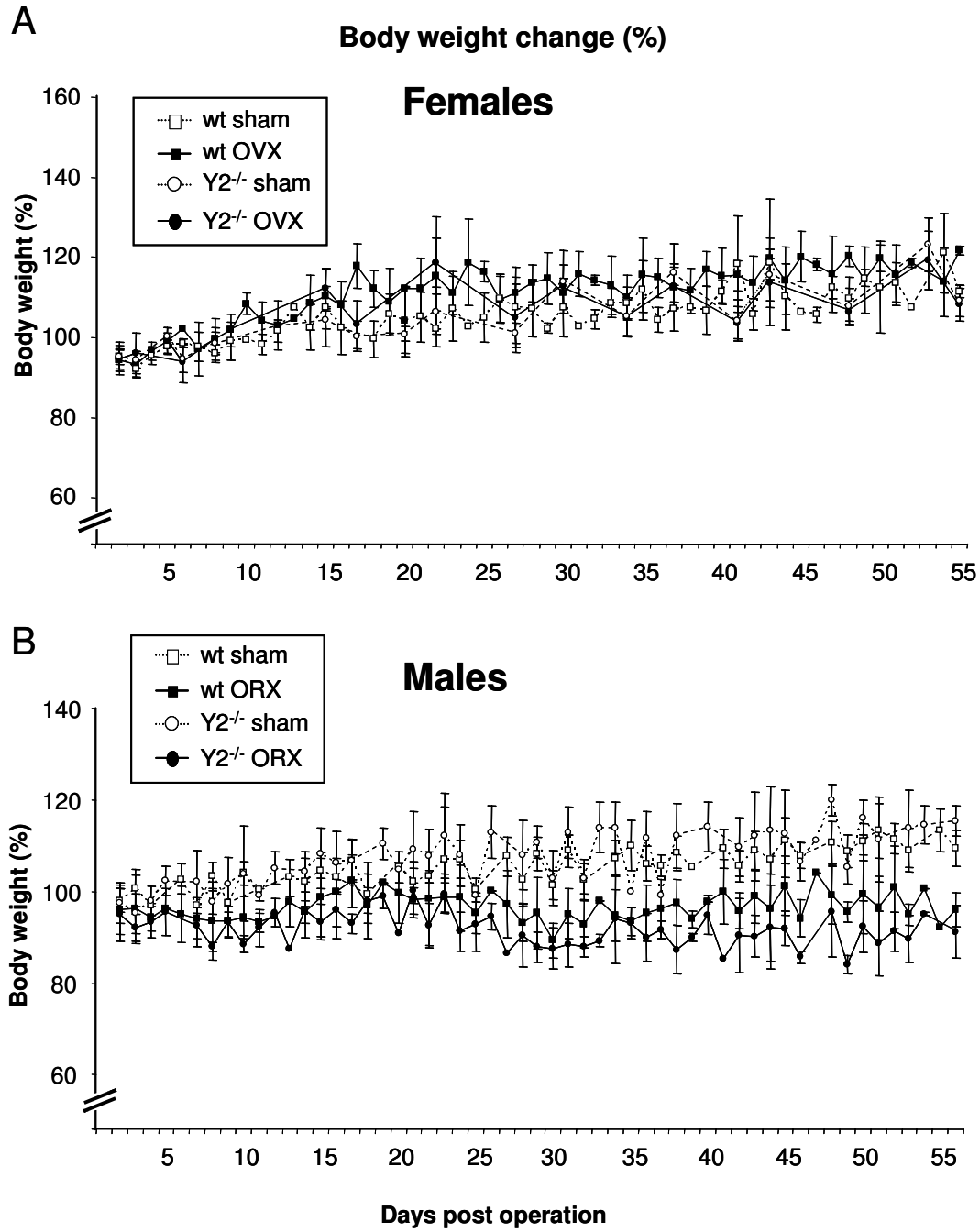

Figure 5.8 Effect of gonadectomy on body weight change in wild type and germline $Y2^{-/-}$ mice.
Female (A), and male (B), wild type and germline $Y2^{-/-}$ mice following gonadectomy (OVX and ORX) or sham-operation. Data are means ± std dev.

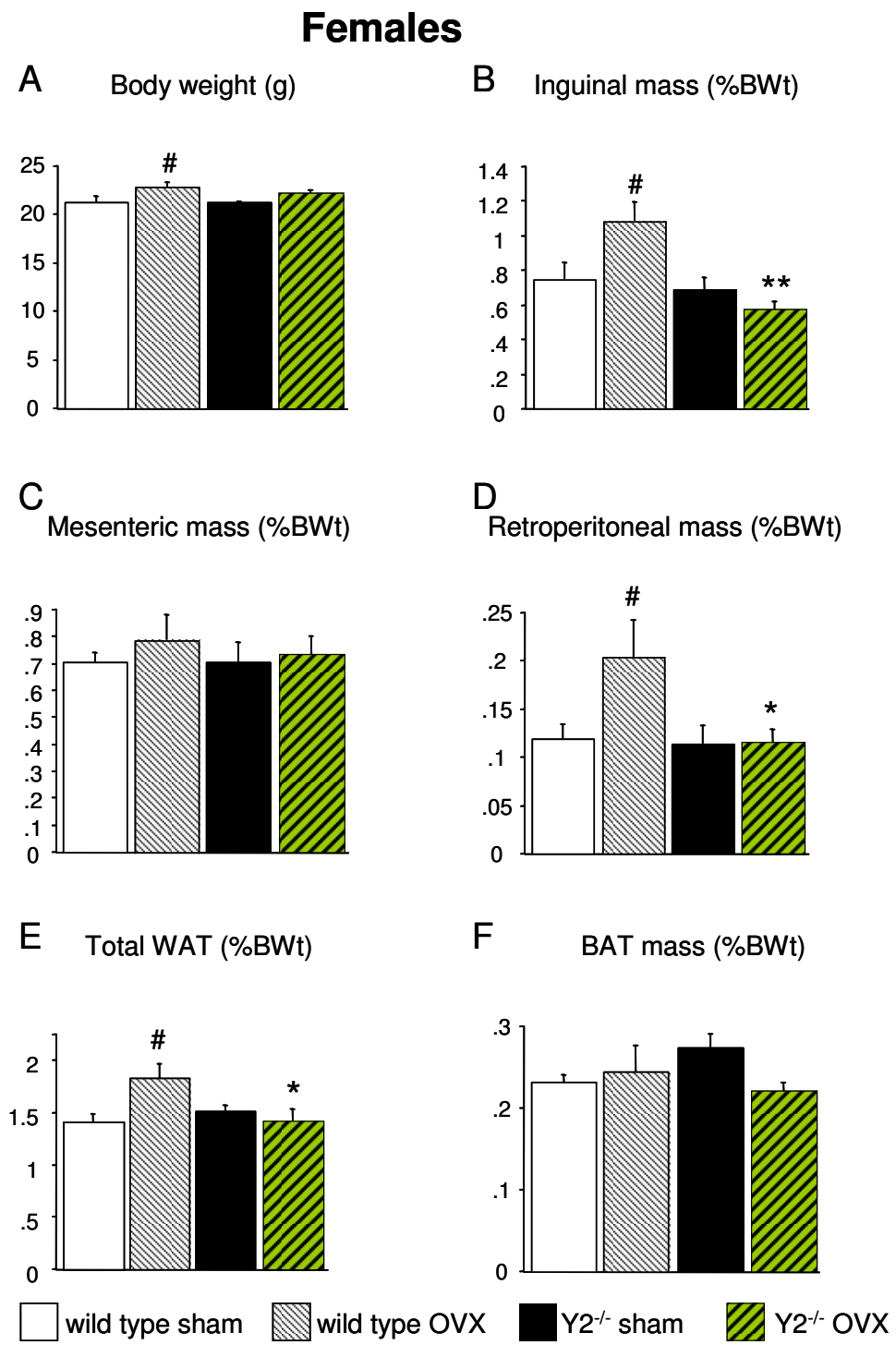

Figure 5.9 Effect of ovariectomy and germline Y2 receptor deletion on body composition of female mice. Body weight (BWt) (A), inguinal (B), mesenteric (C), retroperitoneal (D), total white adipose tissue (E), and brown adipose tissue (F). Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, **P<0.01. Hashes indicate significant differences versus sham within the same genotype #P<0.05.

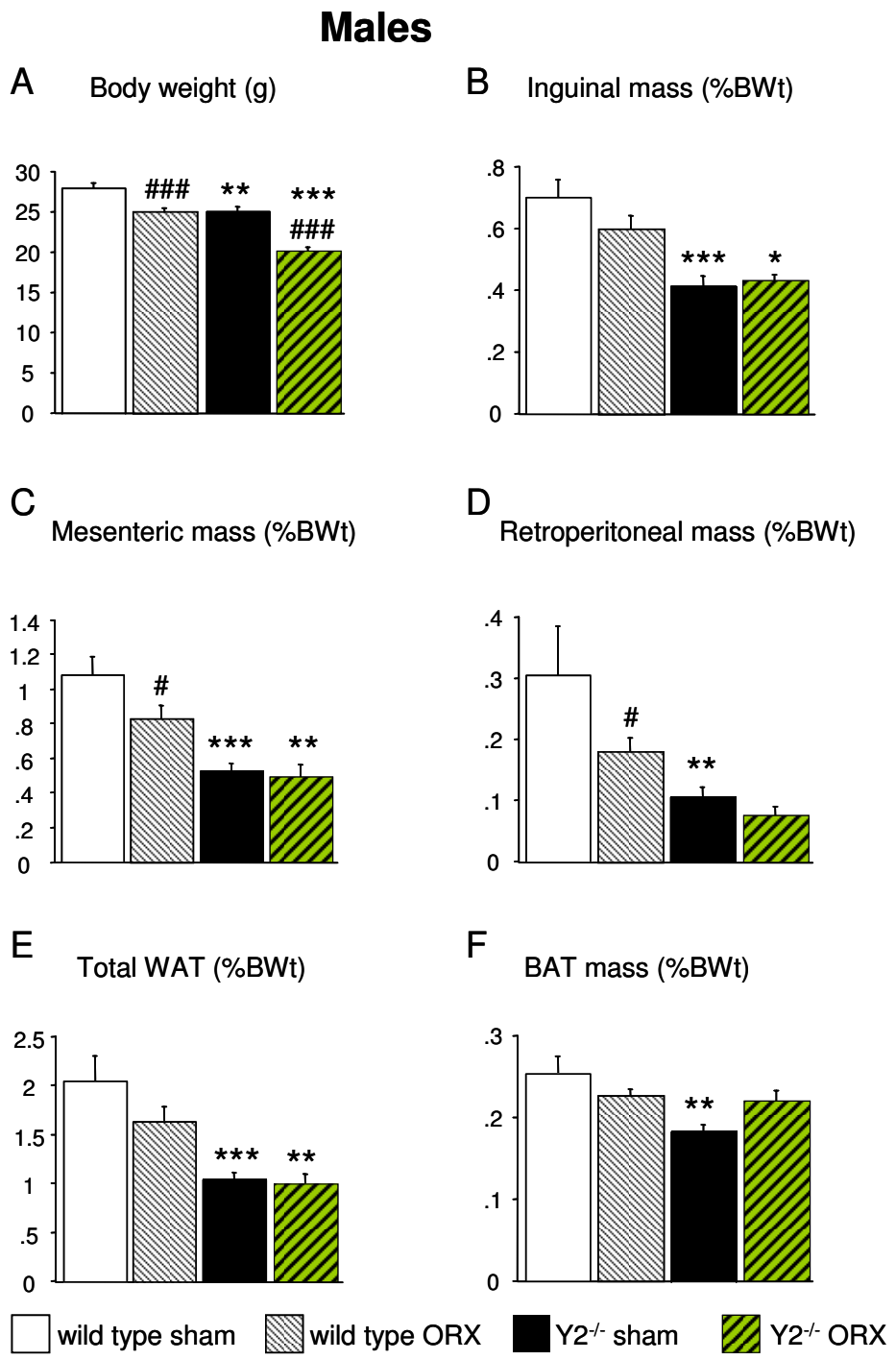

Figure 5.10 Effect of orchidectomy and germline Y2 receptor deletion on body composition of male mice. Body weight (BWt) (A), inguinal (B), mesenteric (C), retroperitoneal (D), total white adipose tissue (E), and brown adipose tissue (F). Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #P<0.05, ###P<0.001.

Serum concentrations of IGF-1 and osteocalcin were unaffected by gonadectomy or germline deletion of Y2 receptors in both sexes. Serum corticosterone was significantly reduced in ovariectomised $Y2^{-/-}$ compared with wild-type mice (Table 5-3), but was significantly higher in male $Y2^{-/-}$ mice compared with wild type, and in orchidectomised compared with sham-operated germline $Y2^{-/-}$ mice (Table 5-4), suggesting a gender-specific change in the levels and gonadectomy-induced response of this hormone.

FEMALES:

	wild type sham	wild type OVX	$Y2^{-/-}$ sham	$Y2^{-/-}$ OVX
IGF-1 (ng/ml)	150 ± 11	151.8 ± 15	133.1 ± 15	137.3 ± 19
Corticosterone (ng/ml)	100 ± 8	117.3 ± 23	60.2 ± 11	70.5 ± 9.7 *
Osteocalcin (ng/ml)	99.8 ± 9	99.4 ± 6	92.9 ± 11	82.9 ± 5

Table 5-3 Effect of ovariectomy on serum levels of IGF-1, corticosterone, and osteocalcin in female wild type and germline $Y2^{-/-}$ mice.
Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05.

MALES:

	wild type sham	wild type ORX	$Y2^{-/-}$ sham	$Y2^{-/-}$ ORX
IGF-1 (ng/ml)	178.2 ± 18	145.6 ± 7	146.7 ± 7	146.4 ± 21
Corticosterone (ng/ml)	78.8 ± 14	121.7 ± 16	129.5 ± 13 *	185.4 ± 28 * #
Osteocalcin (ng/ml)	84.4 ± 4.5	101.1 ± 5	76.5 ± 6	86.6 ± 5

Table 5-4 Effect of orchidectomy on serum levels of IGF-1, corticosterone, and osteocalcin in male wild type and germline $Y2^{-/-}$ mice.
Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05. Hashes indicate significant differences versus sham within the same genotype #P<0.05.

5.3.4 Germline Y2 receptor deletion and gonadectomy-induced bone loss in the distal femur

16 week old male and female sham-operated germline $Y2^{-/-}$ mice had significantly greater BV/TV at the distal femur than sham-operated wild type (Figure 5.11 A,B,E). As demonstrated in section 5.3.2, gonadectomy at 8 weeks of age significantly reduced BV/TV and Tb.N in wild type mice of both genders (Figure 5.11 A,B,E). Similar to observations in $Y1^{-/-}$ mice, gonadectomy at 8 weeks of age also significantly reduced trabecular bone volume of $Y2^{-/-}$ mice (Figure 5.11 A,B,E). In female $Y2^{-/-}$ mice, as in $Y1^{-/-}$ mice, ovariectomy reduced bone volume only to wild type sham levels, remaining significantly greater than bone volume of ovariectomised wild type mice (Figure 5.11 A,B), associated with greater Tb.N and Tb.Th (Figure 5.11 C,D). Hence germline deletion of Y2 receptors also protected against ovariectomy-induced bone loss and deterioration of bone microarchitecture in the distal femur.

In contrast to observations in ovariectomised female mice, and in line with observations in orchidectomised male $Y1^{-/-}$ mice, orchidectomy of $Y2^{-/-}$ male mice abolished the difference in BV/TV between $Y2^{-/-}$ and wild type mice (Figure 5.11 E), due to reduced Tb.N and Tb.Th (Figure 5.11 F,G).

In female mice, the loss of trabecular bone volume following ovariectomy was associated with increased Oc.S and mineralising surface (MS) (Figure 5.12 B,C), consistent with the characteristic response to estrogen deficiency (Baldock et al., 1998; Rehman et al., 1994; Wronski et al., 1989; Wronski et al., 1986). The increased osteoclast surface in wild type and $Y2^{-/-}$ mice was comparable and therefore not affected by Y2 receptor deletion (Figure 5.12 B). MS was elevated in ovariectomised wild type and $Y2^{-/-}$ mice, reaching statistical significance only in wild type mice (Figure 5.12 C). Similarly in male mice, the loss of trabecular bone volume following gonadectomy was associated with significantly elevated Oc.S in both wild type and $Y2^{-/-}$ mice (Figure 5.13 A). MS was elevated in orchidectomised $Y2^{-/-}$, but not wild type mice (Figure 5.13 B).

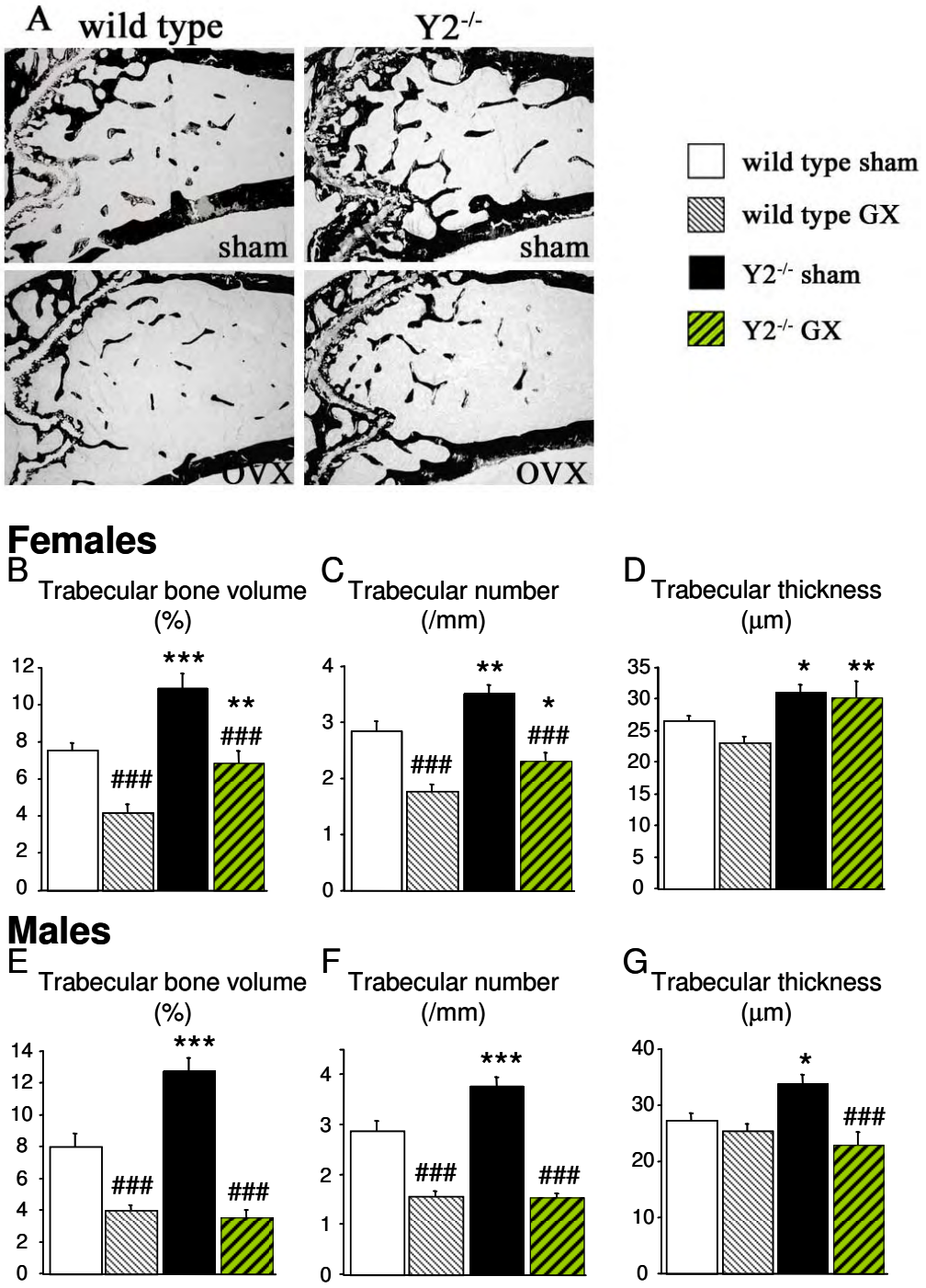

Figure 5.11 Effect of gonadectomy and germline Y2 receptor deletion on trabecular morphology of distal femur.
Female (A-D) and male (E-G) mice. Von Kossa stained sections of distal femur from ovariectomised wild type and $Y2^{-/-}$ mice (A), trabecular bone volume (B,E), trabecular number (C,F), and trabecular thickness (D,G). Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype ###P<0.001.

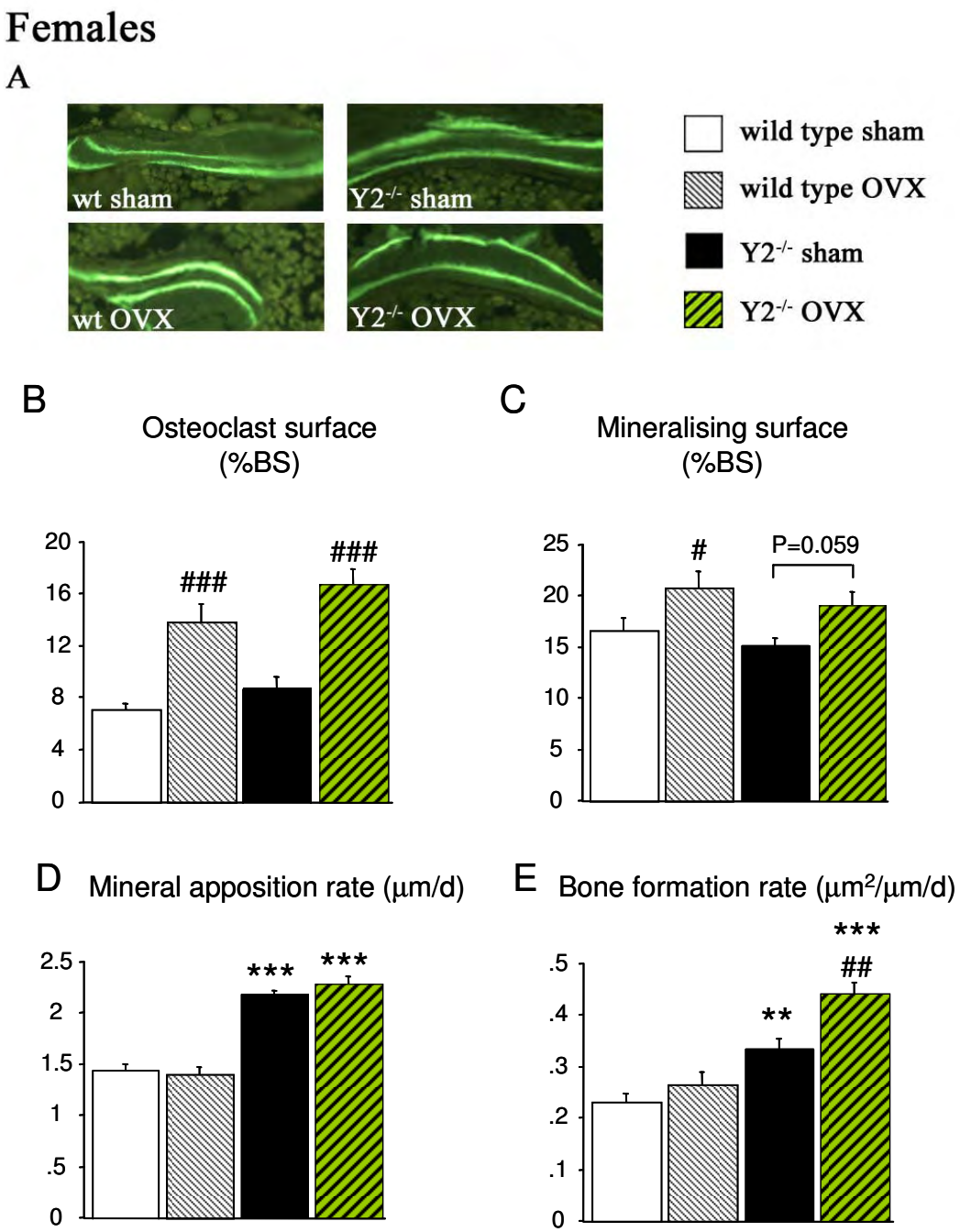

Figure 5.12 Effect of ovariectomy and germline Y2 receptor deletion on parameters of bone resorption and formation in the distal femur.
Double calcein labels (A), osteoclast surface (B), mineralising surface (C), mineral apposition rate (D), and bone formation rate (E). Asterisks indicate statistically significant differences versus wild type equivalent operation **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #<0.05, ##P<0.01, ###P<0.001.

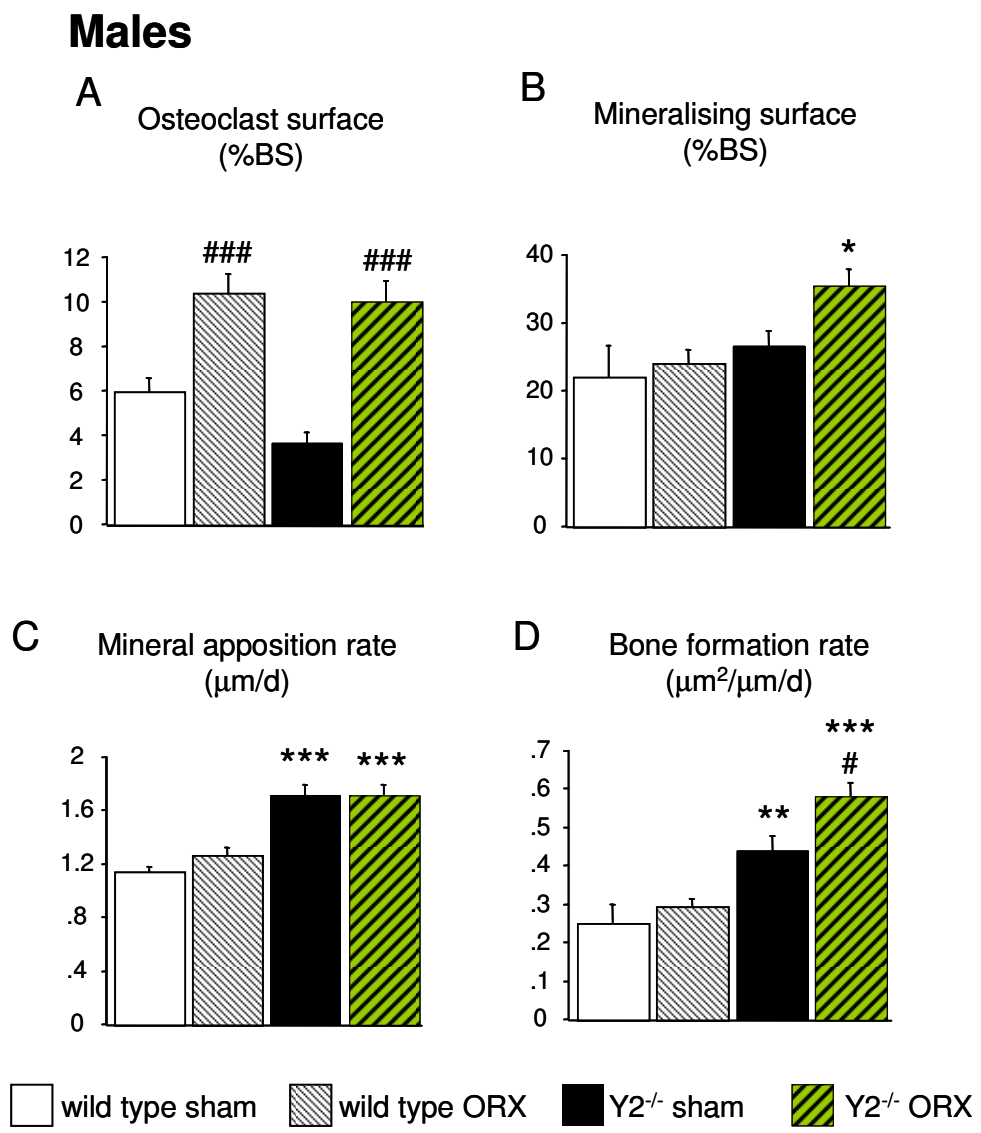

Figure 5.13 Effect of orchidectomy and germline Y2 receptor deletion on parameters of bone resorption and formation in the distal femur.
Osteoclast surface (A), mineralising surface (B), mineral apposition rate (C), and bone formation rate (D). Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype #<0.05, ###P<0.001.

Of importance however, the greater MAR which confers the greater trabecular bone volume in non-operated $Y2^{-/-}$ mice (Baldock et al., 2002), was maintained following gonadectomy in both female and male mice (Figure 5.12 A,D, 5.13 C). Furthermore, BFR, a measure of total osteoblast activity, was elevated in gonadectomised $Y2^{-/-}$ mice of both genders to a level even greater than sham-operated values (Figure 5.12 E, 5.13 D). Together these results demonstrate that unlike observations in gonadectomised $Y1^{-/-}$ mice, the Y2-associated anabolic phenotype persists in the absence of sex hormones in both males and females. Therefore, the preservation of bone micro-architecture post-ovariectomy in female $Y2^{-/-}$ mice was likely the result of Y2-dependent anabolic activity, demonstrating this anabolic pathway to be independent of the presence of sex hormones.

5.3.5 Germline Y2 receptor deletion and gonadectomy-induced bone loss in the lumbar vertebrae

Due to the persistent elevation of MAR in both male and female $Y2^{-/-}$ mice in the absence of sex hormones, histological analysis was extended to the lumbar vertebrae to investigate the response of trabecular bone in this region to gonadectomy in the absence of Y2 receptor signalling. Analysis of the lumbar vertebrae revealed for the first time, the lack of a high trabecular bone mass phenotype in sham-operated male and female $Y2^{-/-}$ mice, with similar BV/TV in sham-operated $Y2^{-/-}$ mice compared with sham-operated wild type (Figure 5.14 B,E). Consistent with this observation, lumbar vertebrae MAR in male and female $Y2^{-/-}$ sham-operated mice was also similar to wild type levels (Figure 5.14 A,D,G), demonstrating regional variation in the skeletal phenotype of the $Y2^{-/-}$ model.

Gonadectomy of wild type mice significantly reduced lumbar vertebrae BV/TV in males with a similar trend in females (Figure 5.14 B,E), corresponding to elevated Oc.S in both male and female mice (Figure 5.14 C,F). Consistent with our findings in the distal femur, deletion of Y2 receptors in male mice did not protect against sex-hormone deficient bone loss in the lumbar vertebrae, with reduced BV/TV in $Y2^{-/-}$ mice following orchidectomy (Figure 5.14 E), while lumbar vertebrae BV/TV was unaffected by ovariectomy in female $Y2^{-/-}$ mice (Figure 5.14 B). Oc.S was elevated in $Y2^{-/-}$ mice following gonadectomy, albeit

to a lesser extent as observed in gonadectomised wild type mice and reaching significance only in males (Figure 5.14 C,F).

Interestingly, despite a lack of elevated MAR in sham-operated $Y2^{-/-}$ mice compared with wild type, MAR was significantly greater in both male and female gonadectomised $Y2^{-/-}$ mice compared with similarly-operated wild type and sham-operated $Y2^{-/-}$ mice (Figure 5.14 A,D,G), indicating that in the absence of sex hormones the anabolic activity of the Y2 pathway may be activated in multiple skeletal sites. Together these data suggest that despite the lack of a high trabecular bone mass phenotype in lumbar vertebrae of sex-hormone sufficient germline $Y2^{-/-}$ mice, attenuation of Y2 receptor signalling in the absence of sex hormones activates osteoblast activity in both sexes, and in females provides some protection against sex-hormone deficient bone loss.

These results from distal femur and lumbar vertebrae of germline $Y2^{-/-}$ mice reveal a resistance of $Y2^{-/-}$ bone to gonadectomy-induced bone loss. In the clinic, however, an effective anabolic treatment for osteoporosis must rebuild osteopenic bone. To assess whether activation of the Y2-mediated anabolic response can reverse bone loss that has already occurred, we utilised a conditional Y2 receptor knockout model, using hypothalamic injection of recombinant adeno-associated viral vector containing the cre-recombinase gene (AAV-cre) to delete the Y2 receptor gene in adult $Y2^{lox/lox}$ mice. Hypothalamic Y2 receptors were deleted in 16 week old $Y2^{lox/lox}$ mice, 8 weeks after gonadectomy, followed by a further 6 weeks to allow the consequences of Y2 gene deletion to develop. Control $Y2^{lox/lox}$ mice were injected with adeno-associated viral vector without the transgene (AAV-empty) therefore remaining genetically equivalent to wild type mice. This model, in which the anabolic pathway is activated following the occurrence of bone loss, is more akin to the typical osteoporotic patient, in whom treatment is initiated after substantial bone has been lost.

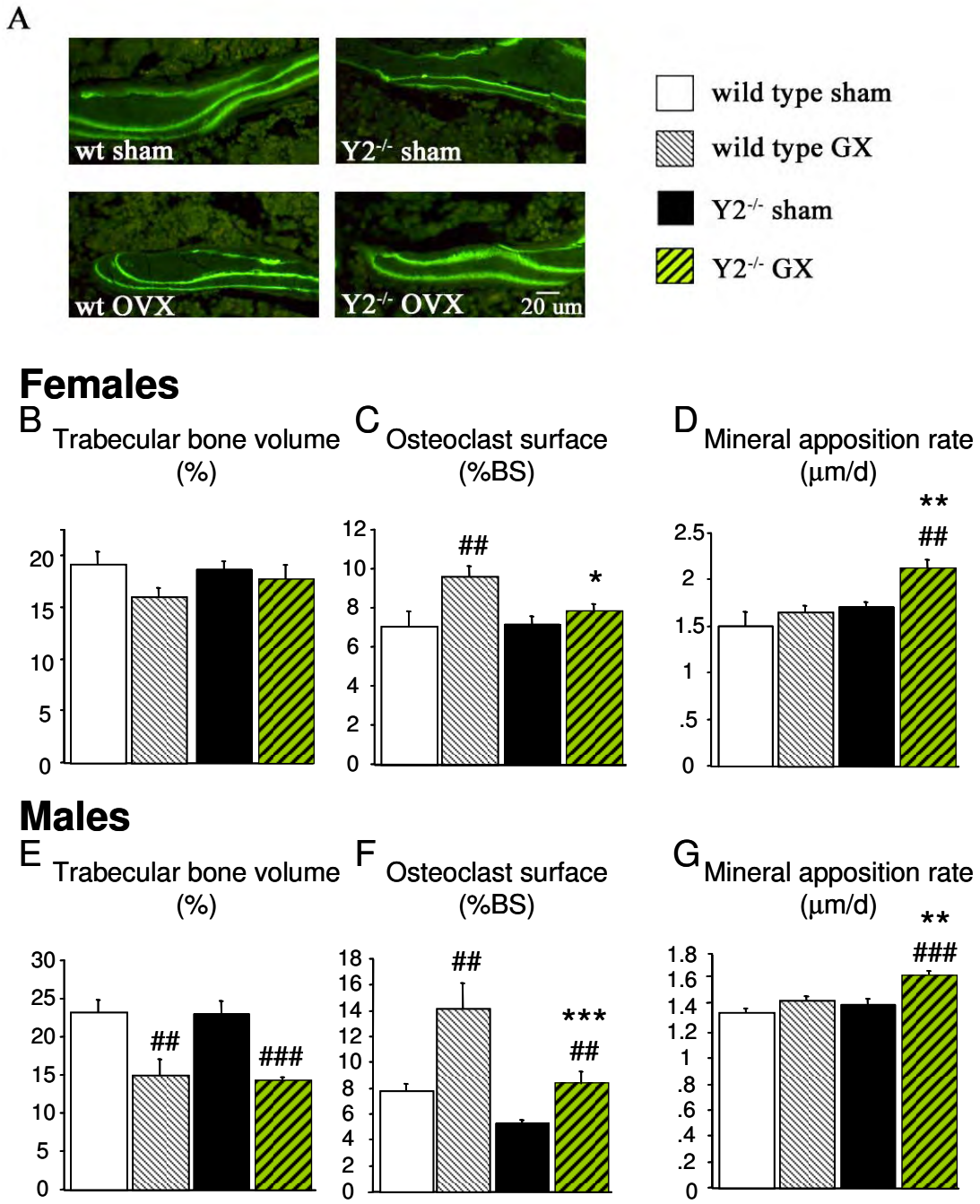

Figure 5.14 Effect of gonadectomy and germline Y2 receptor deletion on trabecular bone and cellular activity of lumbar vertebrae.
Female (A-D) and male (E-G) mice. Double calcein labels in sections of vertebrae from ovariectomised wild type and $Y2^{-/-}$ mice (A), trabecular bone volume (B,E), osteoclast surface (C,F), and mineral apposition rate (D,G). Asterisks indicate statistically significant differences versus wild type equivalent operation *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus sham within the same genotype ##P<0.01, ###P<0.001.

5.3.6 Conditional hypothalamic Y2 receptor deletion and gonadectomy-induced changes on feeding, body weight, adipose mass and biochemical parameters

Investigation of whether conditional activation of this central pathway would affect gonadectomy-induced changes in body weight revealed that in the absence of sex hormones, hypothalamic-specific deletion of Y2 receptors had no significant effect on food intake or body weight change compared with similarly operated AAV-empty recipient mice (Figure 5.15, 5.16, 5.17 A, 5.18 A).

In females, fat pad weights from ovariectomised conditional $Y2^{-/-}$ mice were similar to sham-operated controls (Figure 5.17 B-F). As WAT mass would be expected to increase following ovariectomy prior to the deletion of Y2 receptors, these findings suggest that similar to germline deletion, hypothalamic deletion of Y2 receptors caused a decrease in WAT mass.

In males, BAT was similarly reduced in both orchidectomised models (Figure 5.18 F). However, in contrast to orchidectomised germline $Y2^{-/-}$ mice which were significantly lighter and less fat than similarly operated wild type mice, orchidectomised conditional $Y2^{-/-}$ mice had increased mesenteric, retroperitoneal, and total WAT mass compared with orchidectomised AAV-empty recipient mice (Figure 5.18 C-E), suggesting this population of Y2 receptors to be crucial for sex-hormone regulated fat accumulation in males, and that the effect of their deletion was not revealed in germline $Y2^{-/-}$ mice with global deletion of Y2 receptor signalling.

Conditional deletion of Y2-receptors did not affect serum levels of IGF-1, corticosterone, or osteocalcin, although corticosterone was elevated in both ovariectomised groups compared to conditional $Y2^{-/-}$ sham-operated mice, with the difference reaching significance only in AAV-empty recipient mice (Table 5-5, 5-6).

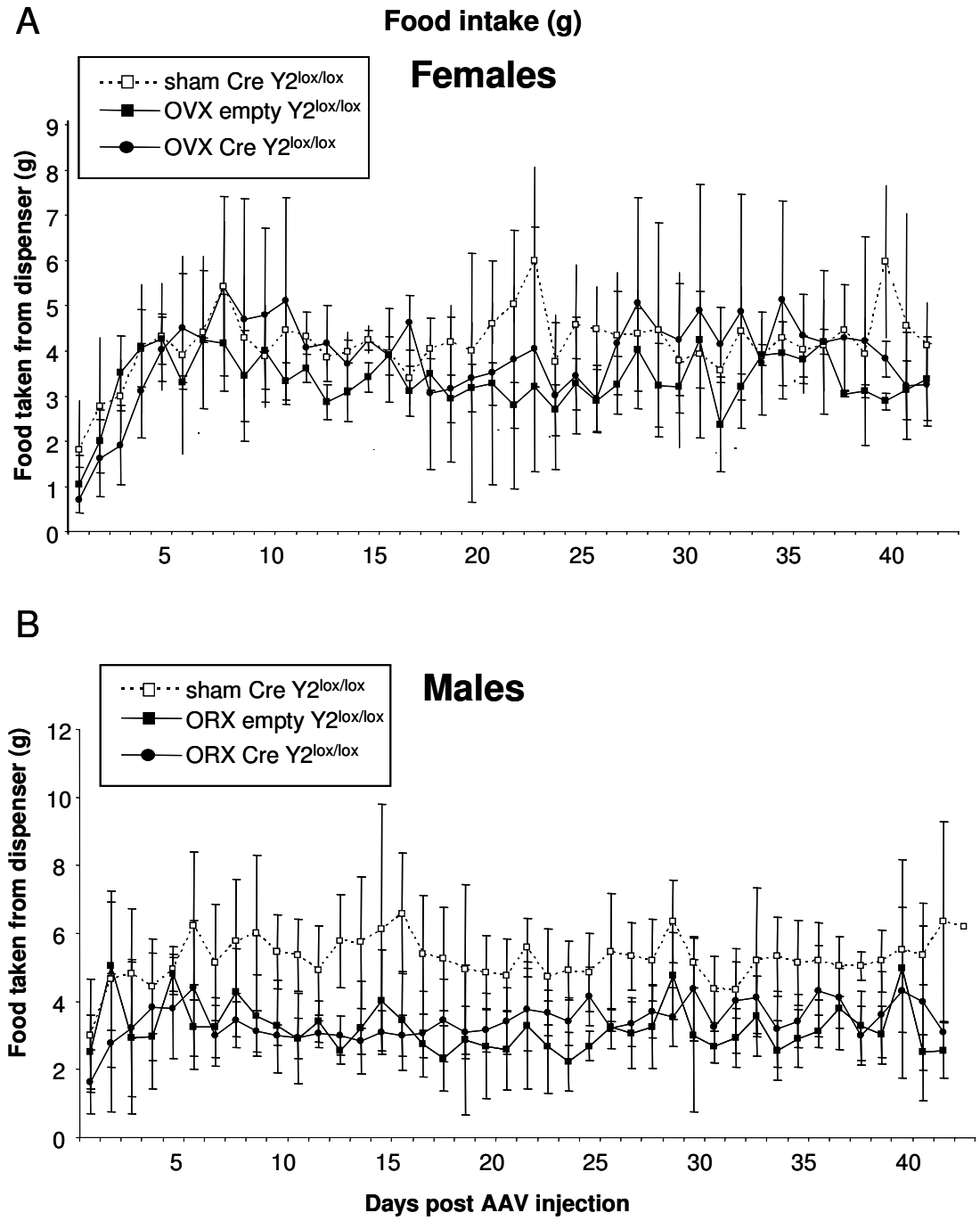

Figure 5.15 Effect of conditional Y2 receptor deletion on food intake of gonadectomised mice. Gonadectomised (OVX and ORX) and sham-operated female (A), and male (B), mice. Data are means ± std dev.

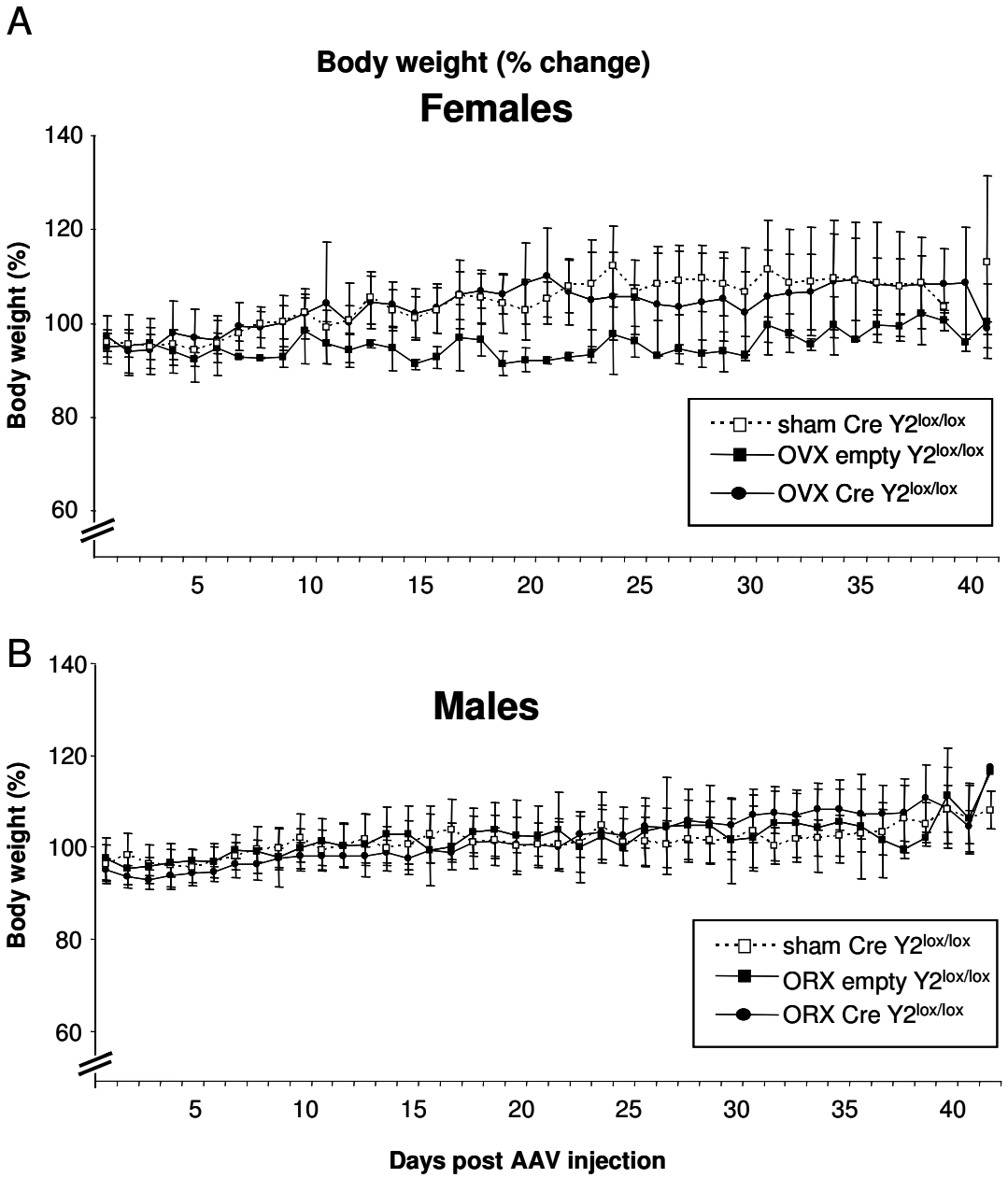

Figure 5.16 Effect of conditional Y2 receptor deletion on body weight of gonadectomised mice. Gonadectomised (OVX and ORX) and sham-operated female (A), and male (B), mice. Data are means ± std dev.

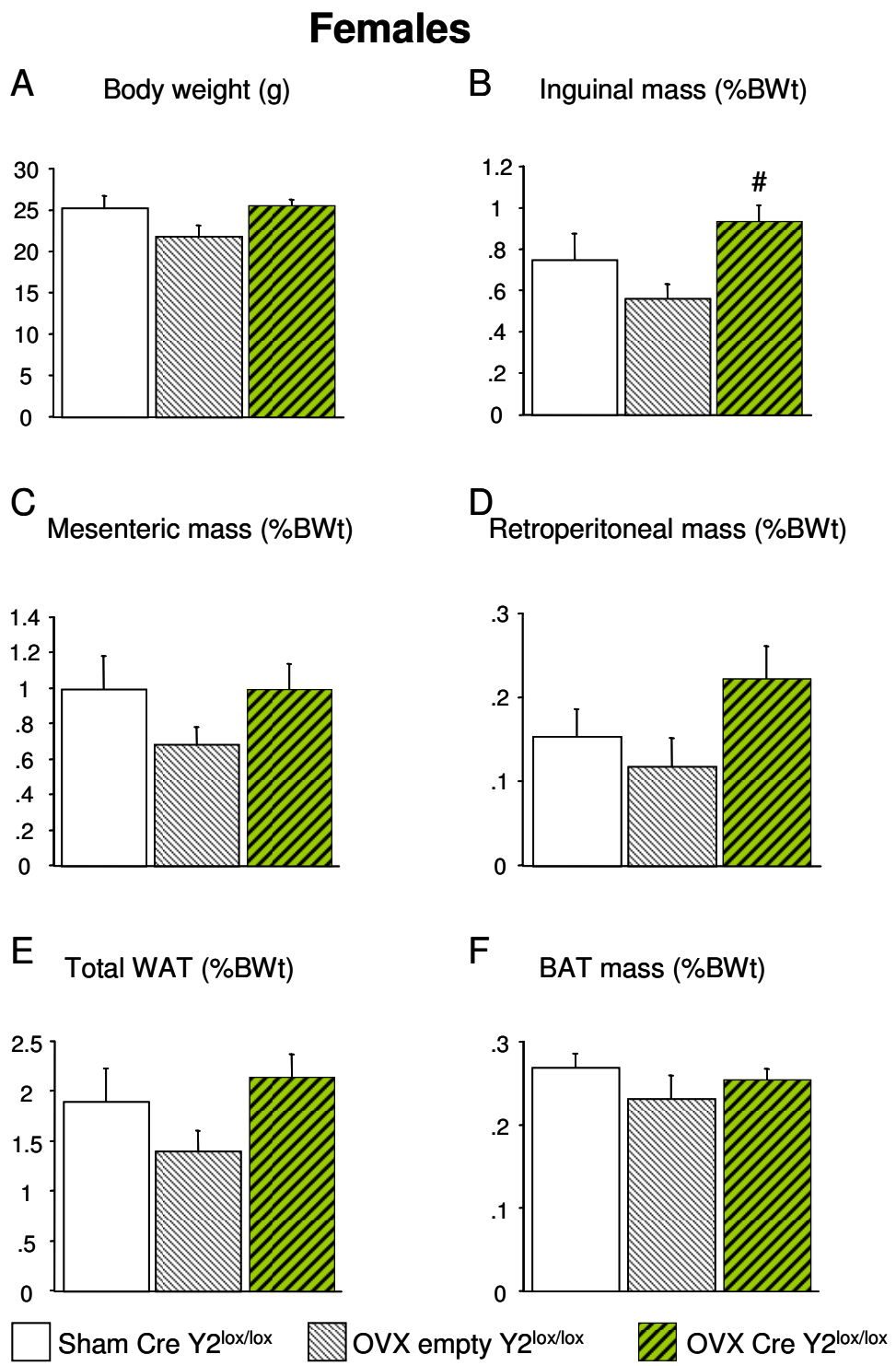

Figure 5.17 Effect of ovariectomy and conditional Y2 receptor deletion on body composition of female mice. Body weight (BWt) (A), inguinal (B), mesenteric (C), retroperitoneal (D), total white adipose tissue (E), and brown adipose tissue (F), of ovariectomised and sham-operated female mice. Hashes indicate significant differences versus OVX empty $Y2^{lox/lox}$, #<0.05.

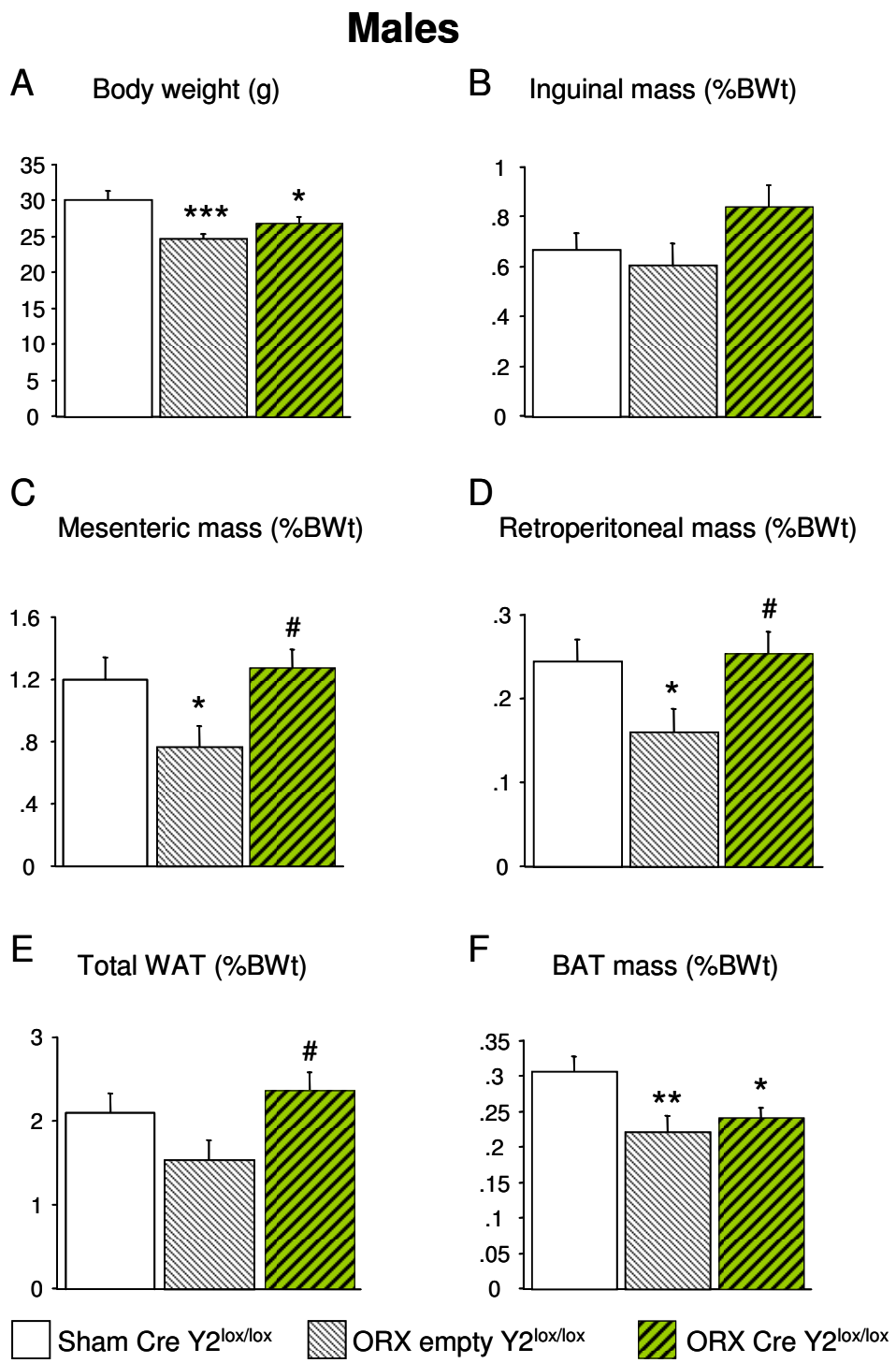

Figure 5.18 Effect of orchidectomy and conditional Y2 receptor deletion on body composition of male mice. Body weight (BWt) (A), inguinal (B), mesenteric (C), retroperitoneal (D), total white adipose tissue (E), and brown adipose tissue (F), of orchidectomised and sham-operated male mice. Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$, *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus ORX empty $Y2^{lox/lox}$, #<0.05.

FEMALES:

	Sham Cre $Y2^{lox/lox}$	ORX empty $Y2^{lox/lox}$	ORX Cre $Y2^{lox/lox}$
IGF-1 (ng/ml)	338.3 ± 32	282.9 ± 24	278.1 ± 25
Corticosterone (ng/ml)	48.1 ± 13	88.4 ± 16 *	81.2 ± 6
Osteocalcin (ng/ml)	29.6 ± 4	44.6 ± 9	38.9 ± 10

Table 5-5 Effect of ovariectomy and conditional Y2 receptor deletion on serum levels of IGF-1, corticosterone, and osteocalcin.
Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$ *P<0.05.

MALES:

	Sham Cre $Y2^{lox/lox}$	ORX empty $Y2^{lox/lox}$	ORX Cre $Y2^{lox/lox}$
IGF-1 (ng/ml)	263.7 ± 24	276.6 ± 19	218 ± 21
Corticosterone (ng/ml)	32.5 ± 9	86.1 ± 15 **	64.7 ± 12
Osteocalcin (ng/ml)	25.7 ± 7	49.2 ± 8	49.6 ± 12

Table 5-6 Effect of orchidectomy and conditional Y2 receptor deletion on serum levels of IGF-1, corticosterone, and osteocalcin.
Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$ **P<0.01.

5.3.7 Conditional hypothalamic Y2 receptor deletion and gonadectomy-induced bone loss in the distal femur

Six weeks after conditional deletion of hypothalamic Y2 receptors in previously gonadectomised female and male $Y2^{lox/lox}$ mice, BV/TV at the distal femur was two-fold greater compared to bone volume in gonadectomised $Y2^{lox/lox}$ AAV-empty recipient mice (Figure 5.19 A,B,E). This difference in bone volume was due to significantly greater Tb.N in male mice (Figure 5.19 F), with significantly greater Tb.Th in both female and male mice (Figure 5.19 D,G), demonstrating that conditional deletion of hypothalamic Y2 receptors can significantly improve outcome following gonadectomy-induced bone loss in both sexes, with marked protection of bone mass and microarchitecture.

Osteoclast surface was significantly increased by gonadectomy in both groups (AAV-cre and AAV-empty) compared with sham-operated mice (Figure 5.20 A, 5.21 A), indicating that as in the germline Y2 receptor knockouts, conditional deletion of hypothalamic Y2 receptors did not inhibit gonadectomy-induced increases in bone resorption at this skeletal site. MS was unaffected by surgery or by conditional deletion of Y2 receptors (Figure 5.20 B, 5.21 B).

Importantly, and consistent with our findings in germline $Y2^{-/-}$ mice, MAR was again significantly elevated in both sham-operated and gonadectomised mice lacking hypothalamic Y2 receptors, compared with AAV-empty controls (Figure 5.20 C, 5.21 C), with a similar pattern also observed for BFR (Figure 5.20 D, 5.21 D), together demonstrating that despite persistent high resorptive activity, conditional deletion of hypothalamic Y2 receptors can prevent further gonadectomy-induced bone loss in both female and male mice through activation of a central Y2-mediated bone anabolic pathway. Furthermore, this anabolic response was strong enough to produce a difference in bone mass compared with AAV-empty recipient mice within just 6 weeks, with substantial benefits to trabecular microarchitecture.

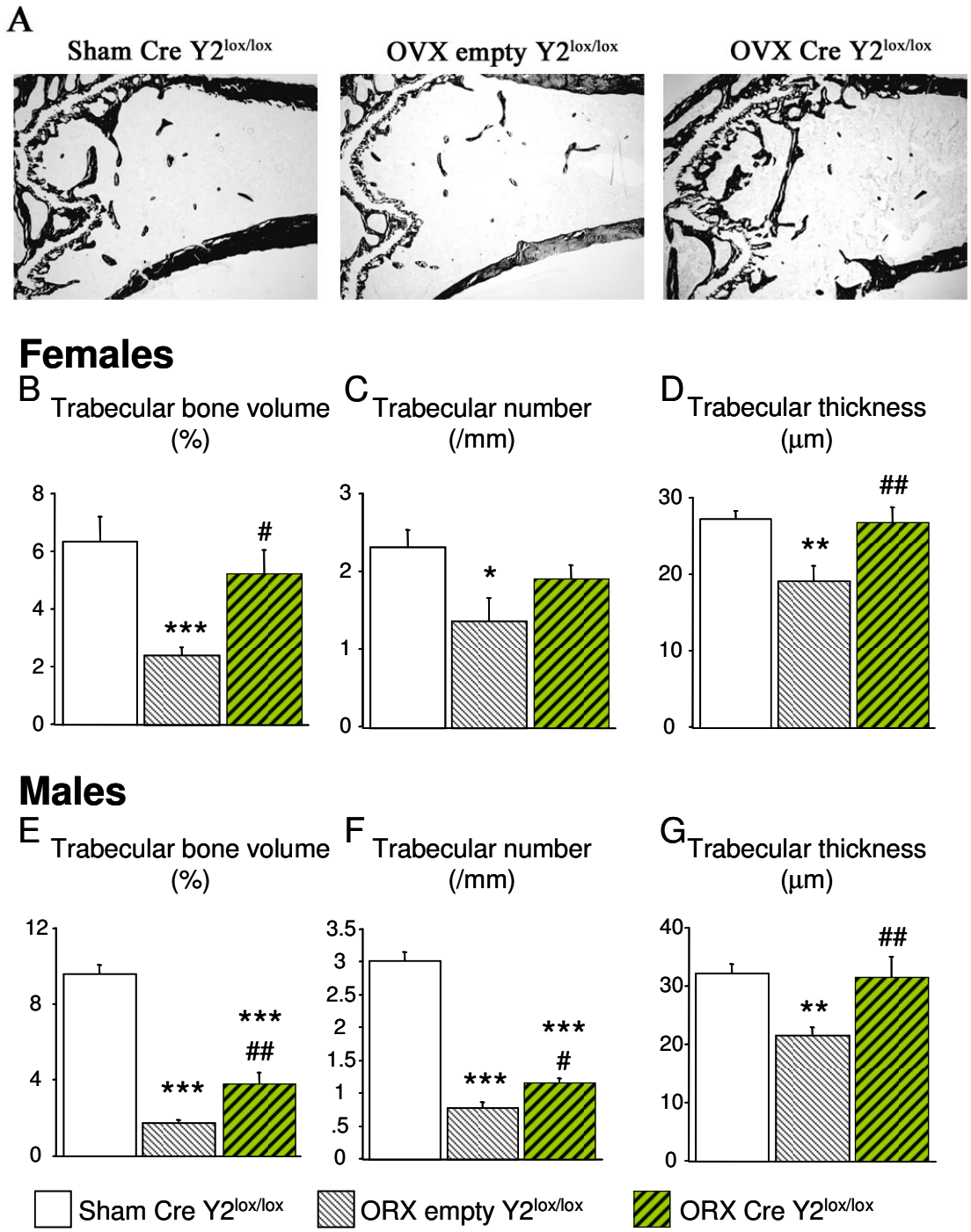

Figure 5.19 Effect of conditional Y2 receptor deletion on trabecular morphology of distal femur of gonadectomised and sham-operated mice.
Female (A-D) and male (E-G) mice. Von Kossa stained section of distal femur from ovariectomised (OVX) mice (A), trabecular bone volume (B,E), trabecular number (C,F), trabecular thickness (D,G). Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$, *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus GX empty $Y2^{lox/lox}$, #<0.05, ##P<0.01.

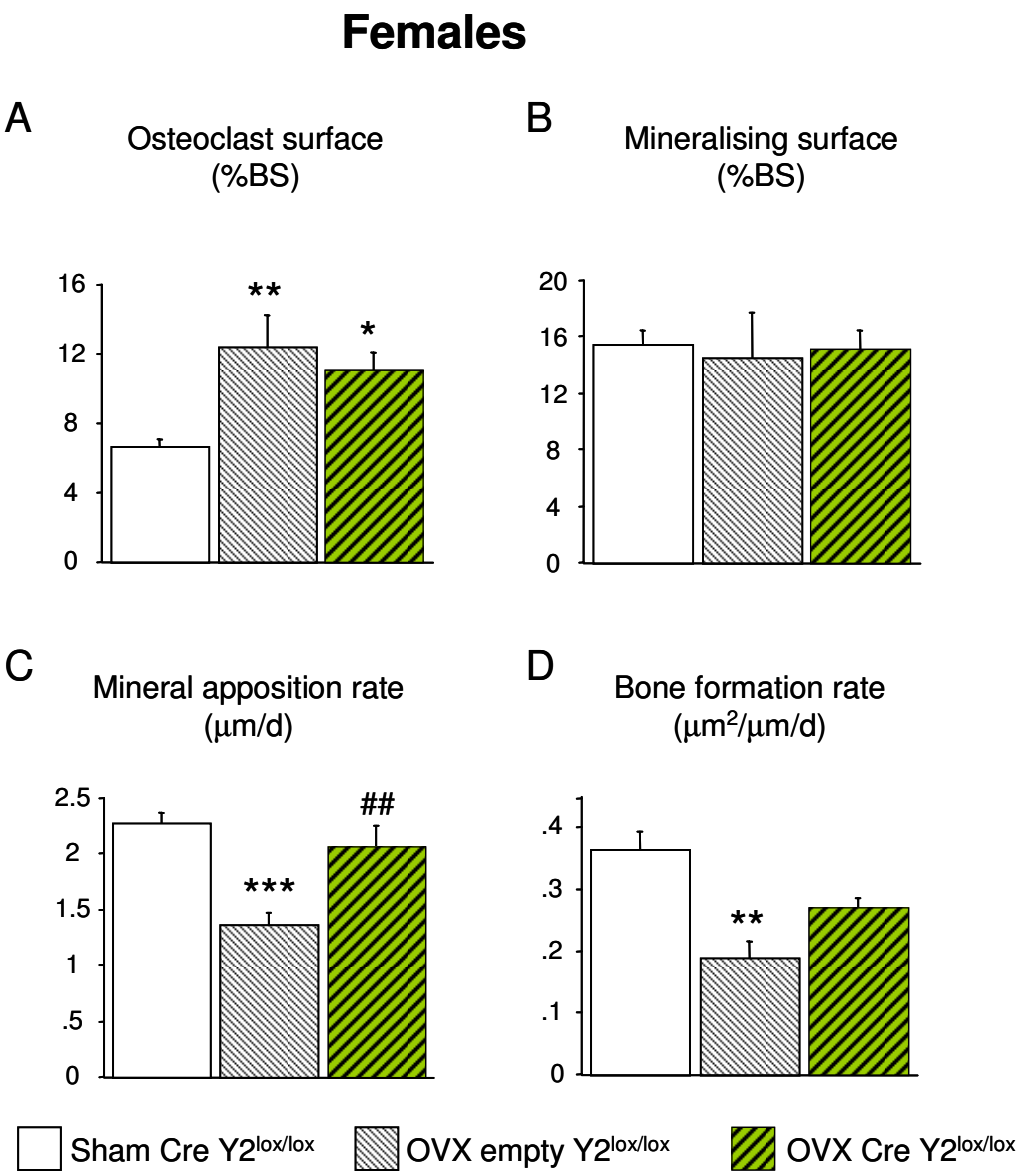

Figure 5.20 Effect of conditional Y2 receptor deletion on parameters of bone resorption and formation in the distal femur of ovariectomised (OVX) and sham-operated female mice.
Osteoclast surface (A), mineralising surface (B), mineral apposition rate (C), bone formation rate (D). Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$, *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus GX empty $Y2^{lox/lox}$, ##P<0.01.

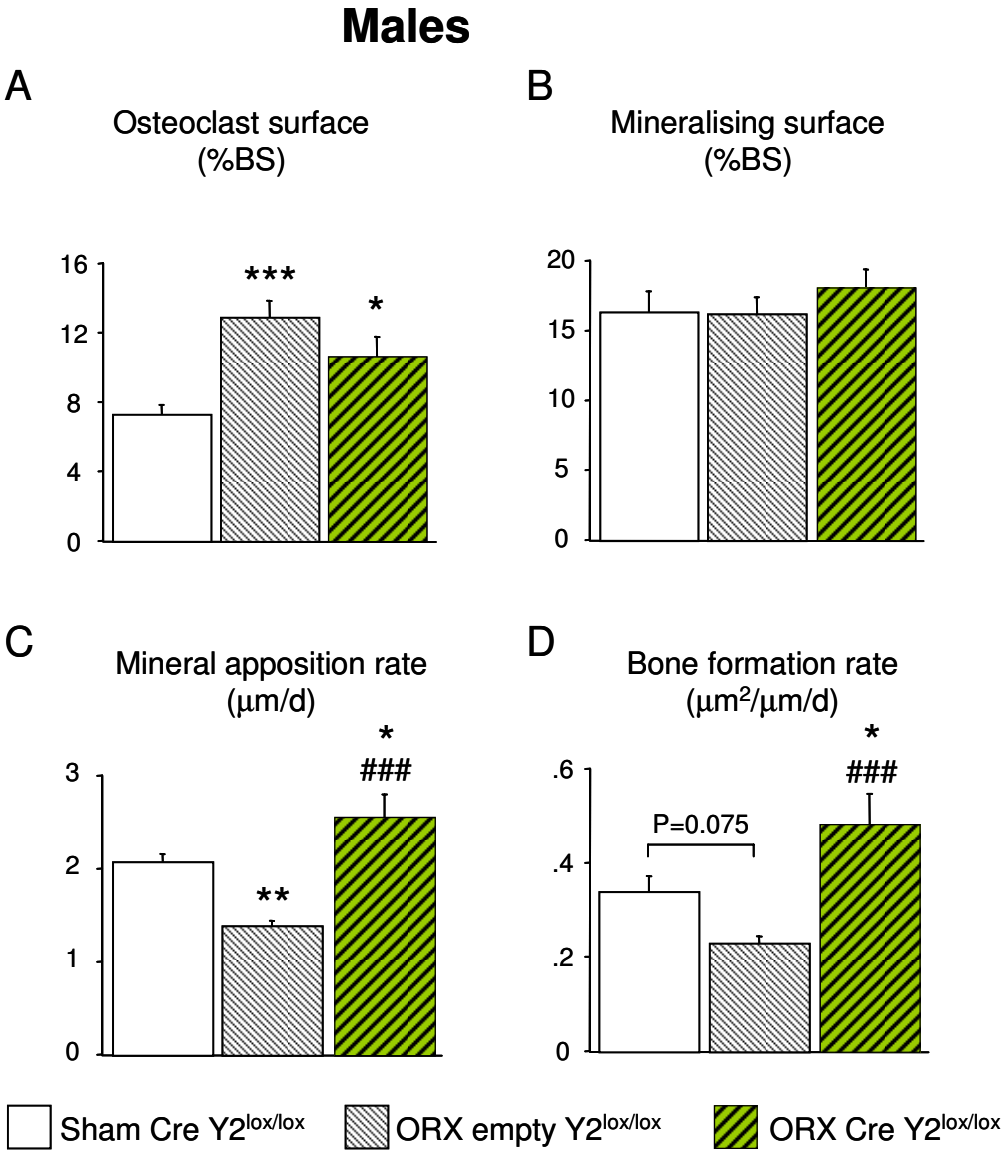

Figure 5.21 Effect of conditional Y2 receptor deletion on parameters of bone resorption and formation in the distal femur of orchidectomised (ORX) and sham-operated male mice.
Osteoclast surface (A), mineralising surface (B), mineral apposition rate (C), bone formation rate (D). Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$, *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus GX empty $Y2^{lox/lox}$, ###P<0.001.

5.3.8 Conditional hypothalamic Y2 receptor deletion and gonadectomy-induced bone loss of the lumbar vertebrae

Analysis of lumbar vertebrae from conditional $Y2^{-/-}$ mice revealed a pattern similar to the distal femur, with conditional deletion of hypothalamic Y2 receptors in gonadectomised $Y2^{lox/lox}$ mice significantly elevating trabecular bone volume compared with AAV-empty controls (Figure 5.22 A,B,E), associated with significantly elevated Tb.Th (data not shown). Osteoclast surface was significantly increased in gonadectomised compared with sham-operated male but not female mice regardless of knockout status (Figure 5.22 C,F). Importantly, and consistent with observations in the distal femur, the greater bone volume after hypothalamus-specific Y2 receptor knockout in gonadectomised mice could be attributed to a significantly greater MAR compared with gonadectomised AAV-empty recipient mice of both genders (Figure 5.22 D,G). These findings demonstrate that conditional deletion of hypothalamic Y2 receptors in the absence of sex hormones elicits an anabolic response in the lumbar vertebrae similar to that of the distal femur, and indicates the activation of a generalised anabolic response in multiple skeletal regions with sex steroid deficiency.

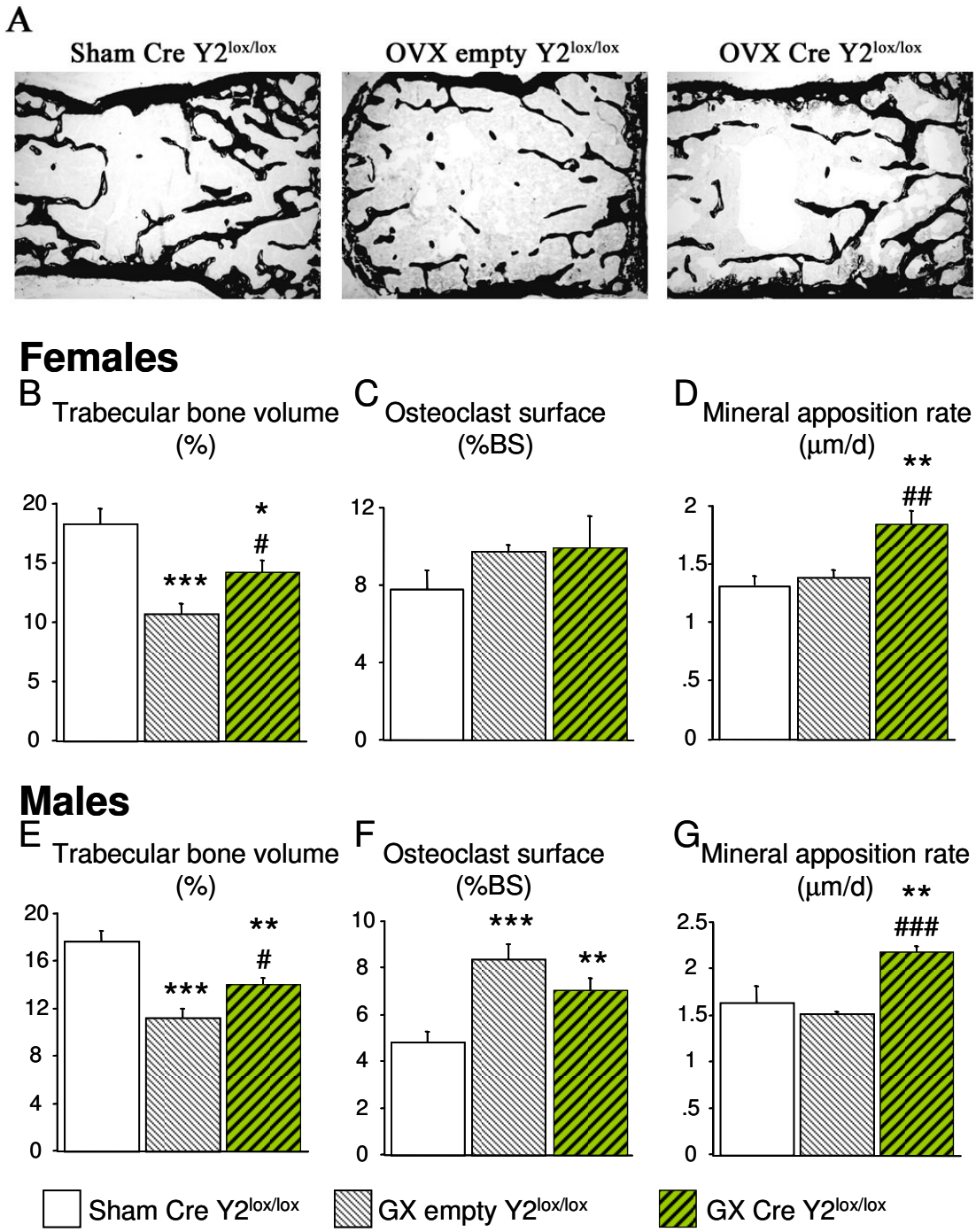

Figure 5.22 Effect of conditional Y2 receptor deletion on trabecular bone and cellular activity of lumbar vertebrae of gonadectomised and sham-operated mice.
Female (A-D) and male (E-G) mice. Von Kossa stained section of distal femur from ovariectomised (OVX) mice (A), trabecular bone volume (B,E), osteoclast surface (C,F), and mineral apposition rate (D,G). Asterisks indicate statistically significant differences versus sham Cre $Y2^{lox/lox}$, *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus GX empty $Y2^{lox/lox}$, #<0.05, ##P<0.01, ###P<0.001.

5.3.9 Effect of Y2 anabolic pathway on progressive bone loss in the distal femur

The consequence of activating the Y2-mediated anabolic pathway in adult mice following the occurrence of significant gonadectomy-induced bone loss in the distal femur is apparent when compared to the effects of gonadectomy in wild type mice. In wild type mice, BV/TV was reduced by 50% in the distal femur 8 weeks after gonadectomy in either sex (Figure 5.23 A,D). This bone volume continued decreasing to 14 weeks post-operation in AAV-empty recipient mice. Importantly, this latter phase of bone loss was blocked in mice in which hypothalamic Y2 receptors were deleted 6 weeks prior to collection, leaving these mice with twice the amount of bone volume compared with control AAV-empty recipient mice (Figure 5.23 A,D). Oc.S was comparably elevated in all gonadectomy groups compared with sham-operated wild type mice (Figure 5.23 B,E). However, MAR was elevated only in mice deficient in hypothalamic Y2 receptors (Figure 5.23 C.F), providing the first demonstration that the central Y2-associated anabolic response can overcome elevated bone resorption induced by sex-hormone deficiency and effectively prevent further bone loss in the distal femur.

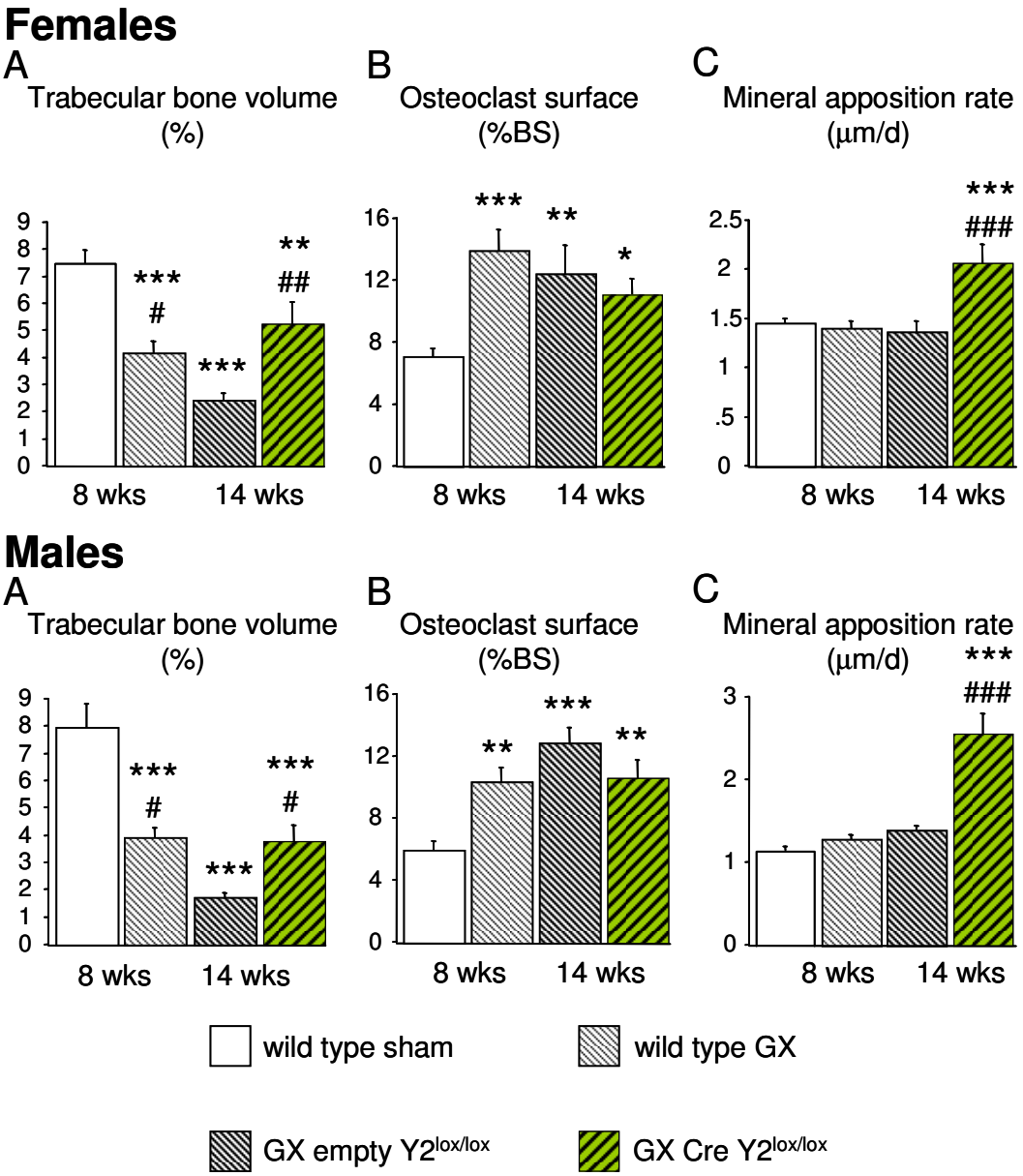

Figure 5.23 Comparison of the effects of gonadectomy after 8 weeks and 14 weeks on bone loss in the distal femur.
Female (A-C) and male (D-F) control and conditonal $Y2^{-/-}$ mice. Trabecular bone volume (A,D), osteoclast surface (B,E), mineral apposition rate (C,F). Asterisks indicate statistically significant differences versus sham-operated wild type, *P<0.05, **P<0.01, ***P<0.001. Hashes indicate significant differences versus GX empty $Y2^{lox/lox}$, #<0.05, ##P<0.01, ###P<0.001.

5.4 DISCUSSION

5.4.1 Role of Y receptors in the regulation of adipose in the absence of sex hormones

Sex hormones play an important role in regulating body fat, with levels of intra-abdominal fat increased up to 49% in post-menopausal compared with pre-menopausal women even when adjusted for age (Toth et al., 2000). Our study also demonstrates a significant increase in WAT content in ovariectomised wild type mice. This response has previously been observed in rodents following gonadectomy, and is associated with hyperphagia and increased deposition of fat even in pair-fed animals, suggesting global alterations in energy homeostasis (Bagi et al., 1997; Liang et al., 2002).

Body weight of female germline $Y1^{-/-}$ mice was significantly greater compared with wild type, associated with greater adiposity consistent with the previous characterisation of ovary-intact $Y1^{-/-}$ mice (Kushi et al., 1998). However, similar to the response to ovariectomy in wild type mice, body weight and fat mass also increased in $Y1^{-/-}$ mice following ovariectomy. Regular monitoring of mice from surgery until collection revealed a noticeably greater increase in body weight in ovariectomised $Y1^{-/-}$ mice compared with all other groups, suggesting that the adipose response in $Y1^{-/-}$ mice to ovariectomy may be amplified over the response observed in ovariectomised wild type mice. However, although deletion of Y1 receptor signalling did not alter the overall adipose response to ovariectomy, the deposition of adipose at discrete sites in ovariectomised $Y1^{-/-}$ mice differed from the response observed in wild type mice, suggesting that Y1 receptors may play a role in the regulation of site specific adipose deposition in the absence of sex hormones in females. The regional distribution of fat is known to be regulated by several factors, for example sex hormones, with greater deposition of fat in gluteo-femoral regions in females while males appear to store more fat in the abdominal region (Blaak, 2001). Central, rather than subcutaneous depots are also thought to be under greater influence by circulating or systemic factors. For example, stress or the administration of corticosterone results in greater deposition of mesenteric adipose compared with other regions (Rebuffe-Scrive et al., 1992). In this study, corticosterone levels were not altered in female $Y1^{-/-}$ mice compared with wild type, while previous studies have demonstrated that levels of the sex

hormones testosterone and estrogen were also not altered by Y1 receptor deletion (A. Sainsbury, personal communication) (Sainsbury et al., 2006). However the different responses of specific fat depots in wild type and $Y1^{-/-}$ mice to ovariectomy suggests Y1 receptors are either directly or indirectly involved in the regulation of adipose deposition in the sex hormone deficient state in female mice.

In contrast to $Y1^{-/-}$ mice, female germline $Y2^{-/-}$ mice were resistant to the increase in body weight and adiposity following ovariectomy indicating an important role also for Y2 receptors in the regulation of adipose in hypogonadal female mice. Conditional deletion of hypothalamic Y2 receptors in ovariectomised female mice resulted in an adipose phenotype similar to sham-operated controls, suggesting that similar to deletion of germline Y2 receptors, central Y2 receptor deletion was also able to reduce adiposity following ovariectomy. Unexpectedly however, in this study adiposity was also not increased in ovariectomised AAV-empty recipient mice, with a continuous trend for reduced body weight in these mice following injection of the virus (Figure 5.17). The reason for this is not clear but indicates that caution should be taken when interpreting the adipose findings from this part of the study.

Studies in humans (Escobar et al., 2004), and rodents (Ainslie et al., 2001; Shimizu et al., 1996), have established a potential role for elevated NPY in the accumulation of WAT that occurs with menopause. The effects of NPY can be reversed by administration of estradiol which decreases levels of hypothalamic NPY (Bonavera et al., 1994), demonstrating an inverse relationship of estrogen on NPY expression and lending explanation to the increased adiposity observed in postmenopausal women. Our findings are supportive of a role for the NPY system in the regulation of WAT content following the removal of gonadal hormones, and strongly suggests a role for Y2 receptors in the control of this process. Estrogen deficiency is also associated with alterations in sympathetic activity, with increased sympathetic tone, circulating levels of noradrenaline, and elevated blood pressure particularly in early post-menopausal women, symptoms which can be attenuated by administration of HRT (Brownley et al., 2004). The NPY system has a known role in the regulation of sympathetic activity, with the colocalisation of NPY and noradrenaline in

sympathetic nerve endings. NPY is a potent and long-acting vasoconstrictor and several lines of evidence demonstrate a role for NPY in modulating the sympathetic nervous activity to control blood pressure (Silva et al., 2002). The vasoactive effect of NPY is mediated by the Y1 receptor present in smooth muscle cells; however, NPY also potentiates the noradrenaline-induced vasoconstriction through the Y1 receptor, suggesting interaction between catecholamines and NPY (Silva et al., 2002). Indeed, sympathetic activity is also modulated by NPY, with inhibition of noradrenaline release from sympathetic nerve terminals by actions of NPY at prejunctional Y2 receptors (Donoso et al., 2006; Silva et al., 2002). These findings together indicate that NPY and the Y receptor system may also play a role or contribute to alterations in sympathetic activity in the postmenopausal state.

In this study orchidectomy of male wild type mice caused a reduction in body weight and adiposity. Male sham-operated $Y1^{-/-}$ mice in this study were heavier than wild type, with some increases in WAT mass depots, consistent with findings from other studies (Kushi et al., 1998; Pedrazzini, 2004; Sainsbury et al., 2006). Investigation of the adipose response of $Y1^{-/-}$ mice to orchidectomy revealed an opposite response to that observed in wild type mice, with increased weights of inguinal, mesenteric, and total WAT mass following orchidectomy of $Y1^{-/-}$ mice, suggesting intact Y1 receptor signalling is required for appropriate adipose deposition in the absence of sex hormones in male mice. In contrast, male germline $Y2^{-/-}$ mice were lighter compared with wild type but did not differ in their adipose response to gonadectomy. Specific deletion of only hypothalamic Y2 receptors however, resulted in increased adipose deposition. These differences in the regulation of WAT mass in orchidectomised male germline and hypothalamic $Y2^{-/-}$ mice are indicative of site specificity, suggesting that Y2 receptors at specific locations within the hypothalamus may be involved in the regulation of adipose in sex hormone deficiency, and are not revealed by ubiquitous deletion of all Y2 receptor signalling.

NPY is one of the most potent orexigenic peptides known, however the precise mechanism of its regulation through its downstream receptors is not entirely understood. Studies using selective Y1 and Y5 receptor agonists and antagonists have demonstrated these receptor subtypes to be important for regulating the effects of NPY on feeding, however

contradicting studies in which Y5 receptor antagonists failed to reduce obesity also exist (MacNeil and Kanatani, 2006). Furthermore, these studies have also revealed differences in the response to activation of central and peripheral receptors, with central injection of the Y2/Y5 agonist PYY(3-36), a PYY metabolite, acting as an orexigen, but acting anorectically when given peripherally (MacNeil and Kanatani, 2006). These findings support the present study in which differential responses of adipose were observed in response to alterations in either central and germline Y receptor signalling.

5.4.2 Role of Y receptors and gonadectomy-induced bone loss

Pre-existing deletion of Y1 receptor signalling in female mice appeared to provide some protection against gonadectomy-induced bone loss in the distal femur with levels only reduced to those of wild type sham-operated mice. This decrease in bone volume was most likely attributable to elevations in bone resorption, probably the direct result of sex hormone deficiency. Male $Y1^{-/-}$ mice however, were not resistant to orchidectomy-induced bone loss with a nearly 4-fold decrease in BV/TV from sham-operated levels, reducing levels to those of orchidectomised wild type mice. In addition to elevated bone resorption as evidenced by elevated Oc.S, the greater MAR of the $Y1^{-/-}$ model was abolished following orchidectomy, likely contributing to the significantly greater loss of bone volume in these mice compared with females. A gender-specific elevation in serum corticosterone levels was also apparent in orchidectomised $Y1^{-/-}$ mice compared with sham-operated mice, which may also have contributed to the greater decrease in bone volume in these mice. Of note, the anabolic activity of the $Y1^{-/-}$ model was also abolished by crossing $Y1^{-/-}$ mice onto the leptin deficient and sterile ob/ob background (section 4.3.4). Although in the study described here, MAR was maintained in female $Y1^{-/-}$ mice following ovariectomy, these data together suggest that the anabolic activity of the $Y1^{-/-}$ model may be in part dependent on the presence of sex hormones. These findings are supported by recent studies by our laboratory demonstrating that the anabolic activity of the Y1-mediated pathway is not mediated by central receptors, with conditional deletion of hypothalamic Y1 receptors failing to stimulate bone formation and trabecular bone volume, suggesting the Y1-anabolic pathway may be more likely to respond to circulating or systemic factors.

In contrast to the $Y1^{-/-}$ model, the elevated MAR in distal femur of the $Y2^{-/-}$ model was maintained both following gonadectomy, and when crossed onto the ob/ob background (section 4.3.5), indicating this pathway to be independent of the presence of sex hormones, and demonstrating that the loss of bone volume seen in these models was the result of elevated osteoclastic activity. Interestingly, histological analysis of the lumbar vertebrae of $Y2^{-/-}$ mice revealed a lack of a high trabecular bone mass phenotype in this region in the presence of normal levels of sex hormones. However, in the absence of sex hormones an anabolic response was activated, resulting in some protection of bone loss in female germline $Y2^{-/-}$ mice, and a significantly greater BV/TV in both male and female conditional $Y2^{-/-}$ mice compared with Y2 receptor replete controls. Unexpectedly, there was also some indication that bone resorption following gonadectomy was blunted in lumbar vertebrae particularly of germline $Y2^{-/-}$ mice, with Oc.S in lumbar vertebrae of gonadectomised $Y2^{-/-}$ mice significantly lower compared with gonadectomised wild type. In contrast, Oc.S in distal femur of gonadectomised germline and conditional $Y2^{-/-}$ mice was equivalent to gonadectomised wild type or AAV-empty recipient mice, suggesting site-specificity in this response. It is possible that the acute resorption response in the lumbar vertebrae in the absence of sex hormones occurred faster in $Y2^{-/-}$ mice, or that the peak Oc.S was simply lower than for wild type, however, these findings suggest that deletion of Y2 signalling may also affect the osteoclast lineage in lumbar vertebrae following gonadectomy.

In the distal femur, while constitutive activation of the bone anabolic response in germline $Y2^{-/-}$ mice did not entirely protect against sex hormone-deficient bone loss, selective activation of only the hypothalamic Y2-associated bone formation response following the occurrence of significant hypogonadal bone loss effectively prevented further bone loss. Of particular interest, this prevention of bone loss occurred despite elevated bone resorption, and thus was specifically attributable to a Y2-associated anabolic response of osteoblastic cells. The end result was a doubling of trabecular bone volume of gonadectomised mice lacking hypothalamic Y2 receptors compared with their counterparts with an intact Y2 receptor gene. Notably, the marked improvement in bone volume seen in this study occurred within just 6 weeks of hypothalamic Y2 receptor deletion, a clear demonstration

of the potency of this central anabolic pathway. Osteoporotic patients at risk of fragility fractures are often only identified after considerable loss of bone has occurred, or after the occurrence of an initial osteoporotic fracture. Therefore the ability to completely halt further bone loss with the added potential of this pathway to actually increase bone formation to restore lost bone mass is of extreme clinical importance. The potential ability of this activated anabolic response to restore lost bone mass requires further investigation using a longer period of time post-operation to investigate this possibility further.

The reason why constitutive activation of the Y2-mediated bone formation response due to germline Y2-knockout did not provide more resistance to gonadectomy-induced bone loss, whereas activation of this pathway in adults prevented any further bone loss is not clear, however there are a number of possible explanations for this finding. Osteoclast surface was reduced at the 22 week time point in ovariectomised conditional $Y2^{-/-}$ female mice compared with 16 week old ovariectomised germline $Y2^{-/-}$ mice. This finding is not unexpected as the gonadectomy-induced increase in resorption is an acute response, which rapidly diminishes (Baldock et al., 1998; Wronski et al., 1989). MAR was also higher in orchidectomised conditional $Y2^{-/-}$ male mice compared with germline knockouts, suggesting that a greater osteoblastic response in the conditional knockouts may be responsible for the protective effect on orchidectomy-induced bone loss. As the anabolic bone formation response was actually active in both male and female gonadectomised germline $Y2^{-/-}$ mice, as evidenced by a greater MAR and BFR, it is possible that the only reason why a protective effect was observed in the conditional knockouts and not in the germlines, was a longer time post-operation, allowing the anabolic response to overcome the acute increase in resorption.

Of some interest, was the observation that female sham-operated conditional $Y2^{-/-}$ mice did not display the high trabecular bone mass phenotype observed in the male distal femur, despite an elevated rate of mineral apposition compared with AAV-empty recipient mice. This suggests that the central Y2-anabolic pathway is not gender specific, as deletion of hypothalamic Y2 receptors was successful in eliciting the osteoblastic bone formation response in these mice. Rather, that the response in female mice was either not great

enough to produce a corresponding increase in trabecular bone volume, or alternatively, that the response was slower compared with the response in male mice, and would therefore require a longer duration to produce a corresponding increase in bone volume. Further studies investigating the longer-term effects of conditional deletion of hypothalamic Y2 receptors would need to be performed to address these questions.

Higher body weight is associated with higher bone mineral density in human studies (Reid et al., 1992a). The greater body weight of $Y1^{-/-}$ mice may contribute to their greater bone volume, however adipose content of these mice increased in both male and female mice following gonadectomy and therefore does not correspond with the observed decrease in bone. The data presented here also show that the increased bone volume of gonadectomised Y2 receptor knockout mice was not due to increased body weight, as body weight and fat mass were actually reduced in germline Y2-knockout mice, and unchanged in conditional knockout mice. IGF-1 is known to affect bone growth and turnover (McCarthy and Centrella, 2001), and Y2 receptors regulate serum IGF-1 concentrations under conditions of elevated NPY-ergic expression (Sainsbury et al., 2002b). Serum IGF-1 was elevated in $Y1^{-/-}$ models compared with wild type, suggesting an endocrine mechanism by which Y1 receptor deletion increases bone mass, but were unchanged by gonadectomy. Serum IGF-1 concentrations however, were unaffected by Y2 receptor deletion. Elevated corticosterone decreases bone mass, however, while this may contribute to the greater bone loss of orchidectomised $Y1^{-/-}$ mice, this also cannot explain the bone differences observed in $Y2^{-/-}$ mice. Ovariectomised wild type mice had greater serum corticosterone compared with ovariectomised $Y2^{-/-}$ mice, which could possibly influence bone volume, however, corticosterone was unchanged in sham-operated $Y2^{-/-}$ compared with wild-type mice suggesting this is unlikely. Moreover, serum corticosterone was actually greater in male germline $Y2^{-/-}$ mice compared with wild type, which would be expected to produce a decrease, rather than the observed increase in bone volume. In conditional $Y2^{-/-}$ mice, serum corticosterone levels in both male and female mice were similar between both gonadectomy groups, again arguing against changes in corticosterone levels being responsible for the bone changes.

5.4.3 Implications of study findings and relevance to osteoporosis

In osteoporosis, excess osteoclastic resorption results in deep resorption pits within bone. As these pits deepen trabecular perforation and loss of trabecular structures occurs. A reduction in bone formation capacity contributes to the deterioration of bone microarchitecture, as lost bone material is not adequately replaced (Inoue et al., 1997; Weinstein and Manolagas, 2000; Yudoh et al., 2001). Anabolic treatments to balance out excess osteoclastic activity are therefore essential. In this study, conditional deletion of hypothalamic Y2 receptors in gonadectomised female and male adult mice resulted in significantly greater trabecular thickness compared with AAV-empty recipient mice, and most importantly, greater trabecular number in orchidectomised conditional knockout mice compared with AAV-empty controls. A similar but non-significant trend was noted in female mice. These data suggest that the Y2 receptor-associated anabolic pathway not only improves total bone mass, but also results in a beneficial microarchitectural outcome compared with gonadectomised wild type mice.

These beneficial effects on bone volume and microarchitecture occurred in the face of elevated resorption. It is possible that even more bone mass could be replaced over a longer period of time after conditional deletion of hypothalamic Y2 receptors. It will also be important to investigate whether concurrent or sequential administration of an anti-resorptive treatment in combination with Y2 receptor deletion would suppress osteoclast activity sufficiently to allow a more effective anabolic response by the $Y2^{-/-}$ pathway. Interestingly, recent studies of co-administration of the only available anabolic therapy, parathyroid hormone (PTH1-34) with anti-resorptive bisphosphonate indicated that the anti-resorptive treatment may reduce the anabolic potential of PTH (Black et al., 2003; Finkelstein et al., 2003). These studies are yet to be performed for the Y2 receptor anabolic pathway, however, the studies so far demonstrate that inactivation of hypothalamic Y2 receptor signalling can prevent continued loss of bone by stimulating bone formation in gonadectomised adult mice, and may present a promising avenue for osteoporosis treatment.

Chapter 6

Effect of central Y2 receptor deletion on bone loss in aging

6.1 INTRODUCTION

With the exception of sex hormone deficiency, aging is the leading contributing factor for the development of idiopathic osteoporosis. Age-related bone loss occurs from around 40 years of age and progresses at a rate of about 0.3% to 1% per year in both men and women (Manolagas and Jilka, 1995; Overton and Basu, 1999; Rehman et al., 1994). This loss of bone occurs with a reduction in the amount of bone formed with each remodelling cycle, but in contrast to the loss of bone mass that occurs in the absence of sex hormones as a result of excess osteoclastic activity, bone loss with aging in humans is thought to result from a progressive decline in the supply of osteoblasts in proportion to the demand for them (Parfitt et al., 1997; Parfitt et al., 1995; Rehman et al., 1994). Furthermore, in both men and women, decreased stability resulting in an increased risk of falls with advancing age contributes to an elevation in the incidence of fragility fractures in patients with weakened bones.

The extent and degree of age-related osteoporosis is influenced by several factors, firstly whether optimal skeletal mass was reached during growth which is itself influenced by genetic factors, nutrition, and lifestyle such as smoking and physical activity, levels of sex hormones and levels of sex hormone-binding globulin (SHBG) which can alter the bioavailability of estrogen (Javaid and Cooper, 2002; Legrand et al., 2001; Lormeau et al., 2004; Olszynski et al., 2004), and decreased calcium intake and age-related vitamin D deficiency which can result in secondary hyperparathyroidism and contribute to accelerated bone loss (Lips, 2001; Mosekilde, 2005; Reginster, 2005). Furthermore chronic inflammatory disorders and drugs such as glucocorticoids can also affect bone cell activity resulting in loss of bone mass (Clowes et al., 2005; Raisz, 2005; Tannirandorn and Epstein, 2000). However, the presence of an age-dependent mechanism of bone loss, distinct from confounding factors such as age-related alterations in hormone levels, has been revealed by the failure of hormone replacement therapy to prevent bone loss indefinitely and the occurrence of bone loss with advancing age in sex hormone-replete men (Orwoll and Klein, 1995; Orwoll and Nelson, 1999)

The mechanisms behind underlying age-related bone loss are not well understood; however, analysis of a murine model of age-related osteopenia has demonstrated a marked decrease in osteoblast progenitor number associated with reduced bone formation and reduced bone mass (Jilka et al., 1996). *Ex vivo* stromal cell cultures from these mice also revealed a reduction in their proliferative and osteogenic capacity (Jilka et al., 1996), suggesting that an impairment in osteoblastogenesis with increasing age may be the underlying cause of age-related bone loss in mice. A reduction in the population of marrow stromal and osteoblastic cells is also evident in aged rats, together with reduced levels of bone matrix proteins such as osteocalcin and type I collagen mRNAs expressed by individual osteoblastic cells, which together are likely to contribute to the reduction in bone formation previously reported (Ikeda et al., 1995; Inoue et al., 1997; Roholl et al., 1994; Wronski et al., 1989; Wronski et al., 1985). In human bone marrow, an association of decreased osteoblastogenesis with increasing age has also been confirmed (D'Ippolito et al., 1999). The mechanisms underlying this defect in osteoblast formation are not known; however, some studies have proposed that replicative senescence of mesenchymal stem cells with age, or a decrease in the responsiveness of bone cells to growth factors, hormones and cytokines with increasing age may be involved (D'Ippolito et al., 1999; Rajaram et al., 1997; Yudoh et al., 2001).

In the previous chapter, conditional deletion of hypothalamic Y2 receptors elicited an anabolic response in adult gonadectomised male and female mice and completely prevented further bone loss. This resulted in significantly greater trabecular bone mass in the distal femur and lumbar vertebrae compared with AAV-empty vector injected controls, and demonstrated the Y2-mediated anabolic pathway to function independent of the presence of sex hormones. In this chapter, the response of bone in aged mice to activation of the central Y2-mediated anabolic pathway is investigated. C57/BL6 mice develop a senile osteoporosis-like bone phenotype with decreases in both cortical and trabecular bone (Cao et al., 2003a; Ferguson et al., 2003; Halloran et al., 2002). In this study, deletion of hypothalamic Y2 receptors was performed in 14 month old C57/BL6-129/SvJ $Y2^{lox/lox}$ mice. Distal femora were assessed for trabecular and cortical changes following collection

of mice at 18 months of age to determine whether attenuation of hypothalamic Y2 receptor signalling was able to activate an anabolic response in aged mice, and the effect of this on age-related changes in bone mass.

6.2 METHODS

6.2.1 Study design

Conditional deletion of hypothalamic Y2 receptors was performed in 14 month old male and female $Y2^{lox/lox}$ mice by bilateral injection of recombinant adeno-associated virus (AAV) vector containing the cre-recombinase gene (AAV-cre). Control mice were injected with the viral vector containing an empty cassette (AAV-empty). Whole body bone mineral density was measured by DXA at the time of injection (baseline) and 6, 10, and 14 weeks after injection to monitor any change in bone density that occurred as a result of Y2 receptor deletion. Mice were collected 16 weeks following hypothalamic injection, at 18 months of age.

6.2.2 Adeno-associated virus injection

Anaesthesia and hypothalamic injection of AAV-cre and AAV-empty were performed as described in materials and methods (section 2.4.3, 2.4.4). Bone density was measured in all mice following anaesthesia prior to injection of the virus.

6.2.3 Anaesthesia for bone densitometry

For measurements of bone density 6, 10, and 14 weeks after AAV injection, mice were anesthetised as described in materials and methods (section 2.4.1).

6.2.4 Bone densitometry and body composition analysis

Whole body lean mass, fat mass, bone mineral content (BMC) and bone mineral density

(BMD) was measured in anesthetised mice using a PIXImus DXA as described in materials and methods (section 2.5.1). BMD and BMC were also measured for femur and tibia as described in materials and methods (section 2.5.1).

Following collection and fixation, the excised left femora were scanned as described in materials and methods (section 2.5.1). In addition to scanning the entire femur, femoral scans were analysed in linear thirds, including all mineralised tissue within the distal, shaft and proximal thirds.

6.2.5 Histomorphometry

Following fixation, the right femur was bisected transversely at the midpoint of the shaft, and the distal half embedded, undecalcified, in MMA as described in materials and methods (section 2.3.5). 5μm sagittal sections were analysed as described in materials and methods (section 2.3.6). BV/TV, Tb.N, and Tb.Th were calculated (section 2.3.9.1) from von Kossa stained sections (section 2.3.7.1). Oc.S was estimated (section 2.3.8.3) from TRAP stained sections (section 2.3.7.2). MAR was estimated from unstained sections (section 2.3.8.2).

6.2.6 Peripheral Quantitative Computed Tomography and Micro Computed Tomography

As the pQCT and μ-CT techniques are both non-invasive, the same left femur which was analysed by DXA was also used for analysis by pQCT and μ-CT. Isolated femurs were assessed by pQCT as described in materials and methods (section 2.5.2), with non-invasive cross-sections taken from both the distal femur metaphysis and the femoral diaphysis to determine bone mineral content and volume at the distal femur and at the shaft. Volumetric mineral content, density and area were determined for total, trabecular, and cortical bone. Trabecular parameters were excluded for analysis of the diaphyseal region due to the very low trabecular content within this region. Cortical thickness, periosteal and endocortical circumferences were also determined at both sites, and polar moment of inertia was

calculated at the femoral diaphysis.

Micro-CT was also performed on a cross-section of the distal femur metaphysis as described in materials and methods (section 2.5.3) to determine 3-dimensional parameters of trabecular bone morphology.

6.2.7 Statistics

For multiple bone densitometry measurements performed on the same animal over time, comparisons were made using factorial ANOVA, followed by ANOVA-repeated measures. All analyses between the genotypes were performed using two-tailed unpaired t-tests. StatView version 4.5 (Abacus Concepts Inc, CA) was used for all statistical analyses, and $p < 0.05$ was accepted as being statistically significant

6.3 RESULTS

6.3.1 Bone densitometry and body composition of aged conditional Y2 knockout mice

Bone densitometry of mice was assessed at baseline, and at 6, 10, and 14 weeks following conditional deletion of hypothalamic Y2 receptors in 14 month old mice using DXA, to monitor changes in whole body bone density over time, and to determine an appropriate time point for tissue collection. There was no evidence of change in BMD or BMC at any of the time points measured (data not shown). It was therefore decided to initiate calcein labelling following the final DXA 14 weeks after AAV-injection, and mice were subsequently collected 2 weeks later at 18 months of age.

Body weight, lean mass and fat mass were unchanged in male and female conditional $Y2^{-/-}$ mice 14 weeks following hypothalamic injection of AAV-cre compared with AAV-empty recipient mice (Figure 6.1 A-C, Figure 6.2 A-C). Whole body BMD was significantly lower in male conditional Y2 knockouts compared with controls (Figure 6.1 D); however, no

change in BMC was observed (Figure 6.1 E), suggesting a difference in bone size between the two genotypes. Consistent with this was a slight but non-significant increase in bone area of male conditional $Y2^{-/-}$ mice (Figure 6.1 F). Whole body BMD, BMC and bone area were not significantly different between female controls and conditional $Y2^{-/-}$ mice at the final DXA scan 14 weeks after injection of AAV (Figure 6.2 D-F). However, overall changes in body composition were similar between male and female conditional $Y2^{-/-}$ mice, with a non-significant reduction in fat mass and a trend for increased bone area in $Y2^{-/-}$ mice of both genders (Figure 6.1, Figure 6.2).

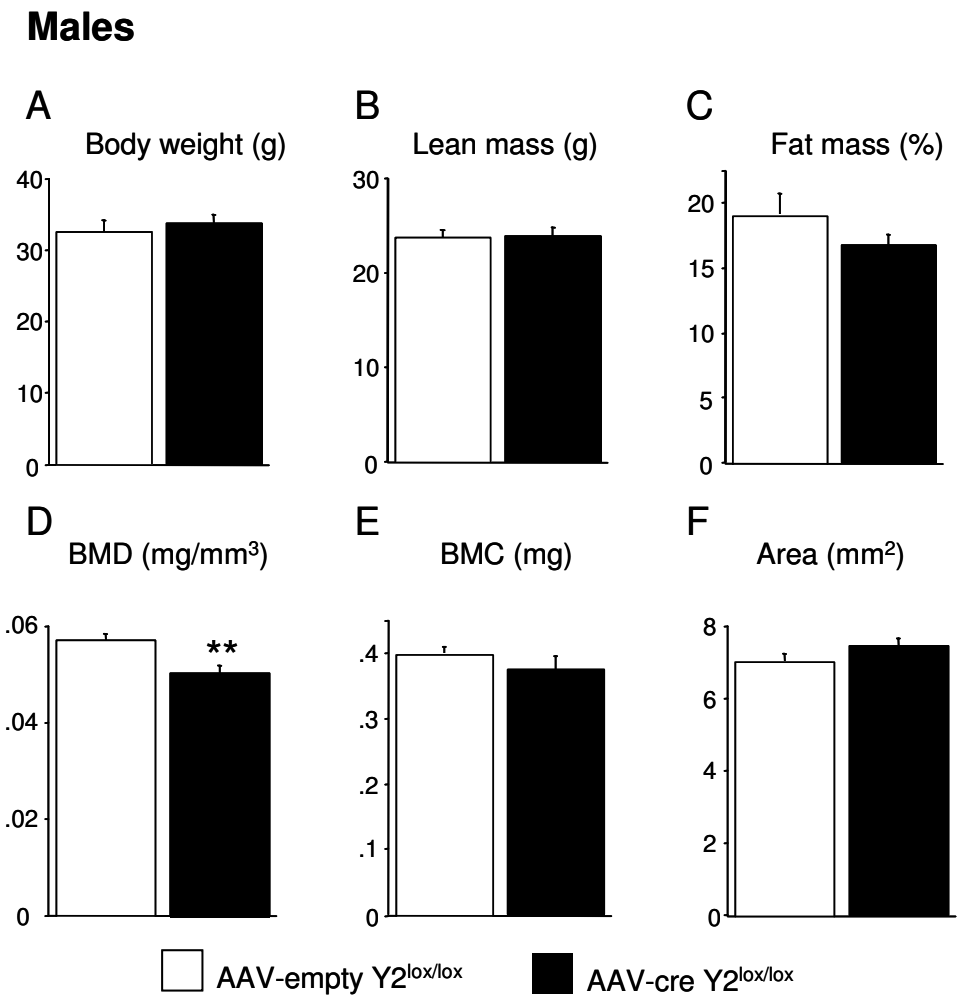

Figure 6.1 Effect of hypothalamic Y2 receptor deletion on body composition and bone mass of aged male mice.
Body weight (A), lean mass (B), fat mass (C), whole body BMD (D), BMC (E) and area (F) measured by DXA 14 weeks following injection of virus. **$p<0.01$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

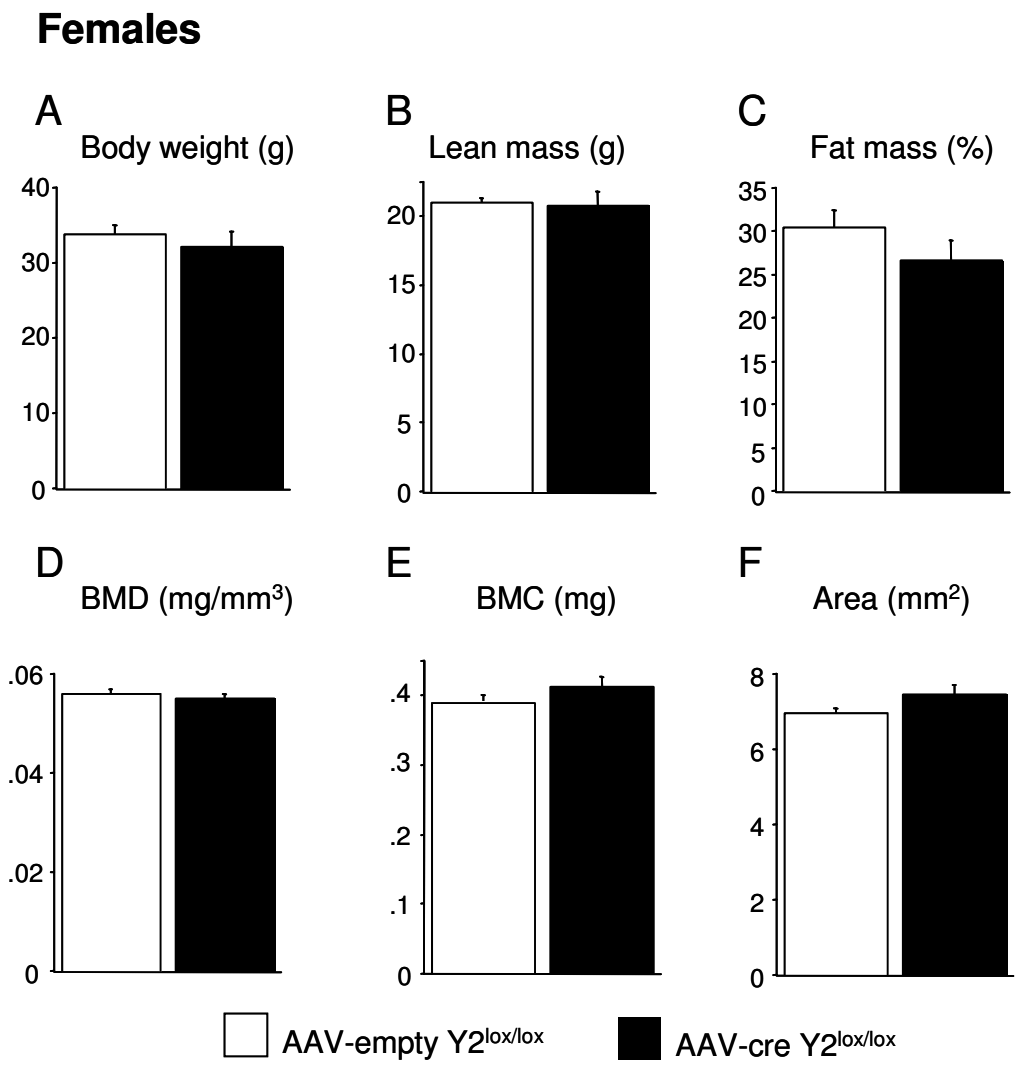

Figure 6.2 Effect of hypothalamic Y2 receptor deletion on body composition and bone mass of aged female mice.
Body weight (A), lean mass (B), fat mass (C), whole body BMD (D), BMC (E) and area (F) measured by DXA 14 weeks following injection of virus. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

Bone densitometry of the right leg, from the hip joint to the ankle joint, and the lumbar vertebrae were also assessed 14 weeks following AAV injection. Leg BMD was significantly lower in both male and female conditional $Y2^{-/-}$ mice compared with controls (Figure 6.3 A, Figure 6.4 A). BMC was unchanged in either sex (Figure 6.3 B, Figure 6.4

B). However as area was unchanged in male $Y2^{-/-}$ mice compared with control, the non-significant reduction in leg BMC likely contributed to the reduced BMD of these mice (Figure 6.3). In contrast, area was significantly greater in female conditional $Y2^{-/-}$ mice accounting for the lower BMD of these mice (Figure 6.4 C).

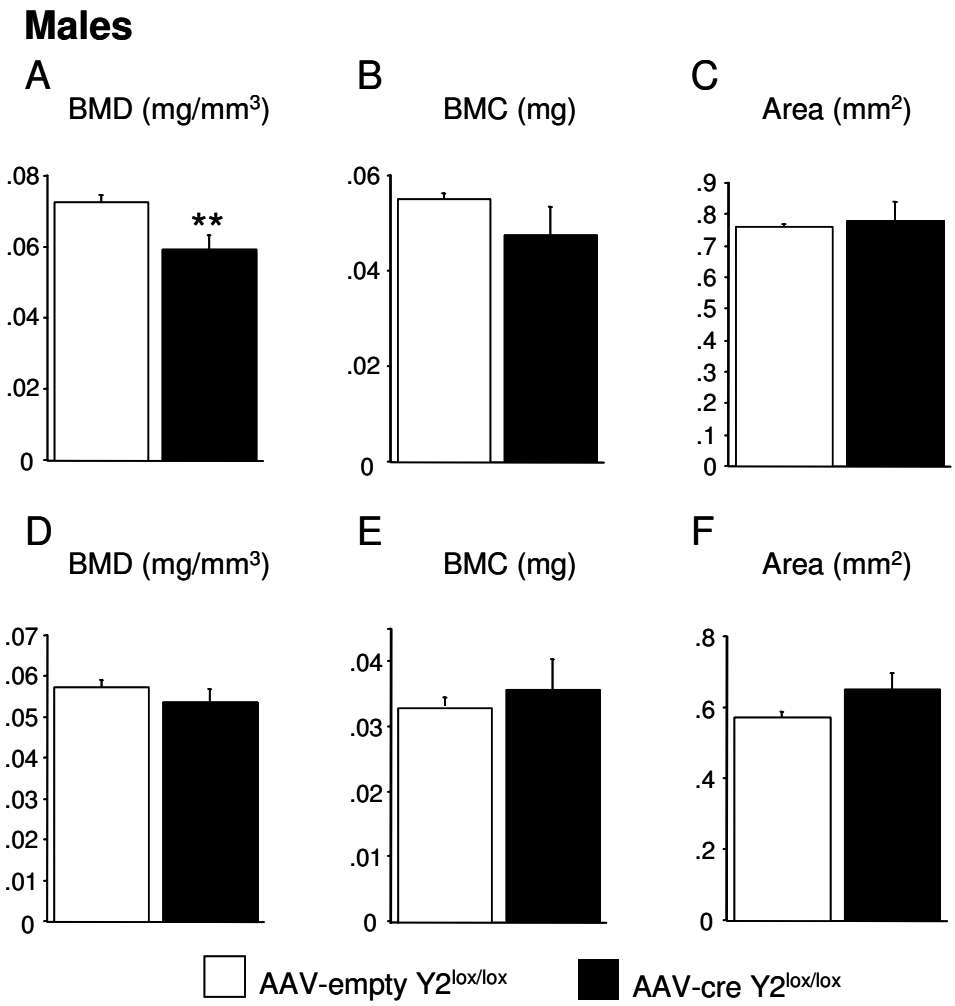

Figure 6.3 Effect of hypothalamic Y2 receptor deletion on bone mass of whole leg and lumbar vertebrae of male mice.
Leg including femur and tibia (A-C) and lumbar vertebrae (D-F) 14 weeks following injection of virus; BMD (A,D), BMC (B,E) and area (C,F) measured by DXA. **p<0.01. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

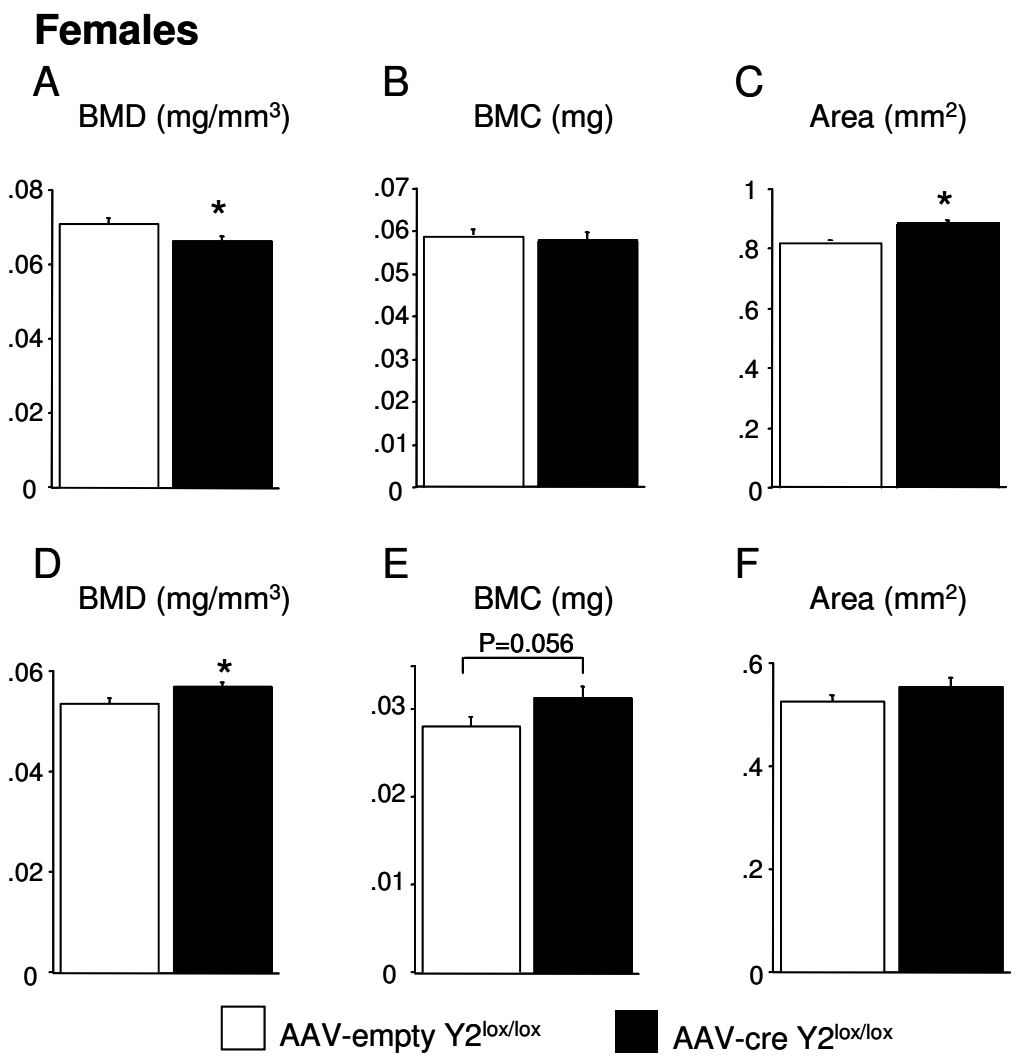

Figure 6.4 Effect of hypothalamic Y2 receptor deletion on bone mass of whole leg and lumbar vertebrae of female mice.
Leg including femur and tibia (A-C) and lumbar vertebrae (D-F) 14 weeks following injection of virus; BMD (A,D), BMC (B,E) and area (C,F) measured by DXA. *$p<0.05$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

In male conditional $Y2^{-/-}$ mice, lumbar BMD, BMC and area were unchanged compared with controls (Figure 6.3 D-F). Lumbar vertebrae BMD was significantly greater in female conditional $Y2^{-/-}$ mice, with a similar but non-significant pattern for BMC ($p=0.056$) (Figure 6.4 D,E).

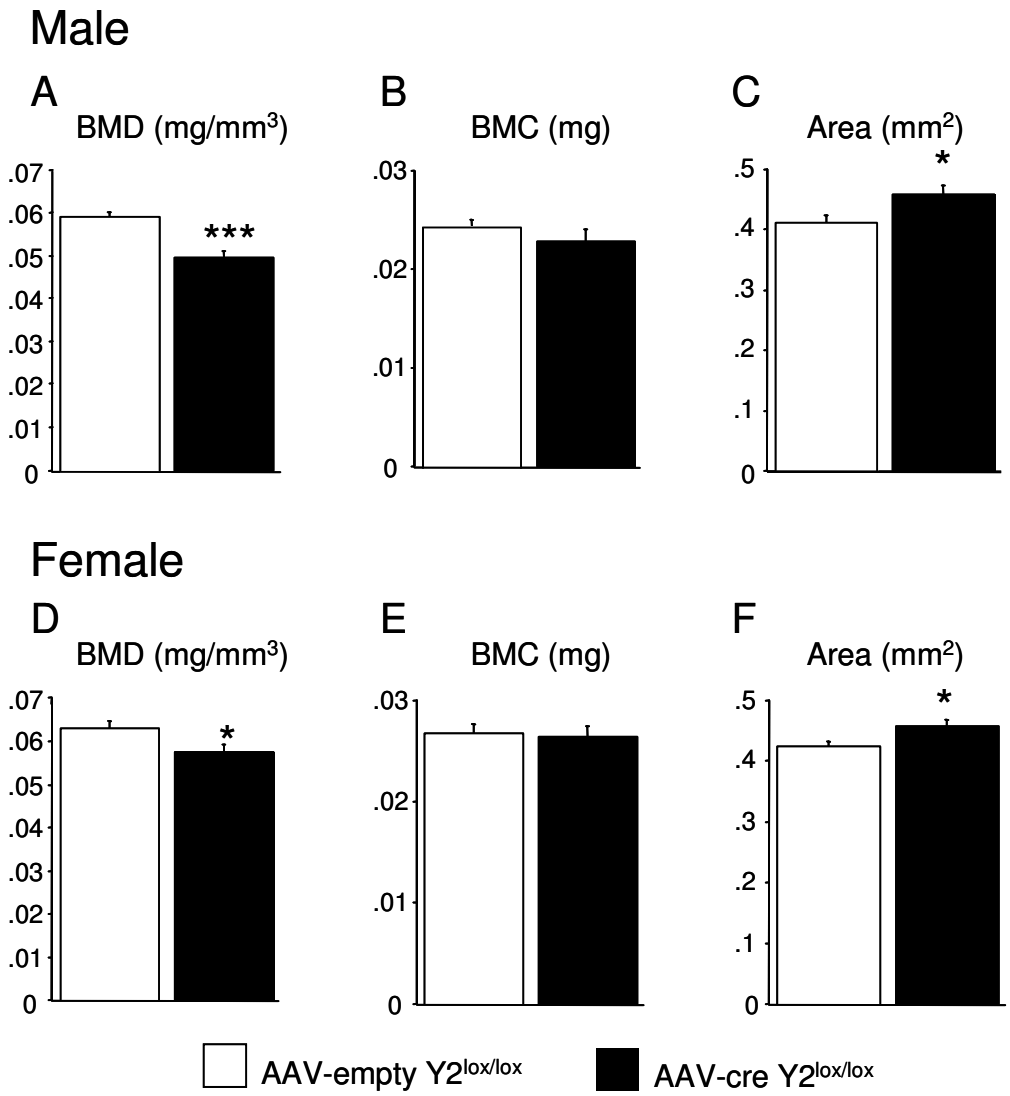

Figure 6.5 Effect of hypothalamic Y2 receptor deletion on bone mass of excised femur.
Male (A-C) and female (D-F) mice; BMD (A,D), BMC (B,E) and area (C,F) measured by DXA. *p<0.05, ***P<0.001. Numbers of mice used; AAV-empty: 8, AAV-cre: 7 for both male and female mice.

DXA analysis was also performed on excised femora at the study endpoint. Whole legs (femora + tibia) were scanned and were analysed with tibia excluded from the analysis. In addition to analysis of the entire femur, femoral scans were analysed in linear thirds, corresponding to the distal, shaft and proximal thirds. Femoral BMD was significantly reduced in both male and female conditional $Y2^{-/-}$ mice compared with AAV-empty

recipient mice (Figure 6.5 A,D). BMC was not different in either gender (Figure 6.5 B,E), however area was significantly elevated in the conditional knockouts (Figure 6.5 C,F), accounting for the reduced BMD seen in these mice. Analysis of linear thirds revealed a similar pattern, with reduced BMD corresponding to a greater area in the distal, shaft, and proximal femur of both male and female $Y2^{-/-}$ mice, but no change in BMC (data not shown), demonstrating a consistent change along the entire femur. These initial DXA data suggest that conditional deletion of hypothalamic Y2 receptors was not able to produce an anabolic response of great enough magnitude to increase the bone mass measuements. However, the greater bone area of these mice suggests an alteration in bone cell activity in response to attenuation of hypothalamic Y2 receptor signaling. In order to investigate this further, distal femora were processed for histomorphometry.

6.3.2 Histological analysis of distal femur trabecular morphology and cellular activity

In concordance with the densitometry data above, BV/TV and Tb.N were significantly reduced in male conditional $Y2^{-/-}$ mice compared with controls (Figure 6.6 A,B). These findings suggest that opposite to our previous findings in younger mice, conditional deletion of hypothalamic Y2 receptors in male aged mice actually had a deleterious effect on trabecular bone mass. However, further examination of the data revealed that while absolute trabecular bone volume was not significantly different between the two genotypes, total volume was significantly greater in conditional $Y2^{-/-}$ compared with control mice (data not shown, $p<0.05$), demonstrating that the apparent decrease in BV/TV and Tb.N in these mice was actually confounded by differences in total volume.

A trend for greater BV/TV and Tb.N was apparent in female conditional $Y2^{-/-}$ mice, however this was not significant compared with controls (Figure 6.6 D,E). Importantly, and in contrast to observations in male mice, total volume in female mice was not different between the two genotypes (data not shown).

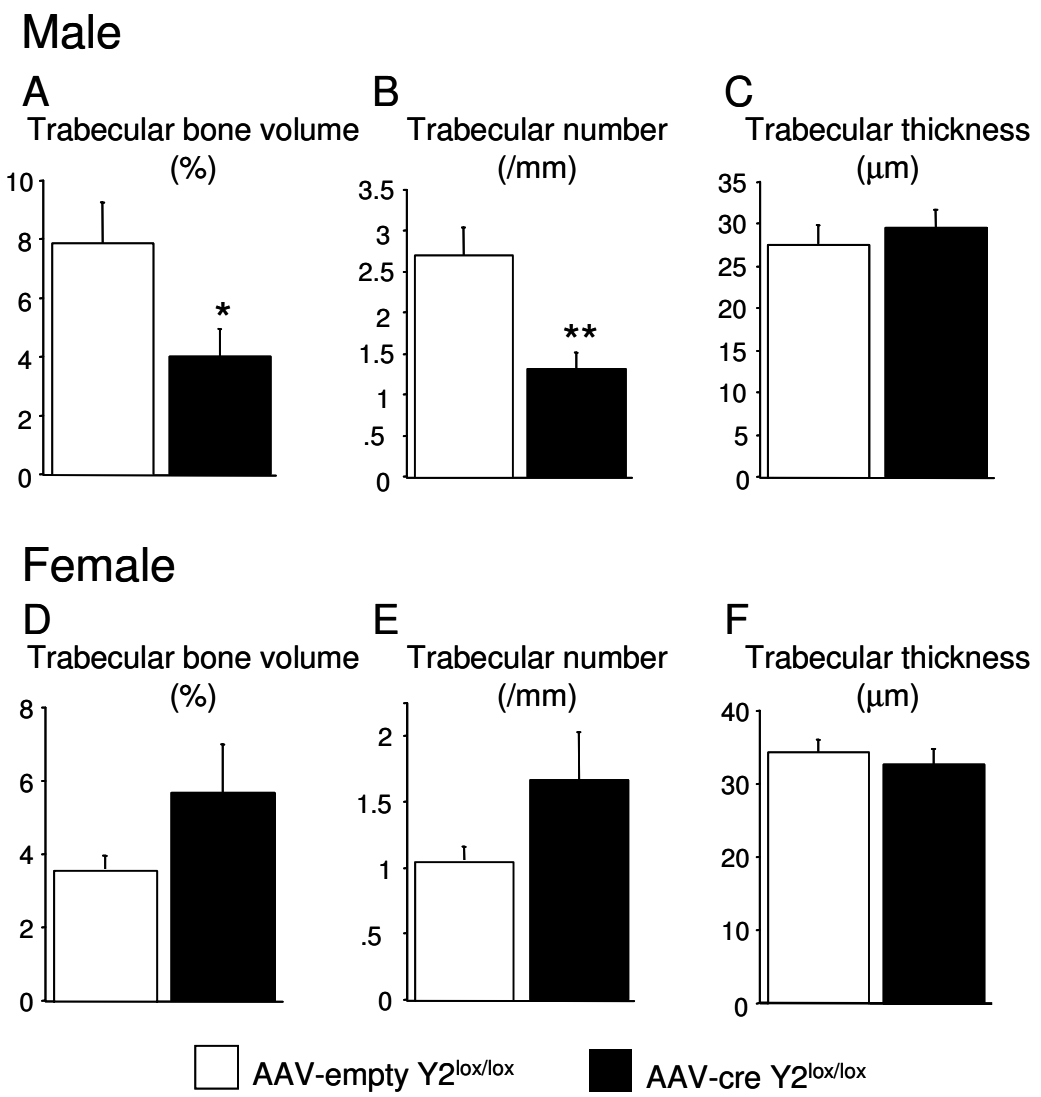

Figure 6.6 Effect of hypothalamic Y2 receptor deletion on trabecular bone morphology of distal femur. Male (A-C) and female (D-F) mice; Trabecular bone volume (A,D), trabecular number (B,E) and trabecular thickness (C,F) measured by histomorphometry. *p<0.05, **p<0.01. Numbers of mice used; AAV-empty: 8, AAV-cre: 7 for both male and female mice.

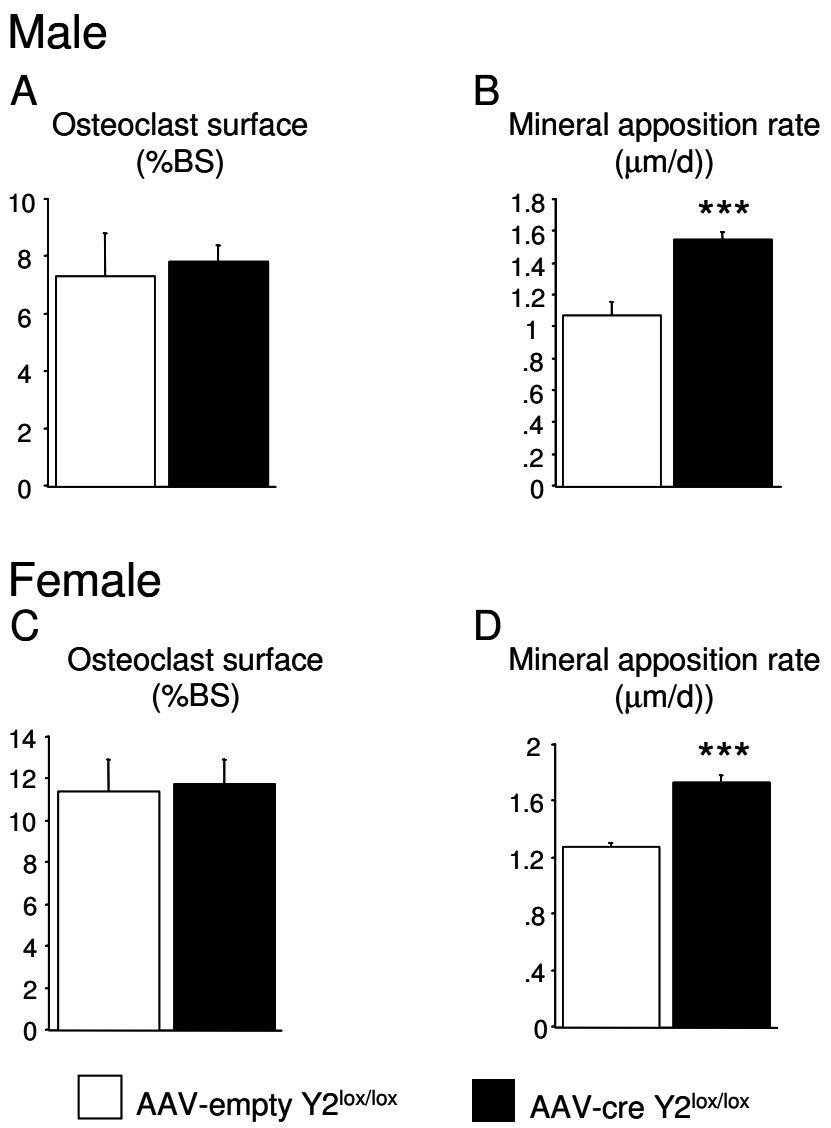

Figure 6.7 Effect of hypothalamic Y2 receptor deletion on trabecular bone cell activity of distal femur. Male (A-B) and female (C-D) mice; Osteoclast surface (A,C), and mineral apposition rate (B,D) measured by histomorphometry. ***$p<0.001$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7 for both male and female mice.

Oc.S was not different between the genotypes in male or female mice (Figure 6.7 A,C). Of considerable interest however, was the observation that MAR was significantly greater in both male and female conditional $Y2^{-/-}$ mice compared with controls (Figure 6.7 B,D), demonstrating that conditional deletion of hypothalamic Y2 receptor signalling in aged mice was successful in activating a bone formation response, which was present even 16

weeks after injection of the cre-recombinase. However, the lack of a significant elevation in trabecular bone volume in these mice suggests that the anabolic response was not great enough to produce a corresponding increase in bone mass.

The analysis of the sagittal histological sections of distal femur from this study also revealed what appeared to be trabecularisation of the cortical bone, particularly in bones from aged conditional $Y2^{-/-}$ mice. These were obviously areas of high remodelling activity with marked indices of bone resorption and formation, as evidenced by TRAP staining and calcein labelling, respectively (Figure 6.8). Further analysis of the trabecular and cortical bone was therefore undertaken to further characterise the effects of this remodelling activity on cortical and trabecular bone microarchitecture.

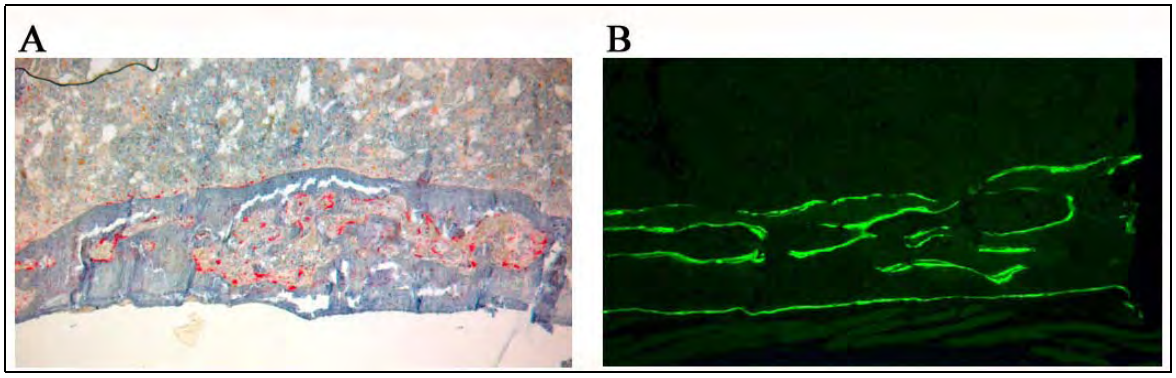

Figure 6.8 High remodelling activity within femoral cortical bone of conditional $Y2^{-/-}$ mice.
TRAP staining (A) and calcein labelling (B) within cortical bone of femurs reveals areas of high bone cell remodelling activity.

6.3.3 Micro-CT analysis of distal femur trabecular morphology

Further investigation of the trabecular phenotype of the aged conditional $Y2^{-/-}$ mice was performed using μCT to scan a single 0.8mm slice in the distal femoral metaphysis. In confirmation of the histomorphometry findings, BV/TV measured by μCT was also significantly reduced in male conditional $Y2^{-/-}$ mice compared with controls, again the result of significantly elevated total volume, or endocortical volume, with no change in

absolute bone volume (Table 6-1). Again in support of our findings by histomorphometry, no significant differences in parameters of trabecular morphology were observed in female conditional knockouts by μCT (Table 6-2).

MALES:

	Total volume (mm^3)	Bone volume (mm^3)	BV/TV (%)	Tb.N (/mm)	Tb.Th (mm)	Tb.Sp (mm)
AAV-empty $Y2^{lox/lox}$	0.62	0.03	0.05	4.5	0.03	0.23
AAV-cre $Y2^{lox/lox}$	0.79 *	0.02	0.02 *	5.84	0.03	0.21

Table 6-1 Distal femur trabecular bone morphology of male mice measured by micro-CT.
Measurements of total (endosteal) volume, absolute bone volume, trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). *$p < 0.05$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

FEMALES:

	Total volume (mm^3)	Bone volume (mm^3)	BV/TV (%)	Tb.N (/mm)	Tb.Th (mm)	Tb.Sp (mm)
AAV-empty $Y2^{lox/lox}$	0.74	0.004	0.01	5.58	0.02	0.22
AAV-cre $Y2^{lox/lox}$	0.68	0.004	0.01	5.13	0.03	0.22

Table 6-2 Distal femur trabecular bone morphology of female mice measured by micro-CT.
Measurements of total (endosteal) volume, absolute bone volume, trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

Trabecular and cortical morphology of the distal femoral metaphysis was visualised following 3-dimensional reconstruction of the μCT scans, revealing what appeared to be a greater marrow cavity most noticeably in male conditional knockouts compared with controls (Figure 6.9). Although from the image trabecular volume appeared to be reduced in male conditional $Y2^{-/-}$ mice, histomorphometric and μCT analysis had demonstrated this difference to be not significant. Images obtained from μCT reconstruction also revealed

what appeared to be a thinning of the cortical bone, particularly in male conditional $Y2^{-/-}$ mice, while cortical bone of female $Y2^{-/-}$ mice appeared to be distinctly more porous compared with controls (Figure 6.9).

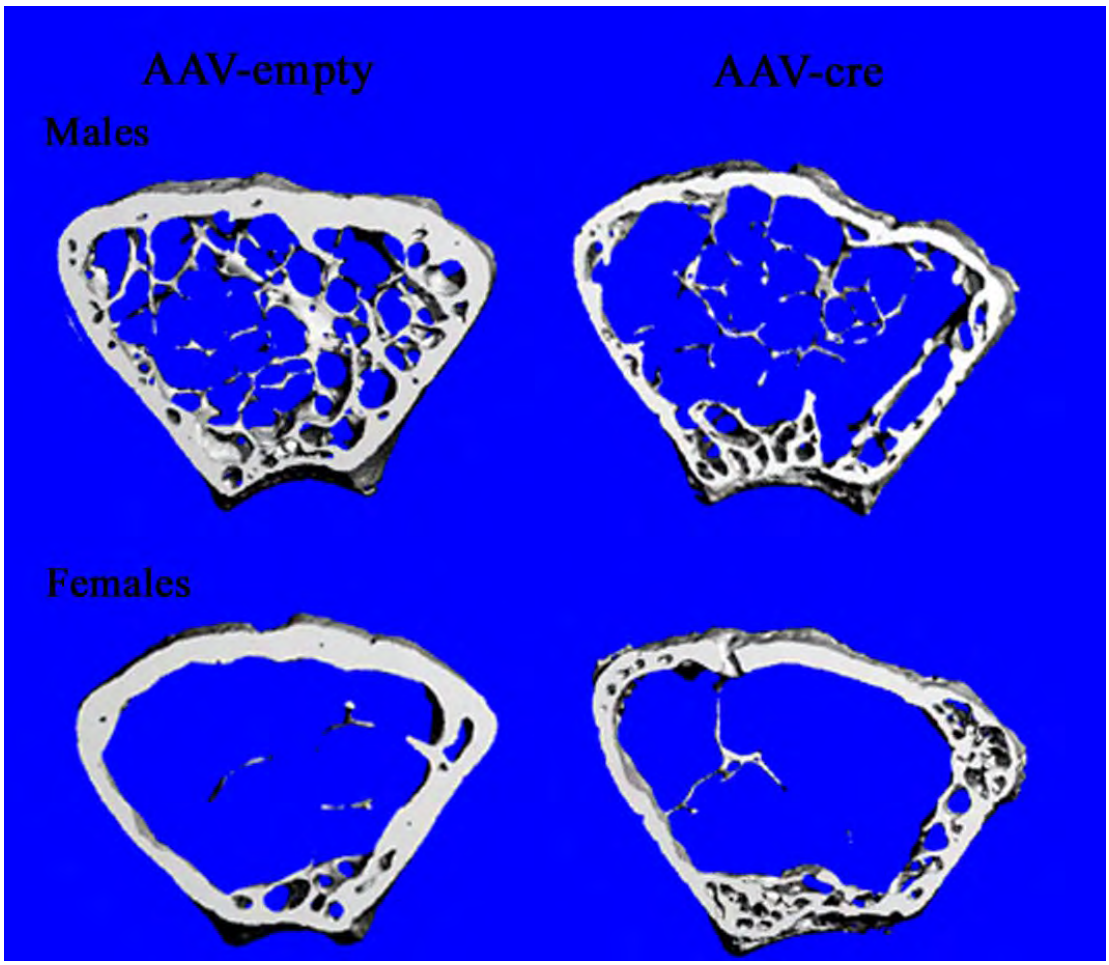

Figure 6.9 Micro-CT images of distal femur from male and female AAV-empty and AAV-cre injected $Y2^{lox/lox}$ mice.

6.3.4 Analysis of cortical bone using peripheral quantitative computed tomography

Scans of the distal metaphysis and the diaphysis were performed on femurs using pQCT to further investigate the effect of conditional deletion of hypothalamic Y2 receptors on cortical bone in aged mice.

In male mice, total mineral content and total bone density were significantly reduced at the distal metaphysis of conditional $Y2^{-/-}$ mice compared with controls (Table 6-3). This decrease in total content and density was associated with a reduction in cortical content, cortical density, cortical thickness, and a greater endocortical circumference, with no change in trabecular content or density, demonstrating that in accordance with our above data, the reduced mineral content of the male conditional knockouts was not the result of a change in trabecular morphology, but rather due to changes in cortical parameters with a greater endocortical circumference contributing to a greater marrow cavity. Analysis of the femoral shaft of male mice revealed similar reductions in total content and density and a significant increase in total area in conditional $Y2^{-/-}$ mice compared with control (Table 6-4). Again this was associated with significantly reduced cortical content, density, and thickness, and a significant increase in both periosteal and endocortical circumferences, demonstrating marked changes in cortical structures in the diaphyseal region again resulting in a greater marrow cavity.

Analysis of the metaphyseal region of female femurs also revealed a significant reduction in total mineral content and density in conditional $Y2^{-/-}$ mice compared with controls (Table 6-5). As in the males, and in accordance with our histomorphometry and μCT data, this was not associated with a change in trabecular bone, but rather the result of decreased cortical content, density, and thickness. No change in periosteal and endocortical circumference was observed at this site. In the diaphysis of female mice, total density was reduced, while total area was increased in conditional $Y2^{-/-}$ mice compared with controls (Table 6-6). Again, cortical density, and thickness were reduced, with a significant increase in both periosteal and endocortical circumferences, similar to observations in the diaphyseal region of male mice.

Importantly however, polar moment of inertia (Ip), an index of bone strength or resistance to torsional loading (Hasegawa et al., 2000), was not significantly different between wild type and conditional $Y2^{-/-}$ mice of either gender, indicating that the greater periosteal circumference of the $Y2^{-/-}$ model sufficiently compensated for the endosteal expansion, and

therefore did not result in a loss of bone strength. Male conditional $Y2^{-/-}$ mice in fact actually had a trend for greater Ip compared with controls (AAV-empty; 0.076 ± 0.03 versus AAV-cre; 0.9 ± 0.07 mm^4, p=0.07), indicative of an improvement in Ip, and thus bone strength in the male conditional knockouts compared with controls.

MALES (DISTAL METAPHYSIS):

	TOT CNT (mg/mm)	TOT DEN (mg/mm^3)	TOT AREA (mm^2)	TRAB CNT (mg/mm)	TRAB DEN (mg/mm^3)	TRAB AREA (mm^2)	CRT CNT (mg/mm)	CRT DEN (mg/mm^3)	CRT THK (mm)	PERI CIRC (mm)	ENDO CIRC (mm)
AAV-empty Y2$^{lox/lox}$	1.25	395.66	3.17	0.34	154.03	2.23	1.13	855.56	0.24	6.31	4.81
AAV-cre Y2$^{lox/lox}$	0.93 **	266.44 **	3.58	0.34	120.44	2.81 *	0.77 **	701.91 **	0.18 **	6.69	5.56 *

Table 6-3 pQCT measurements of the femoral distal metaphysis from male mice
Measurements for total (TOT), trabecular (TRAB), and cortical (CRT) content (CNT), density (DEN), and area (AREA), cortical thickness (CRT THK) and periosteal (PERI) or endosteal (ENDO) circumferences (CIRC) for the distal femoral metaphysis of male mice. *$p < 0.05$, **$p < 0.01$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

MALES (DIAPHYSIS):

	TOT CNT (mg/mm)	TOT DEN (mg/mm^3)	TOT AREA (mm^2)	CRT CNT (mg/mm)	CRT DEN (mg/mm^3)	CRT THK (mm)	PERI CIRC (mm)	ENDO CIRC (mm)
AAV-empty Y2$^{lox/lox}$	1.58	752.19	2.1	1.68	1217.85	0.34	5.14	3.01
AAV-cre Y2$^{lox/lox}$	1.35 ***	551.14 ***	2.48 *	1.43 ***	1105.5 ***	0.28 ***	5.58 *	3.84 ***

Table 6-4 pQCT measurements of the femoral diaphysis from male mice
Measurements for total (TOT) and cortical (CRT) content (CNT), density (DEN), and area (AREA), cortical thickness (CRT THK) and periosteal (PERI) or endosteal (ENDO) circumferences (CIRC) for the femoral diaphysis of male mice. *$p< 0.05$, ***$p< 0.001$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

FEMALES (DISTAL METAPHYSIS):

	TOT CNT (mg/mm)	TOT DEN (mg/mm^3)	TOT AREA (mm^2)	TRAB CNT (mg/mm)	TRAB DEN (mg/mm^3)	TRAB AREA (mm^2)	CRT CNT (mg/mm)	CRT DEN (mg/mm^3)	CRT THK (mm)	PERI CIRC (mm)	ENDO CIRC (mm)
AAV-empty Y2$^{lox/lox}$	1.28	378.51	3.4	0.26	108.03	2.36	1.26	881.33	0.25	6.53	4.98
AAV-cre Y2$^{lox/lox}$	0.98 **	289.13 *	3.41	0.3	117.01	2.55	0.93 **	727.11 ***	0.22 *	6.54	5.16

Table 6-5 pQCT measurements of the femoral distal metaphysis from female mice
Measurements for total (TOT), trabecular (TRAB), and cortical (CRT) content (CNT), density (DEN), and area (AREA), cortical thickness (CRT THK) and periosteal (PERI) or endosteal (ENDO) circumferences (CIRC) for the distal femoral metaphysis of female mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

FEMALES (DIAPHYSIS):

	TOT CNT (mg/mm)	TOT DEN (mg/mm^3)	TOT AREA (mm^2)	CRT CNT (mg/mm)	CRT DEN (mg/mm^3)	CRT THK (mm)	PERI CIRC (mm)	ENDO CIRC (mm)
AAV-empty Y2$^{lox/lox}$	1.58	748.43	2.11	1.67	1225.35	0.33	5.15	3.07
AAV-cre Y2$^{lox/lox}$	1.39 p=0.08	587.14 **	2.38 **	1.48	1131.57 *	0.29 *	5.46 **	3.66 ***

Table 6-6 pQCT measurements of the femoral diaphysis from female mice
Measurements for total (TOT) and cortical (CRT) content (CNT), density (DEN), and area (AREA), cortical thickness (CRT THK) and periosteal (PERI) or endosteal (ENDO) circumferences (CIRC) for the femoral diaphysis of female mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Numbers of mice used; AAV-empty: 8, AAV-cre: 7.

6.4 DISCUSSION

6.4.1 Trabecular bone

The previous chapter investigated the actions of the Y2-anabolic response in a model of sex hormone-deficient bone loss, demonstrating that activation of the anabolic pathway following gonadectomy completely prevented further bone loss in mature male and female mice. In this study, the actions of the Y2-anabolic response were investigated in a second model of osteoporosis: aging-induced bone loss. Contrary to expectations, conditional deletion of hypothalamic Y2 receptors in aged mice was associated with an overall decrease in bone mass, with reduced measurements of whole body BMD by DXA in both male and female mice, associated with an increase in bone area. Histomorphometric and μCT analysis demonstrated that deletion of Y2 receptors did not affect absolute trabecular bone volume, with reduced BV/TV in male conditional knockout mice biased by an increase in total area. Of note however, trabecular MAR was significantly greater in both male and female conditional $Y2^{-/-}$ mice compared with control, despite lack of a measurable effect on trabecular bone mass. These findings demonstrate that consistent with previous findings, deletion of hypothalamic Y2 receptors was also successful in activating an anabolic response in aged mice.

6.4.2 Cortical bone

Analysis of cortical bone mass by pQCT demonstrated that the reduction in total mineral content and density in the conditional knockouts resulted from alterations in cortical bone, with reduced cortical bone content, density, and thickness measurements in the distal femur and femoral shaft of conditional knockout mice. Periosteal and endosteal circumferences were also increased in the knockouts, revealing an expansion of the cortical shaft. While these changes in cortical bone measurements were evident in both male and female conditional $Y2^{-/-}$ mice, some parameters were not significant in female mice, suggesting a slower rate of cortical changes in females. Reconstruction of the μCT distal femur scans clearly illustrated the greater marrow cavity particularly in male knockout mice compared

with controls. Thinning of the cortices and an expansion of the marrow cavity is a primary factor for osteoporotic fracture in humans, with 3-fold and 2-fold increases in medullary area with increasing age reported in females and males, respectively (Bell et al., 1999; Feik et al., 1997). Similarly a decline in cortical thickness and an increase in endocortical circumference and medullary cavity size, concurrent with decreased periosteal and endosteal bone formation and increased intracortical resorption with increasing age, has previously been reported in C57/BL/6J mice, (Ferguson et al., 2003; Halloran et al., 2002). In the studies presented here, osteoclast surface of trabecular bone was comparable between control and conditional $Y2^{-/-}$ mice. However, while the expansion of endocortical circumference in conditional $Y2^{-/-}$ mice was suggestive of greater bone resorption at this region compared with controls, this parameter was not measured due to the marked trabecularisation of this region. However in contrast to the above studies in which deterioration in cortical structures was observed in aged mice in comparison to younger animals, the changes in cortical parameters observed in conditional $Y2^{-/-}$ mice in this study were in comparison to age-matched AAV-empty recipient controls. Therefore the findings from this study suggest that conditional deletion of hypothalamic Y2 receptor in aged mice may result in either a greater rate of cellular activity within cortical bone or, alternatively, may activate a differential cellular response to that observed with normal aging.

6.4.3 Cortical porosity

An interesting observation was the markedly greater porosity within the cortices of female $Y2^{-/-}$ mice, as can be seen in Figure 6.9. This porosity was specific to female mice, and was present within the cortices of nearly every AAV-cre injected but not AAV-empty injected female, consistent with the reduced measurements of subcortical content, density, and area in female conditional $Y2^{-/-}$ mice compared with control (data not shown). The cortical porosity in female conditional $Y2^{-/-}$ mice is reminiscent of osteoporotic human bone, in which porosity of both trabecular and cortical bone is also observed, and which is likely a significant contributing factor to the weakened bone and increased fracture risk of these patients (Feik et al., 1997; Parfitt, 1984; Wang et al., 2002). Studies in aged C57/BL/6J mice have also demonstrated cortical porosity of the femur in up to 50% of mice over 1

year of age (Ferguson et al., 2003). Of interest, administration of the anabolic agent PTH 1-34 also stimulated intracortical bone remodelling and increased cortical bone porosity in primates and humans (Turner, 2002). Importantly however, and consistent with the findings from this study, the majority of porosity following PTH 1-34 treatment occurred at the endosteal surface, which carries the smallest mechanical or bending stress, and therefore did not compromise bone strength (Turner, 2002). In the present study, cortical porosity was only evident in female conditional knockout mice; however, reduced cortical thickness and density were observed in both male and female conditional $Y2^{-/-}$ mice, concurrent with increased endosteal circumference in male mice at both the distal femur and the femoral shaft, and in the femoral shaft of female mice, suggestive of increased intracortical resorption as is discussed below. It is possible that the porosity seen in bones of the female conditional knockouts may indicate the beginning of endocortical resorption and cortical expansion, already progressed in male mice resulting in their greater endosteal circumference and medullary area.

6.4.4 Cortical expansion

It was expected that conditional deletion of hypothalamic Y2 receptors in aged mice would produce a beneficial response in terms of increasing BV/TV and improving bone microarchitecture. In this study however, while the anabolic response was clearly activated in trabecular bone, the primary response in the distal femur was a marked decrease in cortical bone density and thickness and a significant increase in marrow cavity size. Recent studies in 16-week old germline and conditional knockout mice have revealed that similar to the anabolic response induced within trabecular bone in the absence of Y2 receptor signalling, an anabolic response is also activated in cortical bone, with a significantly greater rate of mineral apposition at the endocortical surface of the distal femur and at the periosteal surface of the mid-shaft in 16 week old germline and conditional $Y2^{-/-}$ mice (Baldock et al., 2006b). Measurements of MAR within the cortical bone of aged conditional $Y2^{-/-}$ mice were also elevated in this study, with significantly greater MAR at the endocortical surface of the distal femur of male $Y2^{-/-}$ mice and at the endocortical surface of the femoral shaft of both male and female conditional $Y2^{-/-}$ mice (data not shown).

However, the accuracy and biological significance of these findings in this study are not clear, as the length of label within the cortices was extremely variable, most likely due to concurrent resorption at the endosteal surface. These MAR measurements were also confounded by the trabecularisation of the cortical bone, and while some calcein label was present within the periosteal surface, often the labels within this region were too close together to obtain accurate measurements. Furthermore, as sagittal sections only permit the measurement of cortical changes at the tension/compression axis, it is not possible using this method to assess bone cell activity within other regions along the cortices. Therefore it would be beneficial to perform histomorphometry on femoral cross-sections to characterise cortical bone resorption and formation throughout the entire circumference of the cortical bone.

6.4.5 Possible mechanism for cortical expansion and consequence on femoral strength

The greater endosteal circumference and porosity of the conditional knockouts suggests significantly greater resorptive activity at this site. It is possible that activation of the bone formation response by deletion of Y2 receptors increases the requirement for free calcium with which to build new bone. As 99% of body calcium is in bone, this would essentially be provided by activating bone resorption (Arnaud and Sanchez, 1990). In younger mice with greater trabecular surface and perhaps with improved calcium absorption efficiency, activation of this anabolic response may not represent such a problem; for example, conditional deletion of hypothalamic Y2 receptors in 16 week old mice activates an anabolic response which successfully increases trabecular and cortical bone mass (Baldock et al., 2005; Baldock et al., 2002). However, in very aged mice, such as those used in this study, little trabecular surface is available for osteoclastic resorption with which to provide free calcium, therefore necessitating the resorption of cortical surfaces. Interestingly, conditional deletion of hypothalamic Y2 receptors in young gonadectomised mice also activated an anabolic response preventing further deterioration of trabecular bone microarchitecture (section 5.3.6). The ability of the central Y2-anabolic pathway to prevent further deterioration in young gonadectomised mice in which bone loss had previously

occurred, but not in aged conditional $Y2^{-/-}$ mice, may therefore relate to age-related defects in calcium absorption efficiency, reducing the ability of aged mice to rapidly respond to sudden requirements for free calcium.

The greater periosteal circumference in the femoral shaft of male and female conditional $Y2^{-/-}$ mice suggests a greater rate of bone formation at this surface. This redistribution of mineral at the periosteal surface is possibly an attempt to compensate for the deterioration of cortical bone at the endosteal surface. Previous studies suggest that a small increase in periosteal apposition can preserve cortical bone strength despite endosteal resorption (Beck et al., 2000; Stein et al., 1998). Bones of a larger diameter are biomechanically stronger and thus a small increase in the diameter of the cortex is able to partially compensate mechanically for the loss of endosteal mass (Beck et al., 2000). The dominant bending and torsional stresses in long bones are greatest on the sub-periosteal surface (Turner, 2002), and expansion of the sub-periosteal surface with loss of endosteal surface is thought to be a direct response to increased strain at that surface (Beck et al., 2000). However, investigations of periosteal expansion in aging humans argues that continuation of periosteal expansion is not entirely a biomechanical response to endosteal bone loss, as expansion of the periosteal surface occurs from about the 3rd decade of life, before significant endosteal bone loss occurs. Furthermore, these changes in endosteal and periosteal surfaces have also been shown to occur in bone sites such as the skull, which are not weight bearing (Parfitt, 1984), suggesting other regulatory factors may be involved. Analysis of the polar moment of inertia in this study suggests strength indices are comparable between aged conditional $Y2^{-/-}$ and control mice, with a trend for increased bone strength in male conditional knockouts, despite a thinning of the cortices and an expansion of the endosteal and periosteal surfaces in the $Y2^{-/-}$ model. It is therefore likely that the expansion of the periosteal surface observed in the conditional knockouts is a response to endosteal resorption and expansion resulting from activation of the Y2-mediated anabolic response and an increased requirement for free calcium.

It might be expected that encouraging the formation of new bone in aged mice would reduce levels of free calcium thereby increasing levels of PTH and contributing to the

increased resorption at the endosteal surface. Similar responses are observed in elderly humans, with elevated PTH resulting from decreased calcium absorption, often due to decreased $1,25(OH)_2D_3$ levels and resulting in the development of secondary hyperparathyroidism, increased bone turnover, and loss of bone mass. It would therefore be of interest to measure serum levels of PTH and/or 25-hydroxyvitamin D3, and also investigate functional parameters of bone resorption, for example serum collagen cross-links, to further assess changes in serum biochemistry resulting from activation of the anabolic response in these aged mice.

6.4.6 Summary

Together these studies have demonstrated for the first time that activation of the Y2-mediated bone anabolic pathway in extremely aged mice successfully produced an anabolic response within trabecular bone, as measured by a greater rate of mineral apposition, but was not sufficient to increase trabecular bone mass. Interestingly, changes in the cortical bone measurements in conditional $Y2^{-/-}$ mice were consistent with elevated endocortical resorption and periosteal formation resulting in a widening of the marrow cavity. It is possible that activation of the anabolic response in these aged mice forced the resorption of endocortical surfaces, by increasing the requirement for free calcium with which to lay down new bone. While it would be expected that endocortical resorption might result in deteriorative effects on bone strength, it is important to note that strength index data suggested that alterations in the cross-sectional area of cortical bone through expansion of the periosteal circumference of conditional $Y2^{-/-}$ mice compensated for the endocortical expansion and thinning of the cortices. These data demonstrate the ability of the central Y2-mediated anabolic pathway to preserve biomechanical strength through the accumulation of periosteal bone, however direct mechanical loading or whole bone bending strength analyses would be required to confirm these findings.

Chapter 7

Mechanism by which bone formation is increased in the absence of Y2 receptor signalling

7.1 INTRODUCTION

The studies presented so far demonstrate that deletion of Y2 receptor signalling activates an anabolic pathway, increasing osteoblast activity and bone formation in the mouse distal femur. Furthermore, conditional deletion of hypothalamic Y2 receptors produces a similar increase in bone formation, and is potent enough to completely prevent the continuous loss of bone induced by sex hormone deficiency, despite elevated resorptive activity. These studies have demonstrated the potency and the central nature of the Y2-mediated anabolic pathway; however, they have not revealed the mechanism by which ablation of either germline or hypothalamic Y2 receptors stimulates osteoblast activity and increases bone formation.

While studies using hypothalamic-specific Y2 receptor knockout models and central delivery of leptin and NPY (Baldock et al., 2005; Ducy et al., 2000), are among the few studies to demonstrate a role for neuronal factors in the regulation of osteoblast activity *in vivo*, numerous other studies have described the ability of various neuronal factors to modify the activity of bone cells *in vitro*, with the demonstration that other neuropeptides such as vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), substance P (SP) and calcitonin gene-related peptide (CGRP) affect the formation, differentiation, and activation of osteoblastic and osteoclastic cells through both direct and indirect mechanisms (Lerner and Lundberg, 2002). These studies together have provided increasing evidence that in addition to the well described effects of endocrine and locally acting factors such as cytokines and growth factors, neuronal factors also play an important role in the regulation of bone cell function. A number of these studies have also demonstrated the presence of nerve fibres and neuronal factors within bone tissue (Bjurholm, 1991; Hill et al., 1991). NPY-immunoreactive fibres were found to be primarily associated with blood vessel walls and, together with the finding that NPY was produced by megakaryocytes within bone marrow, led to the suggestion of a primarily vasoregulatory role in bone as opposed to a role in the regulation of bone cell activity (Ahmed et al., 1993; Ericsson et al., 1987; Hill et al., 1991; Lindblad et al., 1994; Sisask et al., 1996). However,

NPY-immunoreactive fibres were also found within the periosteum, bone marrow and associated with bone lining cells (Ahmed et al., 1994; Hill et al., 1991), and treatment with NPY inhibited the cAMP response to PTH and norepinephrine in some osteoblastic cell lines (Bjurholm, 1991; Bjurholm et al., 1992), suggesting the presence of functional NPY receptors on bone cells and a possible role for NPY in the regulation of osteoblast activity. However, reports regarding the presence of Y receptors in bone have so far been somewhat contradictory. Some studies have reported the presence of NPY receptor transcripts on human osteoblastic and human osteosarcoma-derived cell lines, and mouse bone marrow cells (Nakamura et al., 1995; Togari et al., 1997), while another study from our own laboratory did not detect transcripts for any of the Y receptors in primary cultures of mouse calvarial osteoblasts or long bone preparations (Baldock et al., 2002). Thus a role for NPY in the direct regulation of bone cell activity is still not firmly established.

The mature osteoblast which is responsible for the formation and mineralisation of bone is originally derived from the mesenchymal stem cell (MSC), a multipotential cell type which is also able to give rise to adipocytes and chondrocytes (Beresford et al., 1992; Nuttall et al., 1998; Pittenger et al., 1999). Numerous studies have attempted to isolate and characterise pure populations of MSCs using a variety of methods, however the rarity of MSCs within bone and the lack of specific antibodies to facilitate their identification has so far proven this to be difficult. Stromal or progenitor cells have most frequently been isolated based on their ability to adhere to tissue culture plastic; however, while this method has the advantage of simplicity and is suitable for initial comparisons of cellular differentiation, haematopoietic cells actually constitute a large percentage of these cultures, particularly those established from murine models, due to their ability to also adhere to plastic, and bind to stromal cells via adhesion molecules and extra cellular matrix proteins (Bearpark and Gordon, 1989; Phinney et al., 1999; Simmons et al., 1992; Whetton and Graham, 1999). Other studies have removed haematopoietic cells from plastic adherent cultures by negative depletion resulting in a population of cells which retain the potential to undergo adipogenic, chondrogenic and osteogenic differentiation (Baddoo et al., 2003; Wieczorek et al., 2003). It is important to note however, that the characterisation of these cells was performed following a period of culture in the presence of contaminating

haematopoietic cells, which may have altered their phenotype. More recently, isolation of a pure population of multipotential and highly proliferative MSCs from mouse bone was achieved using negative depletion of haematopoietic cells followed by fluorescence-activated cell sorting (FACS) to select cells positive for the cell surface marker stem cell antigen-1 (Sca-1), a marker previously used to identify haematopoietic stem cells (Short et al., 2003). Further sorting of the haematopoietic depleted Sca-1^{-} cell population using an antibody recognising CD51, or the αV subunit of the vitronectin receptor, eliminated CD51^{-} erythroid precursor cell types and yielded a more mature mesenchymal progenitor cell population, which retained the ability to differentiate down the osteoblastic, adipocytic, and chondrogenic lineages (Paul Simmons, personal communication).

This study examines the downstream mechanism by which deletion of Y2 receptors results in a greater rate of bone formation and a greater bone volume. This was approached initially by culturing plastic adherent stromal cells from flushed bone marrow from wild type and germline Y2$^{-/-}$ mice. The method established by the Simmons laboratory mentioned above was also utilised to isolate MSC and progenitor cells to investigate the numbers of progenitor cells within the bones of these mice. Finally, we have further investigated the presence of Y receptors within our stromal cell cultures, to investigate the possibility of a direct mechanistic pathway to regulate osteoblast activity.

7.2 MATERIALS AND METHODS

7.2.1 Isolation of bone marrow stromal cells

Bone marrow stromal cells (BMSCs) from 5 to 9 week old male wild type and germline Y2$^{-/-}$ mice were isolated from flushed bone marrow and cultured in α-MEM control media as described in materials and methods (section 2.6.1). The non-adherent cell population was removed after 72 hours by a medium change. The cells were then continuously cultured for a further 4 days. On day 7, when the cells had reached about 70% confluence, cells were detached with trypsin as described in materials and methods (section 2.6.2), and sub-

cultured into α-MEM control media (section 2.6.2). Cells were changed into differentiation media 2 days later (day 0 of experiment) (section 2.6.4).

7.2.2 Assessment of bone marrow stromal cell proliferation

Differences in proliferation between the two genotypes was estimated by counting numbers of viable cells cultured in α-MEM control media at days -1, 0, 1, 2, 3, and 6 of the experiment (that is, starting from the day after subculture), as described in materials and methods (section 2.6.3). Four wells were counted for each genotype at each time point.

7.2.3 Differentiation of bone marrow stromal cell cultures

Differentiation of plastic-adherent stromal cells into adipocytes and mineral-producing osteoblasts was achieved by culturing cells in adipogenic and osteogenic media, respectively, from day 0 of the experiment as described in materials and methods (section 2.6.4). All differentiation experiments were performed in triplicate wells for each genotype. Media was changed 3 times weekly, for 21 days of culture. Osteoblast differentiation and mineralisation of extracellular matrix was visualised by ALP and von Kossa staining, and extent of mineralisation assessed using a Leica QWin Imaging system (section 2.6.5.3). Formation of adipocytes was visualised using Oil red-O staining (section 2.6.5.4), and the extent of adipogenic differentiation assessed by counting the number of cells containing well-stained oil droplets (section 2.6.5.5).

7.2.4 RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis of cultured bone marrow stromal cells

RNA was isolated from cultured BMSCs in 12-well plates using Trizol® Reagent as per the manufacturers instructions (section 2.6.6). RT-PCR was performed with Taq DNA polymerase using 1μl of cDNA synthesised from 1μg of total RNA with oligo$(dT)_{20}$ using the SuperScript™ III First-Strand Synthesis System for RT-PCR (section 2.6.7, 2.6.8), using specific primers and annealing temperatures outlined in Table 2-1.

7.2.5 Isolation of mesenchymal stem and osteoprogenitor cells from bone tissue

Two populations of mesenchymal progenitor cells were isolated from bone preparations from wild type and germline $Y2^{-/-}$ mice; MSCs and a more mature mesenchymal progenitor cell type, both with the potential to differentiate down the osteogenic, adipogenic, and chondrocytic lineages (Short et al., 2003)(Paul Simmons, personal communication). Previous studies have demonstrated that only a small percentage of the total number of mouse mesenchymal progenitors are present within bone marrow, with the majority more densely associated with the surrounding bone tissue (Short et al., 2003). The protocol utilised here therefore isolated MSCs and a population of more mature mesenchymal progenitor cells from crushed and collagenase-digested compact bone. However, it should be noted that MSCs and progenitor cells within the bone marrow would also contribute to this population.

Both the MSC and the more mature mesenchymal progenitor population were isolated from femurs, tibias, and iliac crests from 8 to 15 week old male mice, by extraction of bone cells, and depletion of contaminating haematopoietic cells, followed by cell sorting based on the marker Sca-1 as described in materials and methods (section 2.7). Sca-1^+ cells represent a multipotential population of immature MSC cells (Short et al., 2003). Sca-1^- cells were further sorted based on the marker CD51, and Sca-1^-CD51^+ cells were collected. These cells are a more mature progenitor cell type which retain the capability to differentiate into osteoblastic, adipocytic, and chondrogenic cell types (Paul Simmons, personal communication).

7.2.6 Culturing and colony formation of sorted MSC and progenitor cells

Sca-1^+ and Sca-1^-CD51^+ cells from wild type and $Y2^{-/-}$ mice were cultured in control media containing 20% FBS, initially in 5%O_2, 10%CO_2, 85%N_2 (Air Liquide, Melbourne, Australia) at 37^0C for 3 days, then changed into regular culture conditions at 37^0C and 5% CO_2 (section 2.7.5).

Formation of colonies by MSC and progenitor cells was assessed by evaluation of colony forming units (CFUs) on day 7 of culture, identified by toluidine blue staining (section 2.7.6). Number of positively-stained colonies containing >5 cells were counted for each experimental group.

7.2.7 Statistical Analysis

Statistical differences in the extent of mineralisation or adipocyte differentiation based on values obtained from the image analysis software (Leica Microsystems, Heerbrugg, Switzerland), were analysed using two-tailed unpaired t-tests between data from the two genotypes at equivalent time-points using StatView version 4.5 (Abacus Concepts, San Francisco, CA, USA). Statistical differences in the number of MSC and progenitor cells types between the two genotypes and between the two different cell types were also analysed using two-tailed unpaired t-tests. For all statistical analyses, $P<0.05$ was accepted as being statistically significant.

7.3 RESULTS

7.3.1 Establishing BMSCs and assessment of growth

The aim of this study was to investigate the mechanism by which deletion of Y2 receptors increases osteoblast activity. To examine whether isolated bone cells from germline $Y2^{-/-}$ mice exhibit differences in proliferation and mineralisation *in vitro*, we isolated plastic adherent bone marrow stromal cells (BMSCs) from wild type and germline $Y2^{-/-}$ mice and cultured them under osteogenic and adipogenic conditions. Similar numbers of cells were obtained from flushed bone marrow from wild type and $Y2^{-/-}$ mice (with approximately 10^8 cells obtained per mouse from each genotype), and were subsequently plated at equal density.

Cells from the two genotypes appeared to grow at the same rate in culture, with no noticeable difference in cell morphology, and were both approximately 70% confluent at

the time of trypsinisation and sub-culturing on day 7. Cell proliferation was estimated by counting viable cell number daily from day 8 of culture (day -1 of the experiment) up until day 3, and then again on day 6. Cell numbers in cultures from both wild type and $Y2^{-/-}$ mice increased over the initial stage of culture (Figure 7.1), with no significant difference in population growth between the two genotypes, indicating that stromal cells from $Y2^{-/-}$ and wild type mice are similar in their proliferative capability *in vitro*, and suggesting that differences in cellular proliferation are therefore not likely accountable for the greater bone formation of the $Y2^{-/-}$ model *in vivo*.

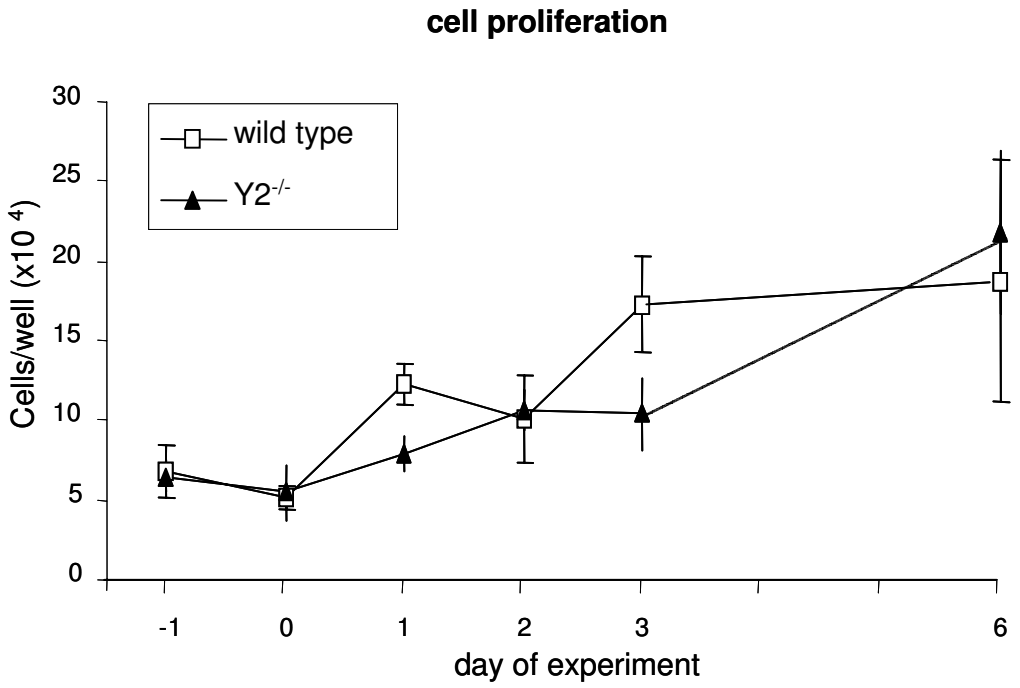

Figure 7.1 Effect of Y2 receptor deletion on proliferation of BMSCs in early culture in control media. Representative data from one of three experiments.

7.3.2 Osteogenic and adipogenic differentiation of BMSCs

Differentiation of BMSCs from wild type and $Y2^{-/-}$ mice was assessed under osteogenic and adipogenic conditions. Cultured stromal cells from wild type and $Y2^{-/-}$ mice under control

and osteogenic conditions stained positively for ALP at early stages of culture (data not shown). However, as previous studies have demonstrated the presence of tissue-nonspecific alkaline phosphatase in cells of the adipocytic lineage, including in adipocyte precursor cells in human bone marrow (Ali et al., 2006; Ali et al., 2005; Bianco et al., 1988), ALP staining was not further assessed and von Kossa staining for mineral was subsequently employed to evaluate the osteogenic capacity of these cells. Under osteogenic conditions, there was a time-dependent increase in the extent of mineralisation in cultures from both genotypes, with a greater increase observed in cultures from $Y2^{-/-}$ mice compared with wild type (Figure 7.2 A). These observations were confirmed by measurements of total mineral area using Leica QWin analysis software, with significantly greater mineralisation in stromal cell cultures from $Y2^{-/-}$ mice at days 18 and 21 (Figure 7.2 B). Of note was an apparent difference in the distribution of mineral between the two genotypes, with a more evenly distributed pattern of mineralisation in the $Y2^{-/-}$ cultures in comparison with a more central localisation of mineral in cultures from wild type mice (Figure 7.2 A).

Surprisingly, in one of three experiments, significant mineralisation was observed in stromal cell cultures from $Y2^{-/-}$, but not wild type mice, cultured in normal media without supplementation of ascorbic acid and β-glycerol phosphate (Figure 7.3). While these observations were not apparent in every experiment performed, possibly due to differences in phosphate concentrations in the medium, these findings together with the above results suggest an increased capability of osteoblastic cells from $Y2^{-/-}$ mice to produce mineral *in vitro*.

Cells cultured under adipogenic conditions also underwent a time-dependent increase in adipocyte differentiation as evidenced by increasing numbers of Oil Red-O stained cells from about day 6 of the experiment (Figure 7.4), with the number of adipocytes in cultures from $Y2^{-/-}$ mice significantly greater at days 18 and 21 of culture than wild type cultures (Figure 7.4 B). Together these results indicate that cultures of stromal cells from $Y2^{-/-}$ mice have an enhanced capability to undergo both osteoblast and adipocyte differentiation *in vitro*.

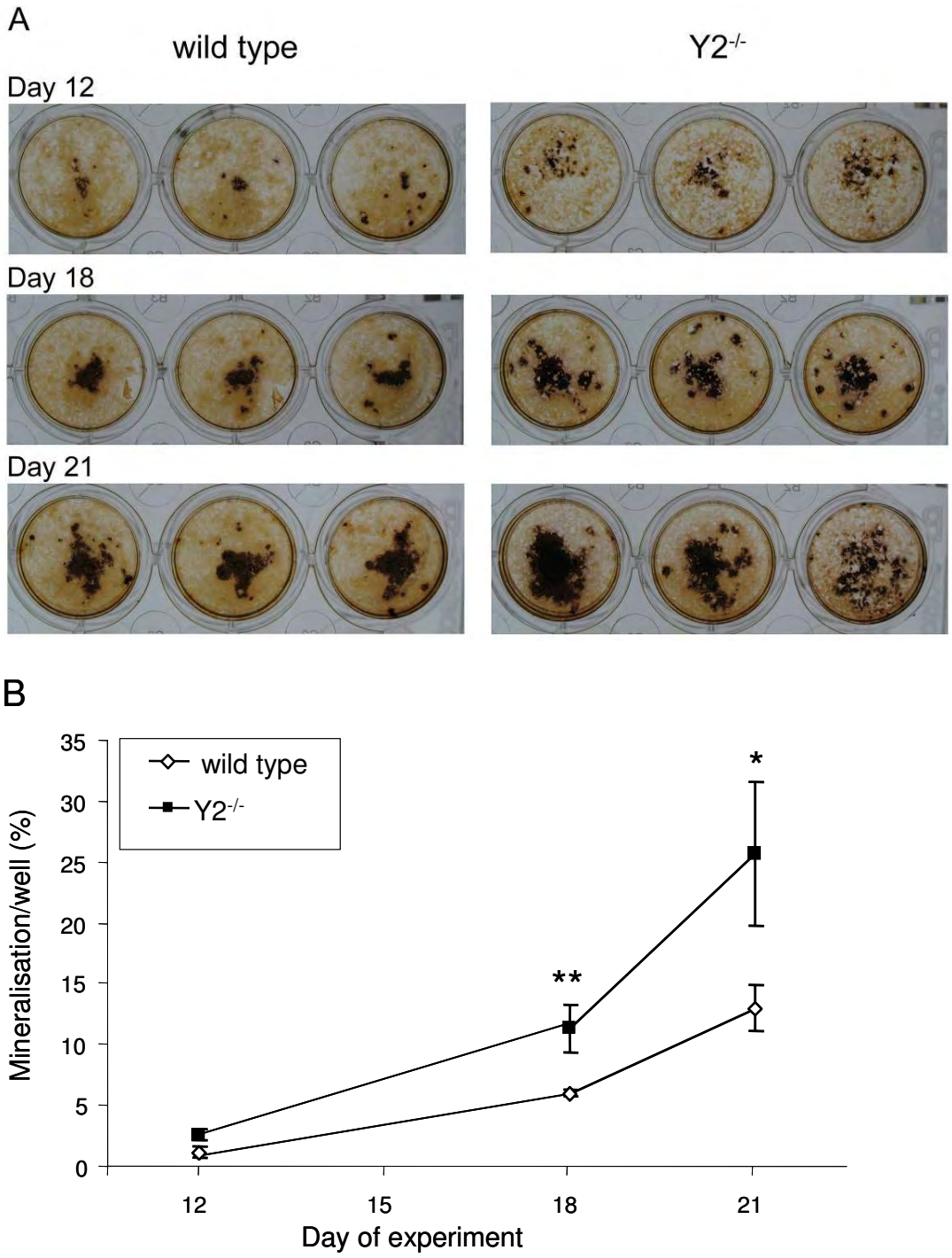

Figure 7.2 Effect of Y2 receptor deletion on osteoblast differentiation and mineralisation of BMSCs under osteogenic conditions.
Overviews of wells with von Kossa staining days 12-21 of culture (A), representative graph showing quantification of mineral (B). *p<0.05, **p<0.01. Representative data from one of three experiments with similar results.

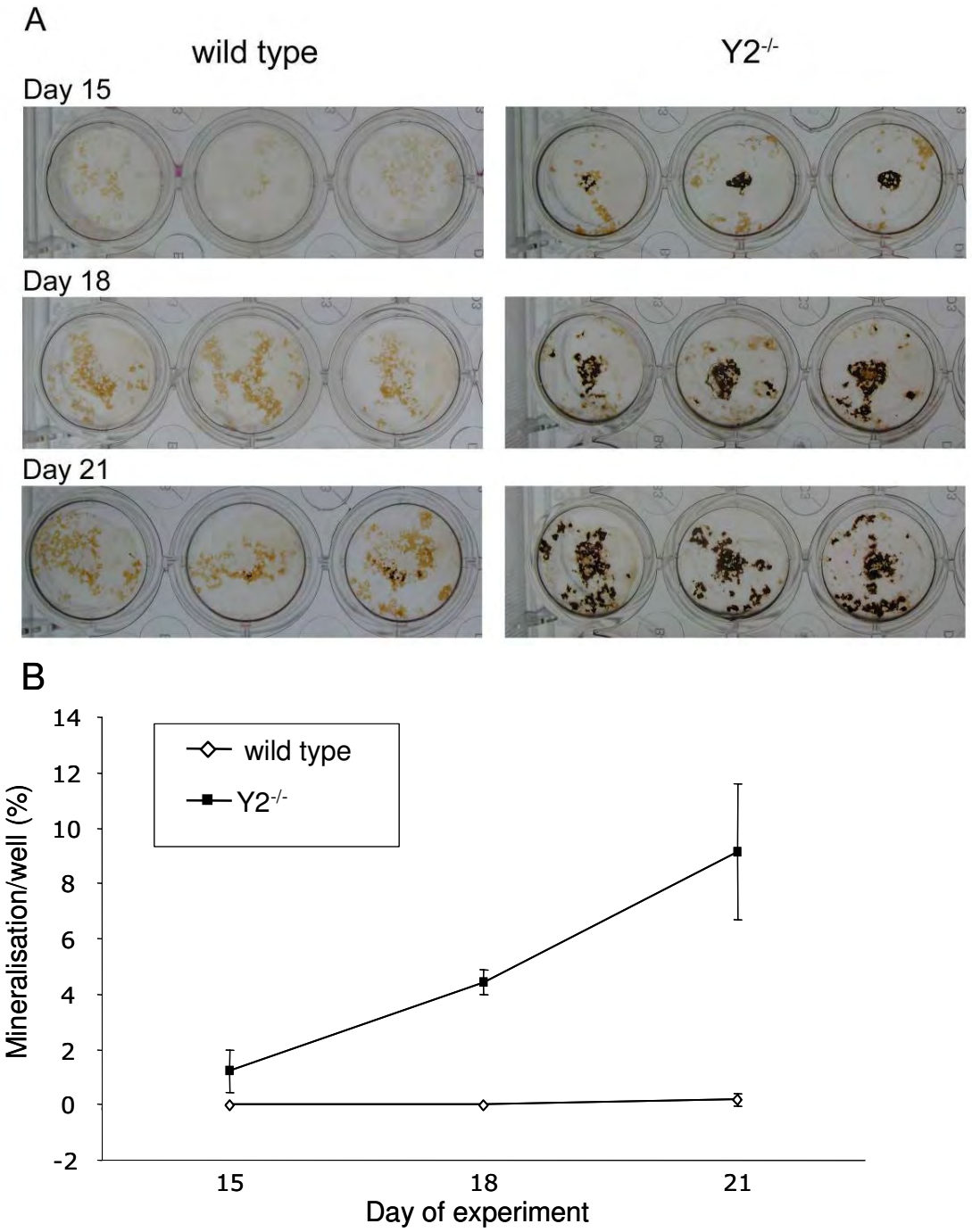

Figure 7.3 Effect of Y2 receptor deletion on mineralisation of BMSCs cultured in control media.
Overviews of wells with von Kossa staining days 15-21 of culture (A), quantification of extent of mineralisation (B). *$p<0.05$, **$p<0.01$. This result was observed in only one of three repetitions of this experiment.

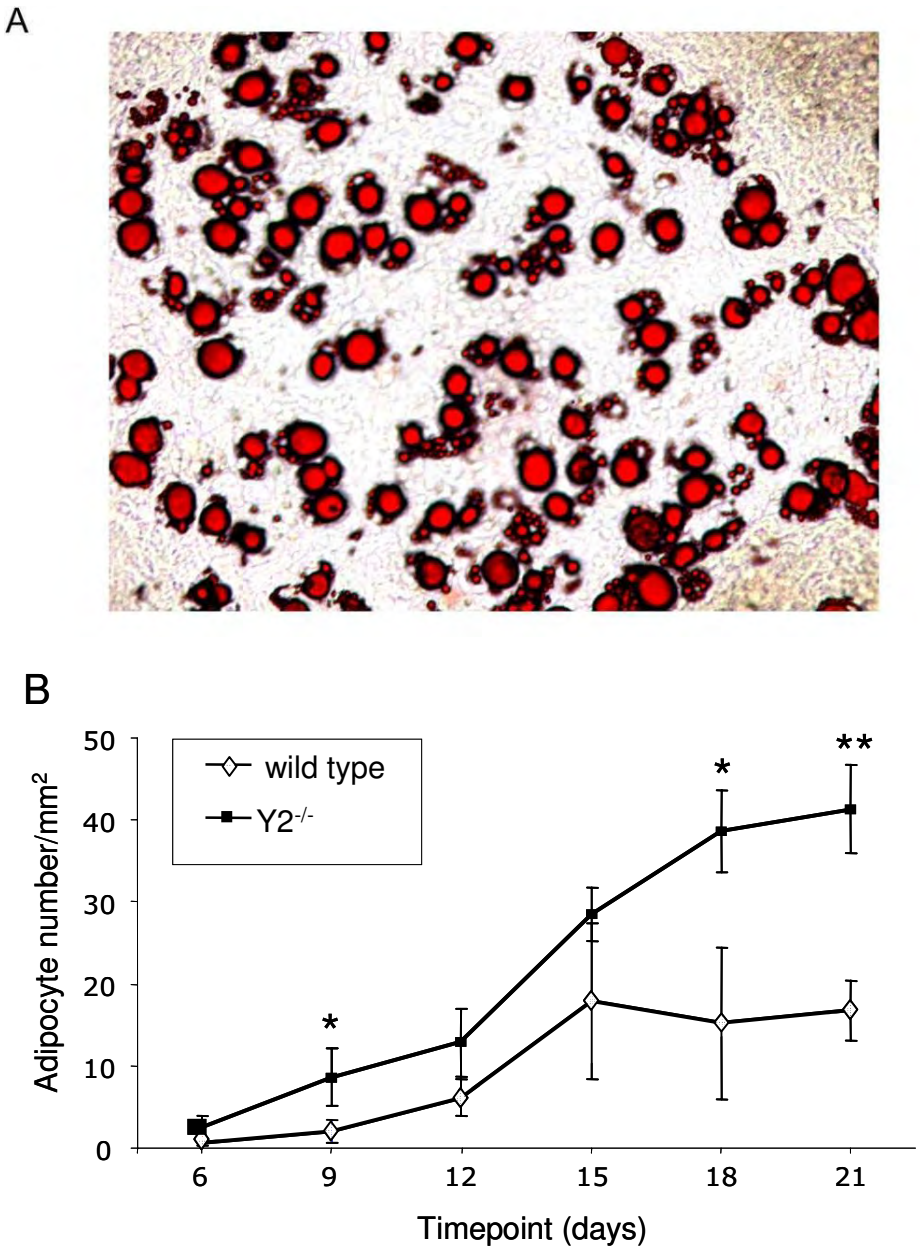

Figure 7.4 Effect of Y2 receptor deletion on adipocyte differentiation of BMSCs under adipogenic conditions. Positive Oil Red-O stained lipid (A), representative graph showing quantification of adipocyte number (B). *p<0.05, **p<0.01 Representative data from one of three consistent experiments.

Greater mineralisation and adipocyte differentiation in stromal cells from $Y2^{-/-}$ mice could result from a greater rate of proliferation; however, results from the earlier study make this possibility unlikely (see above). The increased osteoblast and adipocyte differentiation of $Y2^{-/-}$ mice could possibly be due to an intrinsic difference in the ability of progenitor cells from these mice to undergo osteoblast or adipocyte differentiation, perhaps as a result of altered levels of key transcription factors. As osteoblasts and adipocytes are derived from a common mesenchymal precursor, an alternative possibility could be a difference in the actual population of mesenchymal progenitors present within the bone and/or bone marrow of $Y2^{-/-}$ mice. This possibility was addressed in the next set of experiments.

7.3.3 Isolation of MSCs and progenitor cells from wild type and $Y2^{-/-}$ mice

In order to characterise the population of mesenchymal progenitor cells present in the bone tissue of $Y2^{-/-}$ mice we utilised FACS following depletion of haematopoietic cells to isolate two different populations of mesenchymal progenitors based on a method established in Paul Simmons' laboratory (Stem Cell Research Centre, Peter MacCallum Research Institute, Melbourne, Australia).

Consistent with the above results after bone marrow collection, similar numbers of cells were obtained from bones of wild type and $Y2^{-/-}$ mice following digestion with collagenase (approximately $4.6x10^7$ cells total for 10 mice collected). Numbers of cells remained similar between the two genotypes following depletion of haematopoietic cells by lineage depletion (approximately $3x10^6$ cells total for 10 mice collected). After negative selection of $CD45^+$ and $CD31^+$ haematopoietic cells, cells were sorted based on the cell surface marker Sca-1. The immature mesenchymal stem Sca-1^+ cells were gated and collected, while Sca-1^- cells were sorted again and the more mature mesenchymal progenitor Sca-1^- $CD51^+$ cells were gated and collected.

While there was a slight but non-significant greater number of Sca-1^+ MSCs in $Y2^{-/-}$ mice relative to wild type, importantly, there was a two-fold greater number of $Sca1^-CD51^+$ mature progenitor cells in bones from $Y2^{-/-}$ compared with wild type mice (Figure 7.5).

These findings demonstrated for the first time a greater number of progenitor cells present within the bone of $Y2^{-/-}$ mice, suggesting that the greater mineralisation and adipocyte differentiation observed in cultures of stromal cells from $Y2^{-/-}$ mice is likely to be due to a greater proportion of progenitor cells present when the cells were initially plated. These findings also indicate for the first time a potential cellular mechanism for the greater rate of bone formation observed in $Y2^{-/-}$ mice *in vivo*.

7.3.4 Assessment of colony formation in MSC and progenitor cells

In order to investigate the ability of the progenitor cells from the two genotypes to form proliferating cell colonies, colony forming unit (CFU) assays were performed on both Sca-1^+ and Sca-1^-CD51^+ cell populations isolated from wild type and $Y2^{-/-}$ mice. The CFU assay is an indication of the number of progenitor cells within an isolated population capable of proliferating and therefore differentiating down the distinct mesenchymal lineages. Cells within the colonies from Sca-1^+ and Sca-1^-CD51^+ populations appeared morphologically different, with a more fibroblastic morphology in the Sca-1^+ cells and a more cuboidal morphology in Sca-1^-CD51^+ cultures, consistent with their proposed role as a more mature osteoprogenitor cell type (Figure 7.6 A). However, no difference in morphology between the two genotypes was apparent (data not shown). Furthermore, although CFU number was significantly greater in Sca-1^+ compared with Sca-1^-CD51^+ cultures ($P<0.001$), consistent with their proposed role as a more immature stem cell type, no difference in CFU number was observed between cultures from wild type and $Y2^{-/-}$ mice (Figure 7.6 B,C), suggesting that the relative abundance of proliferating progenitor cells within these populations were comparable between the two genotypes.

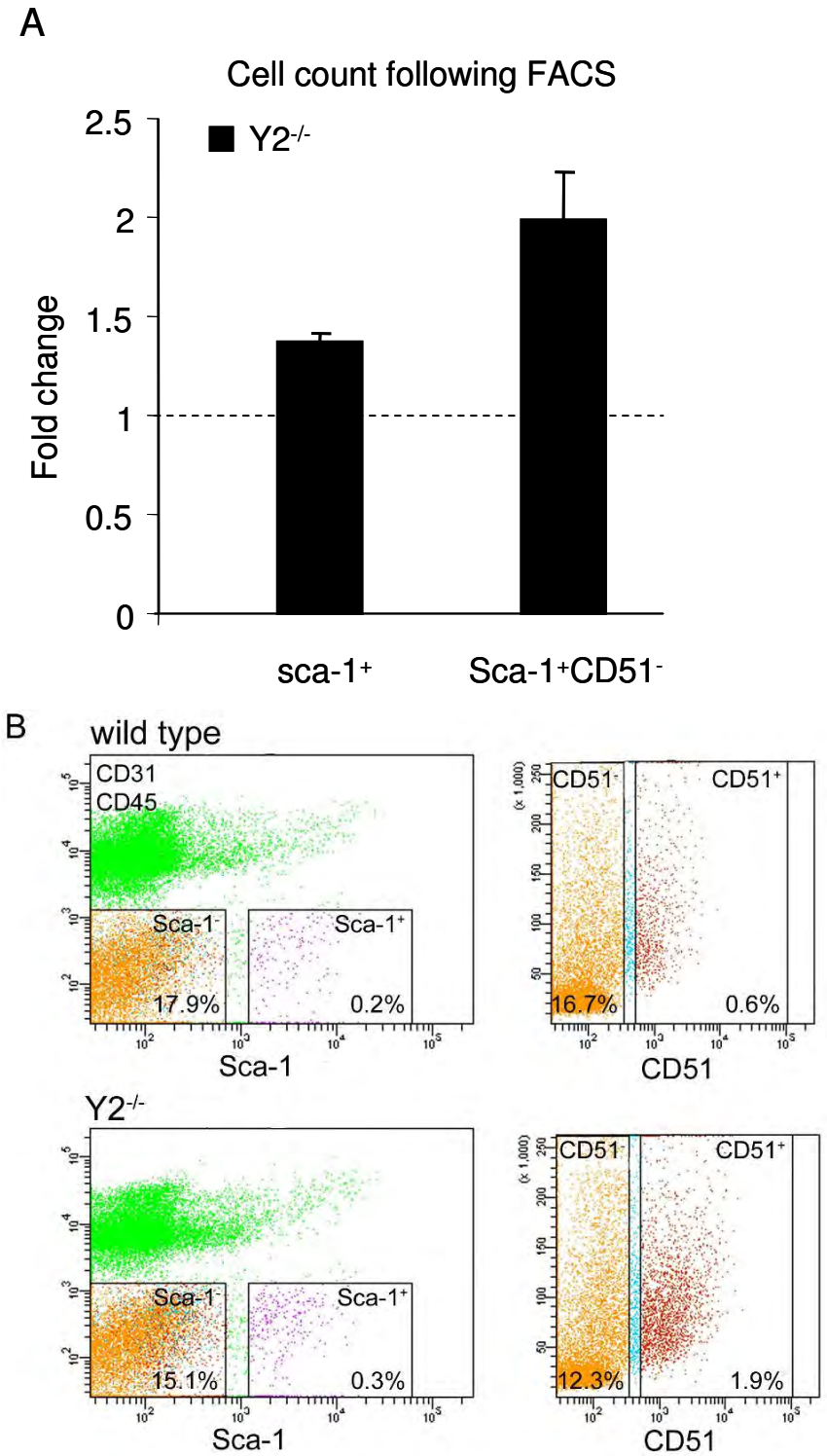

Figure 7.5 Greater number of mature progenitor cells present in bone of $Y2^{-/-}$ mice.
Fold difference of $Sca\text{-}1^{+}$ MSCs and $Sca\text{-}1^{-}CD51^{+}$ mature progenitor cells in $Y2^{-/-}$ bone compared to wild type (dotted line) (A), and proportion (B) of sorted populations in bones of wild type and $Y2^{-/-}$ mice, shows a significant two-fold greater number of $Sca\text{-}1^{-}CD51^{+}$ progenitor cells in $Y2^{-/-}$ bones ($p<0.05$).

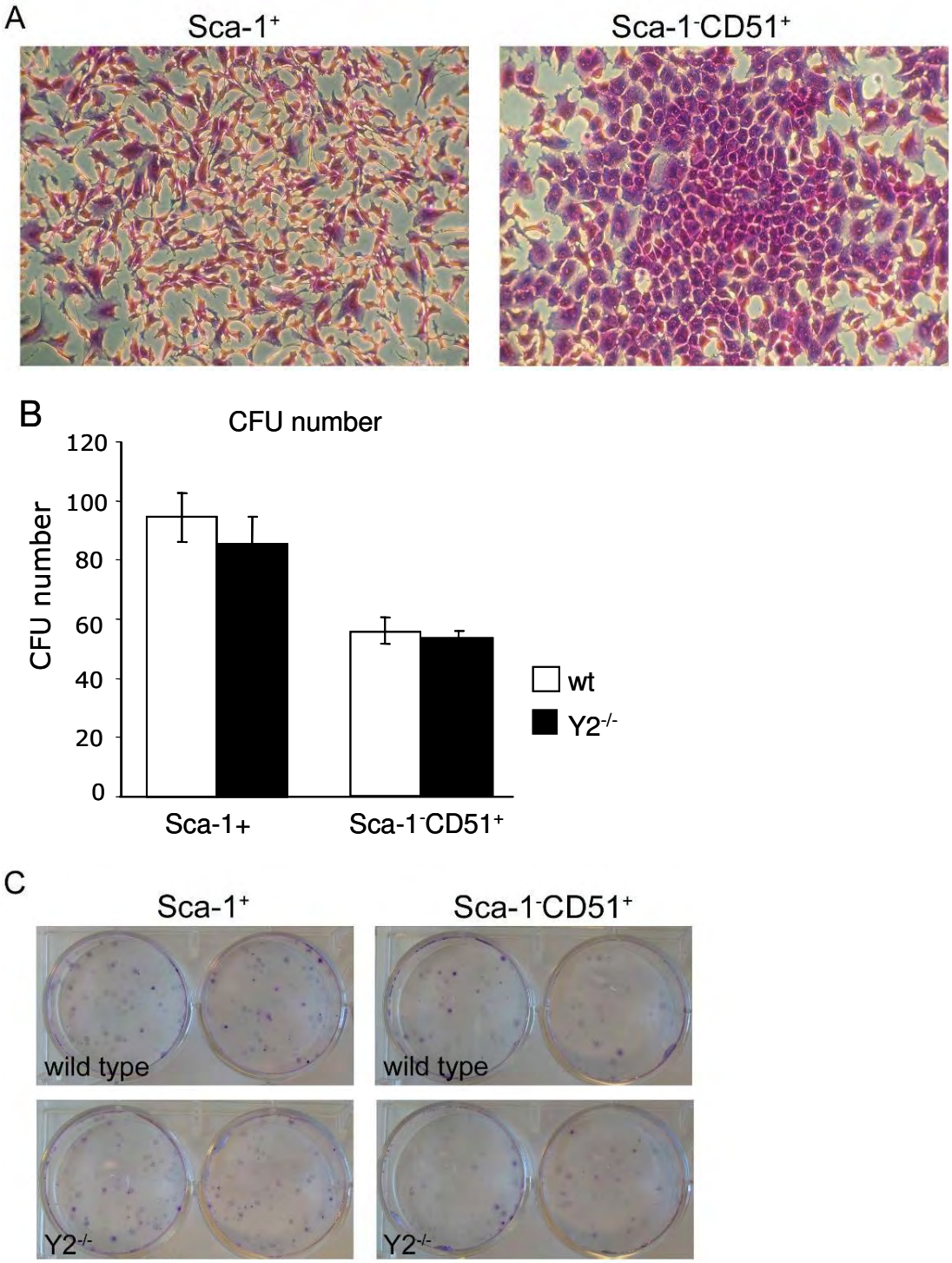

Figure 7.6 CFUs from isolated MSCs and progenitor cells from wt and $Y2^{-/-}$ mice.
Identification of CFUs by toluidine blue staining at 100x magnification (A), CFU number after plating at equal density (B), overview of toluidine blue staining (C).

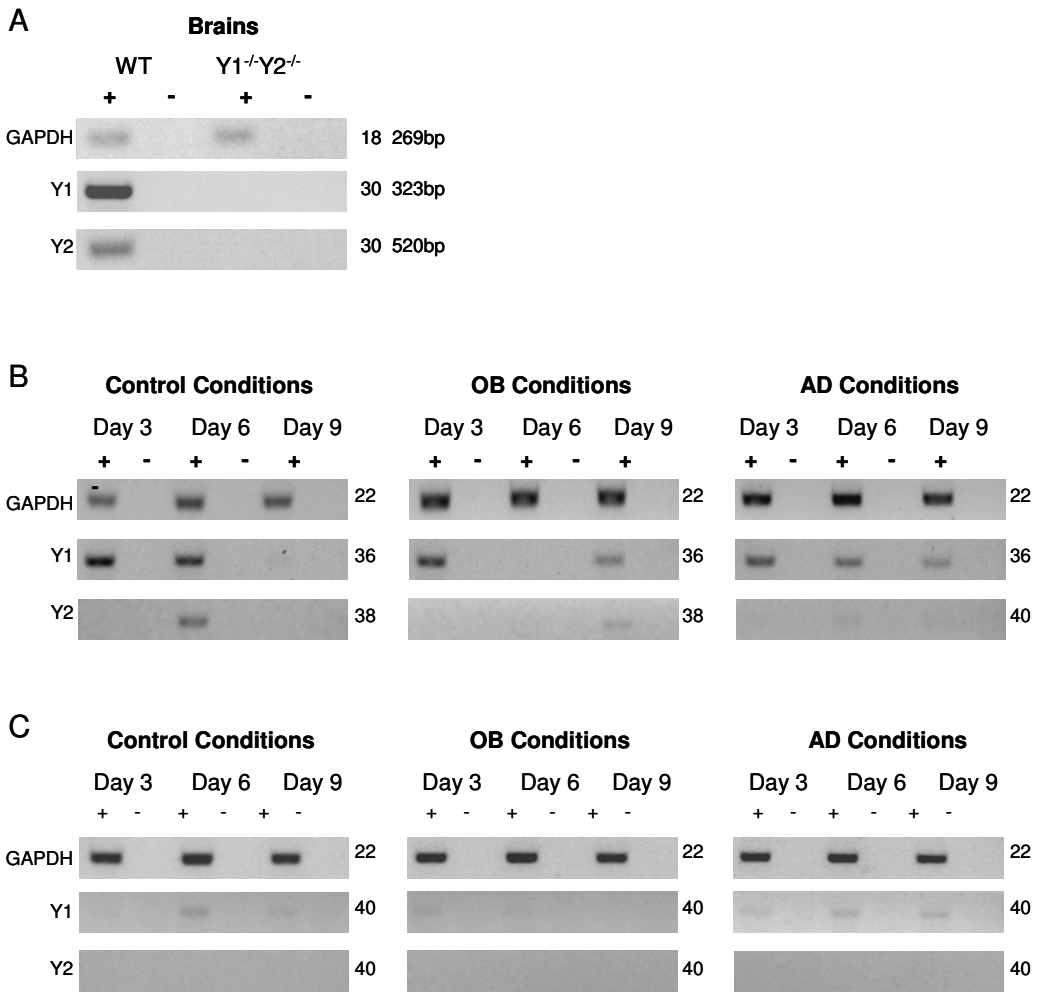

Figure 7.7 Y1 receptor is expressed in cultured bone marrow stromal cells from wild type mice.
Y1 and Y2 receptor mRNA are detected in brain tissue of wild type (WT) but not $Y1^{-/-}Y2^{-/-}$ double knockout mice (A). Y1 receptor is expressed in stromal cell cultures from wild type mice at day 3, 6, and 9 of culture under control, osteoblastic (OB), and adipocytic (AD) conditions (B); and is down-regulated at these time points in stromal cells from $Y2^{-/-}$ mice (C). Y2 receptor is expressed at day 6 of culture under control conditions in wild type mice (B). Number of PCR cycles and product size are indicated on figure. Data shows results for PCR performed with (+) and without (-) reverse transcription.

7.3.5 Investigation of Y receptor expression

To further investigate the mechanism by which progenitor cell number might be increased in bones from $Y2^{-/-}$ mice, the expression of different Y receptor transcripts was investigated in BMSC cultures from wt and $Y2^{-/-}$ mice over the first 9 days of culture. Expression of all Y receptor transcripts was demonstrated from brain cDNA using RT-PCR (Figure 7.7 A,

and data not shown). Y1 receptor transcripts were also detected in stromal cell cultures from wild type mice. Importantly, Y1 receptor expression was all but abolished in cultures from $Y2^{-/-}$ mice (Figure 7.7 B,C). Very weak expression of the Y2 receptor was also observed in cultures from wild type mice; however this signal was detected only on day 6 under control conditions (Figure 7.7 B). Expression of Y4, Y5, and y6 receptor transcripts were not detected in cells from either genotype under any conditions (data not shown).

7.4 DISCUSSION

7.4.1 Greater progenitor number in $Y2^{-/-}$ mice

This study investigated the mechanism by which deletion of Y2 receptor signalling increases bone formation by assessing the ability of plastic-adherent bone marrow stromal cell cultures from wild type and $Y2^{-/-}$ mice to undergo mineralisation, and characterisation of the progenitor population within the bone of the two genotypes.

Extraction and culturing of plastic adherent bone marrow stromal cells under osteogenic conditions demonstrated a greater ability of cells from $Y2^{-/-}$ mice to produce mineral *in vitro*. These *in vitro* observations were consistent with our *in vivo* findings. One possible mechanism by which mineralisation could be greater in cultures from $Y2^{-/-}$ mice, could be a greater rate of proliferation, however the similar increase in viable cell number of cultured BMSCs from wild type and $Y2^{-/-}$ mice exclude this as a likely mechanism for the greater osteoblast differentiation and mineralisation of the $Y2^{-/-}$ culture. This is perhaps not surprising, as our *in vivo* data indicated that deletion of Y2 receptors does not result in a change in osteoblast number, but rather a change in the activity of osteoblastic cells (Baldock et al., 2002). However, in addition to the greater mineralisation of cultured stromal cells from $Y2^{-/-}$ mice, these cells also exhibited an increased ability to undergo adipocytic differentiation. This finding was unexpected, as the $Y2^{-/-}$ mice have a lean phenotype, with reduced total white adipose tissue mass in male germline $Y2^{-/-}$ compared with wild type mice (Figure 5.4), and no obvious increase in marrow adipocyte number (Figure 4.4). These new results suggested that stromal cells from $Y2^{-/-}$ mice isolated by

plastic adherence did not undergo osteoblast differentiation at the expense of adipocyte differentiation, but rather demonstrated an enhanced ability to differentiate down either the osteoblastic or the adipocytic lineages under appropriate conditions. This led us to investigate the proportion of mesenchymal progenitor cells within the bones of the $Y2^{-/-}$ model.

Recent studies have demonstrated that only a small percentage of the total number of mesenchymal progenitors are present within the bone marrow, while the majority of mesenchymal cells are present within compact bone (Short et al., 2003). We therefore employed an unpublished method from the laboratory of our collaborator Paul Simmons to isolate two populations of progenitor cells from compact bone of wild type and $Y2^{-/-}$ mice, involving initial depletion of haematopoietic cells followed by cell sorting using the cell surface antigen Sca-1. Previous work by these collaborators had determined that isolation of Sca-1^{+} cells results in a highly enriched population of stromal progenitors with properties of MSCs, ie., a highly proliferative cell type with high plating efficiency for CFU formation, and the ability to undergo differentiation down different mesenchymal lineages (Short et al., 2003). Further sorting of Sca-1^{-} cells and isolation of Sca-1^{-}CD51^{+} cells results in a population of stromal progenitor cells which are devoid of erythrocyte precursors, are able to proliferate in culture, and are also multi-potential. While both these cell types have the ability to differentiate down osteogenic, adipogenic, and chondrogenic pathways, gene array experiments of these two progenitor populations demonstrated an up-regulation of osteoblastic and adipocytic genes in the Sca-1^{-}CD51^{+} population, indicative of a more highly differentiated cell type (Paul Simmons, personal communication).

In this study, FACS analysis of a population devoid of haematopoietic cells from our $Y2^{-/-}$ and wild type mice revealed that while numbers of Sca-1^{+} MSC cells were similar between the two genotypes, numbers of Sca-1^{-}CD51^{+} progenitor cells were 2-fold greater in bones from $Y2^{-/-}$ mice compared with wild type. These findings indicate that there is a larger proportion of progenitor cells in bones from $Y2^{-/-}$ mice which are capable of undergoing differentiation down mesenchymal lineages, suggesting that the greater mineralisation and adipocyte differentiation seen in plastic-adherent stromal cultures from $Y2^{-/-}$ mice is likely

the result of plating a higher proportion of progenitors. It is also possible that the larger number of progenitors contributes to the greater rate of bone formation seen in $Y2^{-/-}$ mice *in vivo*.

Analysis of CFU formation following the plating of these two cell populations at the same density revealed a greater number of CFUs in the immature Sca-1^{+} cells compared with the Sca-1^{-}CD51^{+} progenitor cells; however, no difference in CFU number was observed between the two genotypes. The number CFUs formed in culture is an indication of the relative abundance of MSCs or progenitor cells present within the isolated cell population. These findings therefore indicate that there is no difference between the genotypes in the ability of either cell population to proliferate and therefore potentially differentiate down multiple mesenchymal lineages.

7.4.2 Down-regulation of Y1 receptor expression in $Y2^{-/-}$ mice

This study also investigated the expression of the different Y receptor transcripts in cultures of bone marrow stromal cells from wild type and $Y2^{-/-}$ mice under different differentiation conditions. While the Y2 receptor was very weakly expressed at day 6 in wild type cultures under control conditions, the Y1 receptor was highly expressed in wild type cultures under normal, osteogenic and adipogenic conditions, particularly during the early stages of culture. Interestingly, Y1 receptor expression was virtually abolished in cultures from $Y2^{-/-}$ mice. The detection of Y1 receptor transcripts in stromal cell cultures from wild type mice suggests an important role for these receptors in the direct regulation of bone cell activity, while the marked down-regulation of Y1 receptor expression in the $Y2^{-/-}$ model indicates a possible mechanism by which bone formation is increased in these mice. Expression of transcripts for the Y4, Y5, and y6 receptors were not detected in these stromal cells.

As mentioned in the introduction, previous studies investigating the presence of Y receptors in bone has resulted in contradictory findings. The presence of functional Y receptors in bone cells was first suggested with the finding that NPY treatment inhibited the cAMP response to PTH and norepinephrine in some osteoblastic cell lines (Bjurholm, 1991;

Bjurholm et al., 1992). Expression of a Y receptor which corresponded in sequence to the Y1 receptor was subsequently found in human osteoblastic and osteosarcoma-derived cell lines (Togari et al., 1997), while another study reported the expression of the Y1 receptor in mouse bone marrow cells (Nakamura et al., 1995). Previously, our laboratory did not detect transcripts for any of the Y receptors in primary osteoblastic cultures prepared from neonatal calvaria or long bone preparations from adult mice (Baldock et al., 2002). In this study however, we found strong expression of the Y1 receptor transcript in cultured bone marrow stromal cells. It is not known why we were unable to detect this transcript in our earlier studies. An absence of transcripts in the primary cultures could be due to site- and/or stage-specific differences in the osteoblastic expression of the Y1 receptor; however this does not explain the negative findings using whole bone RNA preparations. Nevertheless, these more recent findings using a different set of receptor-specific PCR primers correlate with the earlier studies from other laboratories, and together indicate that regulation of Y1 receptor activity may affect bone cell function. The observation that Y1 receptor expression was nearly abolished in cultures from $Y2^{-/-}$ mice, suggests a potential role for reduced Y1 receptor signalling within the bone microenvironment in the anabolic bone phenotype of the $Y2^{-/-}$ model. This is in fitting with earlier studies demonstrating a significantly greater rate of bone formation and trabecular bone volume associated with germline deletion of Y1 receptors (chapter 3). Recent studies by our laboratory have demonstrated that unlike the $Y2^{-/-}$ model, the Y1-mediated anabolic pathway does not appear to be regulated via a central mechanism (unpublished data). Therefore it is plausible that deletion of Y2 receptors either directly or indirectly causes an alteration of Y1 receptor mRNA expression within the bone microenvironment with downstream consequences on osteoblast activity. Altered expression of remaining Y receptors within certain brain regions has been observed in germline Y receptor knockout models, indicative of cross regulation between Y receptor subtypes or overlapping functions in different signalling pathways (Lin et al., 2005). As there is limited evidence for expression of Y2 receptor mRNA in bone cells, it is more likely that the lack of Y2 receptors on neurons is the cause for the down-regulation of Y1 receptors in osteoblast precursors. Y2 receptors are known to be expressed on the pre-synaptic side of sympathetic nerve terminals and act in an auto-inhibitory fashion to regulate the release of NPY and other neurotransmitters (King et al., 2000). The lack of this

feedback inhibition on NPY release could lead to elevated levels of NPY (King et al., 2000; Sainsbury et al., 2002a), which might in turn cause an over-stimulation of Y1 receptors on bone precursor cells and subsequently lead to the desensitisation and down-regulation of this Y1 receptor population (Figure 7.8). This would also be consistent with the increased bone formation observed in hypothalamus-specific Y2 receptor knockout mice, whereby sympathetic nervous activity is potentially altered leading to increased transmitter release (including NPY) in the periphery and may cause a similar down-regulation of Y1-receptors on bone forming cells. This is supported by a recent study which provided evidence that nerve fibres within bone tissue are under the control of synaptic transmission from the hypothalamus (Denes et al., 2005). However, it can not be excluded that part of the effect on Y1-receptor down-regulation is caused by NPY produced by cells such as megakaryocytes within bone marrow (Ericsson et al., 1987).

7.4.3 Methods for the isolation of mesenchymal progenitors

A large variation in the abundance of MSCs and variation in cell surface markers of cells within the bone marrow of different strains of mice has been noted by several studies (Peister et al., 2004; Phinney et al., 1999). In one such study, the number of colonies formed from plastic adherent cells from bone marrow of C57/BL6 and SvJ/129 mice were so low they failed to proliferate and were therefore not further tested for differentiation potential (Phinney et al., 1999). The mice used in this study were on a 50:50 C57/BL6-SvJ/129 background, and in contrast to the above study, problems with establishing and culturing plastic-adherent stromal cell cultures were not encountered. Furthermore, these cells successfully underwent differentiation to form mineralising osteoblasts and lipid-filled adipocytes. These results are in concordance with other studies which have also successfully established plastic adherent stromal cell cultures from C57/BL6 mice (Meirelles and Nardi, 2003; Peister et al., 2004). Differences in the ability to culture cells from these strains of mice may relate to differences in the media used or initial plating density.

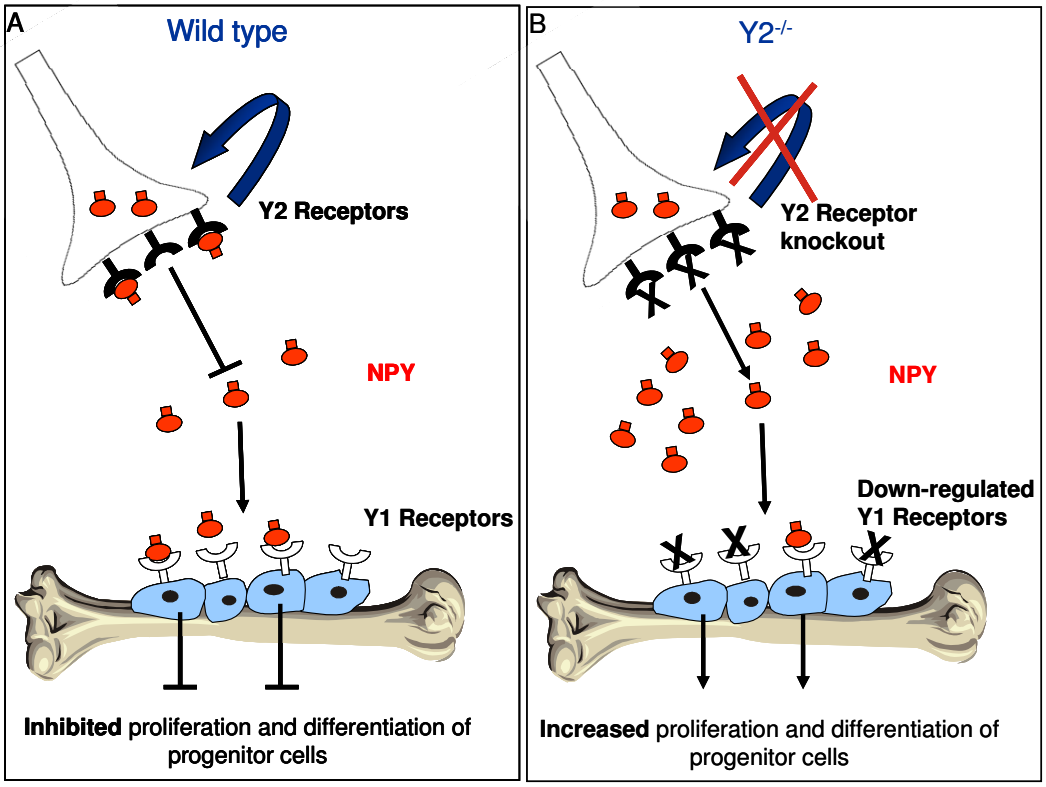

Figure 7.8 Proposed mechanistic model for the greater bone formation in the absence of Y2 receptors.
In wild type mice Y2 receptors which are predominantly pre-synaptically expressed inhibit the release of NPY. Y1 receptor signalling on stromal cells may inhibit or allow a basal rate of proliferation and differentiation of mesenchymal progenitor cells (A). When Y2 is deleted (B), lack of feedback inhibition by Y2 results in increased NPY expression. This may over-stimulate Y1 receptors expressed on bone cells, resulting in their desensitisation and down-regulation. This is also associated with an increase in the number of mesenchymal progenitor cells.

The isolation of a pure population of mesenchymal stem cells has proven difficult due to the rarity of MSCs within bone, the lack of knowledge regarding their location within bone tissue, and the lack of suitable markers for their identification and isolation (Short et al., 2003). Marrow stromal cells isolated by plastic adherence have many characteristics of MSCs as they can differentiate into mineral-producing osteoblasts, chondrocytes, adipocytes, and myotubes (Prockop, 1997). However, multiple studies have shown that cells isolated this way represent a heterogeneous population, contaminated by the presence of haematopoietic and endothelial cells (Phinney et al., 1999; Prockop, 1997). Several different methods have been used by different research groups to isolate a more

homogeneous population of MSCs, for example immunodepletion of haematopoietic cells following plastic adhesion (Baddoo et al., 2003; Wieczorek et al., 2003), isolation of cell populations based on a density gradient (Pittenger et al., 1999), selection of early cycling plastic adherent cells by transfection and selection with a retroviral vector (Kitano et al., 2000), and selection based on 5-flurouracil (5-FU) resistance, Sca-1 antigen expression, and binding to wheat germ agglutinin (WGA) (Van Vlasselaer et al., 1994). However, analysis of cell surface markers following isolation suggests differences in the populations of cells isolated by these different methods. Furthermore, the cells isolated using some of these methods did not retain multi-potentiality or failed to proliferate effectively (Kitano et al., 2000; Pittenger et al., 1999; Prockop, 1997; Van Vlasselaer et al., 1994), indicating the failure of these methods to enrich for a highly proliferative, multipotential progenitor cell type. Thus, to date there is no single established and universally employed method for the isolation of murine MSC or progenitor cells.

Different methods have also been used for the isolation of human MSCs, for example, adherence to plastic and isolation by size (Hung et al., 2002; Lee et al., 2004), or by immunodepletion of haematopoietic cells (Tondreau et al., 2004). Several antibodies have now also been used to facilitate the enrichment of CFUs in aspirates of human bone marrow. The first of these was STRO-1 (Simmons and Torok-Storb, 1991), which has since been used in combination with an antibody to vascular cell adhesion molecule-1 (VCAM-1) to further enrich for CFUs (Gronthos et al., 2003). SB-10/CD166 (Bruder et al., 1997), HOP-26 (Joyner et al., 1997a), and CD49a (Deschaseaux and Charbord, 2000), have also been used to enrich for CFUs prior to culture. A recent study investigating the colony forming efficiencies of cells isolated by these different antibodies, however, demonstrated significant differences in the proportion of CFU-producing cells isolated by the different antibodies, with evidence to suggest differences in the subsets of cells isolated by these antibodies (Stewart et al., 2003). Thus, the search for a defined marker for the isolation of human progenitor cells is also far from over. Furthermore, the majority of these studies have focused on the colony forming or osteogenic ability of the isolated cells and therefore the multi-potentiality of these isolated cells remains to be addressed.

In this study, MSC and progenitor cells were isolated based on the marker Sca-1. Sca-1 is a glycoprotein-I-linked cell surface glycoprotein found on murine haematopoietic, mammary epithelial, and skeletal muscle progenitor cells, primary osteoblasts, stromal cells, and osteoblastic cell lines (Baddoo et al., 2003; Bradfute et al., 2005; Gussoni et al., 1999; Horowitz et al., 1994; Meirelles and Nardi, 2003; Short et al., 2003; Trevisan and Iscove, 1995; Welm et al., 2002; Wieczorek et al., 2003; Wognum et al., 2003). Sca-1 is commonly used as a marker for the isolation of haematopoietic stem cells (HSCs) from mouse bone marrow; however, its biological role is poorly understood. Recent studies have demonstrated that its presence is required for normal HSC activity and lineage fate, while a potential role in the self-renewal and homing ability of HSCs has also been proposed (Bradfute et al., 2005; Ito et al., 2003). Interestingly, mice lacking Sca-1 develop late onset osteoporosis due to a deficiency of osteoprogenitor cells, suggesting that Sca-1 is also required for the appropriate self-renewal of mesenchymal progenitors and identifies a population of immature MSCs with the ability to undergo osteoblast differentiation (Bonyadi et al., 2003).

7.4.4 Summary

This study involved the isolation of two mesenchymal progenitor populations of cells using the cell surface markers Sca-1 and CD51, demonstrating that bones from germline $Y2^{-/-}$ mice have a two-fold greater number of mature mesenchymal progenitor cells compared with wild type mice. These results suggest a likely reason for the greater mineralisation and adipocyte differentiation in $Y2^{-/-}$ stromal cell cultures and a possible mechanism for the greater bone formation of the $Y2^{-/-}$ model. We have also demonstrated a marked down-regulation of Y1 receptor expression in stromal cells from $Y2^{-/-}$ mice, suggesting that down-regulation of Y1 receptor signaling within the bone microenvironment may play an important role in this process.

Despite a greater number of Sca-1^{-}CD51^{+} progenitor cells in bones from $Y2^{-/-}$ mice, numbers of Sca-1 positive MSCs were similar between the two genotypes. While a direct progression from a more immature Sca-1^{+} to a more mature Sca-1^{-}CD51^{+} progenitor cell is

yet to be established (Paul Simmons, personal communication), these findings suggest a greater progression of Sca-1^{+} MSCs from Y2$^{-/-}$ mice subsequently resulting in a greater number of Sca-1^{-}CD51^{+} progenitor cells. It is interesting to note however, that histomorphometry indicated that osteoblast number in distal femora from germline Y2$^{-/-}$ mice were not different from wild type (Baldock et al., 2002). The reason for a lack of increase in osteoblast number in spite of a greater number of progenitor cells is not clear; however, the greater number of progenitor cells may result in a greater turnover rate of the mature osteoblastic cells, which is not detectable by assessment of osteoblast number. This could contribute to a younger population of mature osteoblasts, which may be more active than older ones (Jilka et al., 1996). In the absence of a cell surface marker specific for this type of progenitor cell, the assessment of the location or the number of the Sca-1^{-}CD51^{+} progenitor cells in bone tissue *in vivo* will be impossible.

Deletion of Y2 receptors solely from the hypothalamus also resulted in a greater rate of bone formation and a significant increase in trabecular bone volume (Baldock et al., 2002), demonstrating the control of osteoblast activity in this model to be regulated by a neuronal-mediated pathway. It is yet to be determined whether deletion of hypothalamic Y2 receptors results in a similar increase in mesenchymal progenitor number and down-regulation of Y1 receptor expression in stromal cells. These studies will be essential to investigate whether conditional deletion of Y receptors in adult mice is able to activate a proliferative response of MSCs within the bone, and to determine the downstream mechanism by which the central-Y2 pathway activates bone formation.

Chapter 8

Summary of findings and general discussion

8.1 THE RELATIONSHIP BETWEEN FAT AND BONE

High body weight has long been recognised to have protective effects against bone loss and the development of osteoporosis, with a close correlation between bone density and body weight. Further studies have revealed that fat mass is a major correlate of bone density (Reid et al., 1992a; Reid et al., 1994; Reid et al., 1992b). The protective effects of fat on bone are likely not entirely attributable to a response to greater biomechanical forces due to load bearing, as the correlation between fat mass and bone density also exists for non-weight bearing parts of the skeleton (Reid et al., 1992a). Furthermore, if the greater bone mass associated with increased body weight was solely the result of mechanical loading, the contributions of fat and lean mass to bone density would be expected to be equal (Compston et al., 1992; Reid et al., 1992a; Reid et al., 1994). The protective effects of fat have also been attributed to the role of the adipocyte as the primary site of estrogen production in postmenopausal women; however, the correlation between fat mass and bone density exists also in premenopausal women, in which estrogen production by adipose accounts for only a minor proportion of total estrogen (Reid et al., 1992b). Evidence from recent studies now suggests that adipose may affect bone density through the regulation of bone-active hormones. Adipose mass is a major determinant of insulin, amylin, leptin, and adiponectin, which are all factors able to directly modulate bone cell activity.

Several recent studies and clinical reports have provided evidence of the regulation of bone mass by several factors including CART, leptin, and α-MSH, which interact with each other within the hypothalamus to co-ordinate the regulation of energy homeostasis. Therefore changes in peripheral adiposity could also affect bone mass by altering expression or activity of these neuronal factors. The neuropeptide Y system also interacts with leptin within the hypothalamus to regulate energy balance, modulating feeding behaviour, thyrotropic activity, the HPA axis, and the gonadotropic axis in response to altered leptin signalling. A role also for the Y2 receptor in the regulation of bone formation was subsequently revealed, with a 2-fold increase in bone formation and trabecular bone volume in germline $Y2^{-/-}$ mice (Baldock et al., 2002). The finding that deletion of hypothalamic Y2 receptors produced a similar response within bone demonstrated this to be

a centrally-mediated response (Baldock et al., 2002). Importantly, deletion of Y4 receptor signalling did not result in a discernible bone phenotype (Sainsbury et al., 2003), revealing specificity between the Y receptor subtypes in their control of bone remodelling.

8.2 Y RECEPTOR SPECIFICITY AND INTERACTIONS WITH LEPTIN IN BONE

8.2.1 Bone formation

The studies presented here investigated firstly whether another Y receptor subtype, the Y1 receptor, is involved in the regulation of bone cell activity. Like $Y2^{-/-}$ mice, trabecular bone volume in $Y1^{-/-}$ mice was significantly greater than wild type, also associated with a greater rate of mineral apposition with no change in mineralising surface, demonstrating the activation of an anabolic pathway in the absence of Y1 receptor signalling. Analysis of trabecular bone volume and bone cell activity in compound Y receptor knockouts lacking the Y1 and/or Y2 receptor revealed that the only synergistic elevation in bone volume was observed in male $Y2^{-/-}Y4^{-/-}$ mice, with trabecular bone volume significantly greater than wild type and $Y2^{-/-}$ levels. The lack of synergistic elevation in bone volume in female $Y2^{-/-}Y4^{-/-}$ and $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ mice of both genders was associated with normal serum leptin levels in these mice (Sainsbury et al., 2006), providing support for the hypothesis that the synergistic increase in bone volume in male $Y2^{-/-}Y4^{-/-}$ mice results from additive effects of the leptin and Y2-receptor deficient anabolic pathways initiated in the absence of Y4 receptor signalling. Gender differences in plasma leptin levels have previously been reported in humans, with greater plasma leptin observed in females independent of adiposity (Rosenbaum et al., 1996; Saad et al., 1997). This may relate to differences in fat distribution between genders or to differential responses to sex hormones (Montague et al., 1995; Rosenbaum et al., 1996; Saad et al., 1997). The response of leptin to inflammatory stimuli in rats has also revealed gender specificity, with a greater percentage increase in plasma leptin in female rats compared with males in response to lipopolysaccharide (Gayle et al., 2006). Gender specific differences in the NPY system have also been observed, with greater glucose-induced secretion of PYY from enteroendocrine L cells in female subjects

compared with males (Kim et al., 2005). Interestingly, the Y4 receptor is believed to be involved in the regulation of the gonadotropic axis by leptin, with deletion of Y4 receptors improving the fertility of sterile ob/ob mice (Sainsbury et al., 2002c). However, while in male $Y4^{-/-}$/ob mice fertility was restored completely, fertility was only partially restored in female $Y4^{-/-}$/ob mice (Sainsbury et al., 2002c), suggesting the role of the Y4 receptor in female mice in the regulation of the gonadotropic axis may be reduced compared with males, or that other gender-specific factors are involved in female mice. It therefore appears that the lack of both Y2 and Y4 receptor signalling interacts in a gender-specific manner to reduce serum leptin in male mice only, therefore resulting in a synergistic elevation in bone volume. The reduction in bone volume in $Y1^{-/-}Y2^{-/-}Y4^{-/-}$ male mice from $Y2^{-/-}Y4^{-/-}$ to $Y2^{-/-}$ levels implicates a requirement for functional Y1 receptor signalling in the synergistic response of the $Y2^{-/-}Y4^{-/-}$ model, with wild type levels of leptin in the triple knockout consistent with a role for reduced leptin signalling in the mechanism of the $Y2^{-/-}Y4^{-/-}$ bone response (Sainsbury et al., 2006).

The central antiosteogenic actions of leptin are mediated by altered sympathetic signalling (Takeda et al., 2002). The NPY system is also involved in the regulation of sympathetic activity, with NPY the most prominent neuropeptide in the sympathetic nervous system. It co-exists with noradrenaline in many sympathetic neurons in both the peripheral and central nervous systems, and is co-released with noradrenaline to regulate blood pressure homeostasis (Bleakman et al., 1993). The Y1 and Y2 receptor subtypes are also involved in this process. The predominantly post-synaptic Y1 receptor on blood vessels mediates the actions of NPY on vasoconstriction, while the predominantly pre-synaptic Y2 receptor regulates sympathetic activity by modulating the synthesis and release of noradrenaline. Furthermore, leptin and Y2 receptors are co-expressed on NPY-ergic neurons of the hypothalamus (Baskin et al., 1999; Broberger et al., 1997), suggesting that similar to its role in the control of energy homeostasis, NPY may also mediate the actions of leptin in the control of bone formation acting through either the Y1 and/or Y2 receptor. That possibility was investigated in this thesis by the deletion of Y receptor signalling on the leptin-deficient ob/ob background through the generation of double knockout mice. This model revealed that Y1 receptor signalling was required for the anabolic phenotype of the ob/ob

model, and conversely, that leptin signalling was required for the anabolic phenotype of the $Y1^{-/-}$ model, with the greater mineral apposition rate characteristic of the $Y1^{-/-}$ and ob/ob models attenuated in $Y1^{-/-}$/ob mice. However, these findings do not necessarily necessitate that the leptin and Y1 receptor systems utilise a common downstream signalling pathway to alter osteoblast activity, as it would therefore be expected that deletion of both leptin and Y1 receptor signalling would result in a bone phenotype identical to the $Y1^{-/-}$ and ob/ob models, with no evidence of either additive or deleterious effects. Further investigation is required to address this question and could be approached by investigating whether sympathetic signalling is required for activation of the anabolic response of the $Y1^{-/-}$ model using either a genetic or a pharmacological approach.

In contrast to the $Y1^{-/-}$/ob model, mineral apposition rate in $Y2^{-/-}$/ob mice was not different from either $Y2^{-/-}$ or ob/ob, with reduced trabecular bone volume in the double knockouts relative to $Y2^{-/-}$ resulting from elevated resorption. This may be secondary to the hypogonadism induced by the leptin deficient state. Previous studies have demonstrated that correction of the hypogonadism phenotype of ob/ob mice following replacement of estradiol or testosterone normalises osteoclast number and further increases trabecular bone mass in this model (Ducy et al., 2000). More recent studies, however, have indicated that the elevated resorption phenotype of the ob/ob model results from reduced expression of CART, a negative regulator of osteoclast formation (Elefteriou et al., 2005). Hypothalamic levels of CART in $Y2^{-/-}ob^{-/-}$ mice are reduced to levels similar to ob/ob (Sainsbury et al., 2002b), consistent with the comparable parameters of bone resorption of these models.

While these findings indicate the Y2 receptor and leptin-deficient anabolic responses are mediated by a common pathway or regulatory feedback loop, other studies from our group suggest the control of bone formation by leptin and Y2 receptors occur by distinct pathways, with elevated serum leptin and hypothalamic NPY achieved by injection of adeno-associated viral vector-expressing NPY into wild type and $Y2^{-/-}$ mice reducing bone volume, but unable to abolish the greater osteoblast activity of the $Y2^{-/-}$ model (Baldock et al., 2005). Together these findings reveal that in situations of reduced leptin, the anabolic response of the $Y2^{-/-}$ pathway is not discernible from the regulation of osteoblast activity by

leptin, perhaps indicative of a common regulatory pathway or feedback loop, or alternatively suggesting the requirement of a permissive level of leptin for activation of the Y2-mediated anabolic pathway. However under normal or elevated leptin signalling, the Y2-mediated anabolic pathway is distinct from leptin's actions within bone, with a consistent elevation of bone formation in the absence of Y2.

Distinct actions of the Y2 and leptin-mediated pathways have also been observed in cortical bone. Deletion of Y2 receptor, but not leptin signalling, stimulates osteoblastic activity within the cortices, resulting in greater cortical BMD and BMC in $Y2^{-/-}$ compared with ob/ob mice (Baldock et al., 2006b). The lack of synergistic increase in trabecular bone formation in $Y2^{-/-}$/ob mice in this study could potentially result from an inhibitory feedback loop that prevents further increases in osteoblast activity. However, the synergistic increase in trabecular bone volume in $Y2^{-/-}Y4^{-/-}$ male mice suggests that further increases are possible, and therefore supports the proposal that activation of the Y2-anabolic pathway may require a permissive level of leptin. It should also be noted, however, that leptin deficiency results in numerous developmental defects, with impaired fertility, hypercorticism, hyperglycaemia, hyperinsulinemia, decreased locomotor activity, decreased immune function, and severe obesity characteristic of ob/ob mice (Ahima et al., 1998; Ahima et al., 1996; Friedman and Halaas, 1998; Guo et al., 2004; Legradi et al., 1997; Schwartz et al., 1995; Zhang et al., 1994). Furthermore, the size and weight of ob/ob brains are also reduced, with a generalised reduction in most areas of the brain, a phenotype which is reversible by leptin treatment (Steppan and Swick, 1999; Zhang et al., 1994). These phenotypic abnormalities resulting from leptin deficiency reflect the significant role for leptin in multiple systems during development, emphasising the need for caution when assessing the physiological role of leptin using the leptin deficient ob/ob model.

Nevertheless, the findings from the above studies suggest that the mechanisms by which leptin and Y2 receptor signalling mediate bone formation are distinct. Furthermore, chemical ablation studies have also suggested that leptin uses separate pathways to regulate bone formation and energy homeostasis, with hypothalamic structures important for leptin anorexigenic effects found to be dispensable for its antiosteogenic effects, and structures

essential for leptin antiosteogenic actions dispensable for its anorexigenic effects (Takeda et al., 2002). In an examination of $NPY^{-/-}$ mice, this same group found no skeletal phenotype and therefore concluded that NPY does not play any role in the central control of bone remodelling (Elefteriou et al., 2003). The validity of this conclusion is questionable, however, as $NPY^{-/-}$ mice also lack an obvious phenotype in terms of food intake and energy homeostasis despite the well characterised role of NPY in the regulation of these pathways (Erickson et al., 1996a; Stanley et al., 1986). Moreover, recent findings with $NPY^{-/-}$ mice generated in our laboratory using a different targeting strategy suggest that trabecular and cortical bone formation and volume are increased in the absence of NPY signalling (Baldock et al., 2006a), contradicting the earlier studies and supporting a role for NPY in the control of bone formation.

8.2.2 Bone resorption

Central leptin also modulates adrenergic signalling to regulate bone resorption, with reduced bone resorption in *Adrb2*$^{-/-}$ mice unable to be corrected by central administration of leptin (Elefteriou et al., 2005). This is believed to be mediated indirectly through cells of the osteoblastic lineage, with activation of β-adrenergic signalling stimulating the expression of RANKL (Elefteriou et al., 2005; Takeuchi et al., 2000). Leptin also regulates bone resorption through its downstream effector CART, which inhibits osteoblastic RANKL and therefore inhibits bone resorption by a mechanism which appears to be distinct from sympathetic signalling. CART is elevated in leptin deficient ob/ob mice, consistent with the elevated bone resorption of this model (Ducy et al., 2000; Elefteriou et al., 2005). Osteoclast surface was significantly elevated in $Y1^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice compared with wild type, with the percentage increase compared with wild type greater in the $Y2^{-/-}Y4^{-/-}$ mice than in the $Y1^{-/-}$ model. Thus, alterations in the regulation of osteoclastogenesis may differ in these two models. Levels of hypothalamic CART are reduced to a similar extent in both $Y1^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice (Karl et al., 2004; Sainsbury et al., 2003), suggesting that reduced CART is not the sole mechanism for the elevated Oc.S of these models. Moreover, despite a comparable reduction in hypothalamic CART, Oc.S was not reduced in $Y2^{-/-}$ mice (Sainsbury et al., 2003), suggesting that these moderate

reductions in CART may not be responsible for the observed reduction in bone resorption in the $Y1^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ models. Of note, the greater number of mesenchymal progenitor cells present within the bone of $Y2^{-/-}$ mice would also be expected to result in a corresponding increase in osteoclast number. The reason why this is not observed in $Y2^{-/-}$ mice is not clear. However, as histomorphometric measurements of osteoblast number were not altered in $Y2^{-/-}$ mice (Baldock et al., 2002), possibly due to a greater rate of osteoblast turnover, it is possible that a similar turnover of osteoclastic cells occurs, and is also not detectable by assessment of osteoclast surface or number. It is also possible that alterations in sympathetic activity independent of CART may affect bone resorption in these mice. The role of sympathetic signalling on bone remodelling in the individual Y receptor knockout models is yet to be investigated.

The elevated parameters of bone resorption of $Y1^{-/-}$ mice could also relate to alterations in immune function of this model, as T cells of $Y1^{-/-}$ mice are hyper-responsive to activation (Wheway et al., 2005). There is accumulating evidence that the immune system can regulate bone cell activity. Ovariectomy-induced activation and proliferation of T cells within the bone marrow up-regulates the production of osteoclastogenic cytokines such as TNFα (Weitzmann and Pacifici, 2005; Weitzmann and Pacifici, 2006), and T cells are also a major source of RANKL within bone (Kong et al., 1999a). Therefore, the hyper-responsiveness of T cells may contribute to the increase in bone resorption in $Y1^{-/-}$ mice. However, this proposal is complicated by a defect in the antigen presenting cell (APC) population isolated from $Y1^{-/-}$ spleens (Wheway et al., 2005), as the activation of T cells following estrogen removal is likely to require functional antigen presentation (Cenci et al., 2003). Importantly, the APC population in $Y1^{-/-}$ bone marrow has not yet been investigated. Thus, the observed changes in osteoclast surface in $Y1^{-/-}$ mice may, or may not relate to the immune deficits of this model. Interestingly, *ex vivo* cultures of $Y1^{-/-}$ bone marrow macrophages have a reduced ability to form osteoclasts in the presence of M-CSF and RANKL compared with wild type controls, suggesting a cell- or lineage- autonomous defect in the responsiveness to osteoclastogenic stimuli in this mouse model (S. Allison, P. Lundberg, unpublished data).

Thus it is not obvious why parameters of bone resorption are increased in $Y1^{-/-}$ mice *in vivo* but decreased *in vitro*, or how this may relate to additional influences by leptin, the sympathetic nervous system, or altered production of cytokines due to changes in immune function. The ability of cultured osteoclasts from $Y1^{-/-}$ mice to resorb bone has not yet been investigated, but the elevation in osteoclast surface and loss of trabecular bone volume in gonadectomised $Y1^{-/-}$ mice suggests these osteoclasts are functional. Interaction between the leptin and Y receptor pathways in the regulation of bone remodelling is an area of particular interest in order to determine the mechanisms by which these pathways regulate bone cell activity, but will require further investigation to elucidate the extent of interaction between these models. Together these studies have however, revealed specificity between the Y receptor subtypes in their ability to regulate osteoblastic and osteoclastic responses, with evidence suggesting interaction with leptin in some pathways, and distinct mechanisms of actions in others.

8.2.3 Marrow adipose

In contrast to the unresolved issue of interaction between leptin and the NPY system in bone, the interaction between leptin and NPY in the regulation of energy homeostasis is well established, with attenuation of NPY signalling reducing body weight and the obesity syndrome of ob/ob mice (Erickson et al., 1996b). These effects are mediated through the Y receptor subtypes with deletion of Y1 or Y2 receptor signalling also reducing body weight and adiposity of ob/ob mice (Naveilhan et al., 2002; Pralong et al., 2002; Sainsbury et al., 2002b). The studies presented here revealed for the first time a similar interaction between leptin and Y receptor signalling in the regulation of adipose within the bone microenvironment with reduced marrow adiposity in $Y1^{-/-}$/ob and $Y2^{-/-}$/ob compared to ob/ob mice.

Specificity within the actions of the Y receptor subtypes was revealed with analysis of $Y4^{-/-}$ /ob mice, in which marrow adiposity was not reduced from ob/ob, consistent with the lack of effect in extramedullary adipose (Sainsbury et al., 2002c).

It is interesting to note that $Y1^{-/-}ob^{-/-}$ and $Y2^{-/-}ob^{-/-}$ mice remained heavier, despite a reduction in adiposity compared with ob/ob mice (Naveilhan et al., 2002; Pralong et al., 2002; Sainsbury et al., 2002b). In contrast, total marrow adipocyte volume was reduced from ob/ob in female $Y1^{-/-}$/ob and in both male and female $Y2^{-/-}$/ob mice to levels not significantly different from wild type. The magnitude of the adipose response was therefore greater within the marrow cavity compared with peripheral depots. The similar directional change of marrow and extramedullary adiposity suggests the reduction in marrow adipose in $Y1^{-/-}$/ob and $Y2^{-/-}$/ob mice may simply be a reflection of reduced total body adiposity. The greater response within this region could perhaps be due to a lack of both central and locally-produced leptin signals within the bone microenvironment, with the production of leptin demonstrated by both marrow adipocytes and by osteoblastic cells (Laharrague et al., 1998; Morroni et al., 2004; Reseland et al., 2001). The presence of leptin receptors on osteoblast and stromal cells may also indirectly influence the response of adipocytes within the bone microenvironment to leptin deficiency. It should also be noted that the greater number of mesenchymal progenitor cells present within the bone of germline $Y2^{-/-}$ mice would also be expected to contribute to changes in marrow adiposity, and is further discussed later in this chapter. Importantly, previous studies have revealed site specificity in the marrow adipose response to leptin deficiency, with few adipocytes present within the marrow of the lumbar vertebrae of ob/ob mice, despite significant marrow adiposity of the femur (Hamrick et al., 2004). Although the studies presented in this thesis only examined marrow adiposity within the distal femur, it is recognised that site specificity in the marrow adipose response to leptin deficiency and to Y1 or Y2 receptor deletion may also exist.

8.3 Y RECEPTOR ANABOLIC PATHWAYS IN MODELS OF OSTEOPOROSIS

8.3.1 Sex hormone deficiency

Sex hormone deficiency and aging are two leading risk factors for the development of osteoporosis in humans (Mundy et al., 2003; Orwoll and Klein, 1995). The role of the Y

receptor pathways and their actions within bone in the absence of sex hormones was investigated using gonadectomised Y receptor knockout mice. Interestingly, while trabecular bone volume was reduced following gonadectomy in both genders of germline $Y1^{-/-}$ and $Y2^{-/-}$ mice, there were differential responses between the Y receptor subtypes to sex hormone deficiency. The greater mineral apposition rate of the $Y2^{-/-}$ model was maintained in the sex hormone depleted state, but the bone formation phenotype was markedly reduced in male $Y1^{-/-}$ mice. The reduction in mineral apposition rate in orchidectomised $Y1^{-/-}$ mice was akin to the reduction in mineral apposition rate observed in the hypogonadal leptin deficient $Y1^{-/-}$/ob mice of both genders compared to $Y1^{-/-}$ animals. Thus, the anabolic activity of the Y1-mediated pathway appears to be functional only in the presence of sex hormones; however, the anabolic phenotype persisted in ovariectomised $Y1^{-/-}$ mice, indicating that the situation may be more complex in females. Therefore, the changes in anabolic activity of the $Y1^{-/-}$ model observed in leptin and sex-hormone deficient states may reveal a role for sympathetic activity in the Y1 anabolic pathway, such that reduced sympathetic tone associated with lack of leptin (Takeda et al., 2002), or increased sympathetic tone associated with sex hormone deficiency (Brownley et al., 2004), may modify the osteoblastic response to Y1 receptor deletion.

In contrast to the response observed in gonadectomised male $Y1^{-/-}$ mice, the anabolic activity of the $Y2^{-/-}$ pathway was independent of sex hormones in mice of both sexes. Importantly, conditional deletion of hypothalamic Y2 receptors following the occurrence of significant gonadectomy-induced bone loss activated the anabolic response and completely prevented further bone loss in the face of elevated osteoclast surface, demonstrating the potency of this pathway. Furthermore, these studies also investigated for the first time the actions of the Y2-mediated pathway within the lumbar vertebrae, demonstrating a lack of an anabolic phenotype in $Y2^{-/-}$ mice under normal conditions, which was activated in the absence of sex hormones to prevent bone loss. Thus, this pathway may have the ability to switch on in situations in which bone loss would otherwise occur, such as in the absence of sex hormones.

8.3.2 Aging

The therapeutic potential of the centrally-mediated Y2-anabolic pathway was also investigated in aged mice. The anabolic response was successfully activated in aged animals by conditional deletion of hypothalamic Y2 receptors, as evidenced by elevated mineral apposition rate. Nevertheless, this Y2-associated anabolic response was not able to increase trabecular bone volume. This may be due to substantial loss of trabecular structures associated with aging, such that insufficient template was available on which the osteoblasts could form new bone. The ability of the central Y2-anabolic pathway to produce a successful outcome in trabecular bone following gonadectomy, but not in aged bones may relate to defects in calcium absorption and alterations in PTH levels in the aged models which may be absent in the younger mice.

However these studies did reveal an unusual cortical bone response, with marked increases in endosteal and periosteal circumferences at the distal femur and the femoral midshaft, associated with a marked increase in cortical porosity in female $Y2^{-/-}$ mice. The remarkable changes in cortical circumference occurred within just 16 weeks, demonstrating a significant change in bone cell activity. It is conceivable that this response in aged cortical $Y2^{-/-}$ bones may have resulted from an increased requirement for free calcium following activation of the anabolic response; resulting in an ensuing increase in both resorption and formation at the cortical surfaces. It is also possible that the widening of the cortical shaft could have positive effects on bone strength, and therefore biomechanically compensate for the loss of endocortical bone despite thinning of the cortices (Beck et al., 2000); however, strength testing would be required to confirm this hypothesis.

Interestingly, concurrent studies in our laboratory have demonstrated significantly greater femoral BMD and BMC in germline $Y2^{-/-}$ compared with wild type mice at 7 months of age. However, although this trend still existed at 15 months of age, the differences did not reach significance (P. Baldock, unpublished data). It will be of interest to investigate whether these germline $Y2^{-/-}$ mice also developed wider marrow cavities with age, or whether this phenotype is specifically induced by activation of the anabolic response after

conditional Y2 receptor deletion in extremely aged mice. It is possible that germline $Y2^{-/-}$ mice maintain adequate trabecular template with age, and may therefore maintain calcium homeostasis without resorting to the degree of endocortical resorption observed in the aged conditional knockouts.

The findings from these studies also highlights a potential problem that might arise when administering anabolic agents in aged subjects; with activation of an anabolic response substantially increasing the requirement for free calcium, possibly resulting in hypocalcaemia. However, this issue is not specific to anabolic agents, as it also arises with administration of powerful antiresorptive agents to aged patients with impaired calcium absorption. It is therefore recommended that patients receiving antiresorptive or anabolic treatments must have adequate calcium intake (Boonen et al., 2006).

8.4 MECHANISM OF THE Y2-MEDIATED ANABOLIC RESPONSE

8.4.1 Increased mesenchymal progenitor numbers in $Y2^{-/-}$ bone

Stromal cell cultures from wild type and germline $Y2^{-/-}$ mice revealed an enhanced ability of $Y2^{-/-}$ cells to undergo osteoblast and adipocyte differentiation under appropriate differentiation-promoting conditions. This was initially surprising as the Y2 bone-anabolic pathway is known to be centrally mediated, and therefore it was expected that $Y2^{-/-}$ stromal cells in culture would not differ in their differentiation capabilities. Furthermore, the $Y2^{-/-}$ mice have a lean phenotype with marrow adipocyte number similar to wild type levels. However, further investigation of the mesenchymal population revealed a 2-fold greater number of mesenchymal progenitor cells within the bone of $Y2^{-/-}$ mice, consistent with an enhanced ability of cultured stromal cells from $Y2^{-/-}$ mice to differentiate down either the osteoblastic or adipocytic lineages under appropriate culture conditions, and suggesting a possible mechanism for the greater rate of bone formation of $Y2^{-/-}$ mice *in vivo*.

The identification of markers for the isolation of stem cell populations has been challenging, with various methods employing immunoselection and flow cytometry

adapted by different laboratories to enrich for mesenchymal lineage cells from whole bone marrow. In particular, various antibodies have been used to enrich for osteoprogenitor cells from human marrow. The STRO-1 antibody recognises a CFU enriched population, while the monoclonal antibody SP-10 which is directed against an activated leukocyte cell adhesion molecule (ALCAM) detects marrow stromal cells and osteoprogenitors but not mature osteoblasts in humans (Bruder et al., 1997). The SH2 antibody, which immunoprecipitates CD105 (endoglin, TGFβ-3), and the HOP-26 antibody, which recognises CD63, the cell surface lysosomal enzyme member of the tetraspan glycoprotein family (Bruder et al., 1997; Joyner et al., 1997b; Zannettino et al., 2003), have also been used to identify human early osteoprogenitor cells. Therefore, although no specific osteoprogenitor marker is currently available, a variety of different antibodies have been successfully used to identify populations of osteoprogenitor cells in humans. In addition, the multipotentiality of these cell preparations have not yet been assessed, as the above studies focussed on the identification of a cell type capable of forming mineral in culture.

Techniques to isolate osteoprogenitor or mesenchymal cells from murine bone are even less well established than those used for human, with the majority of methods using a combination of plastic adhesion and depletion of haematopoietic cells to isolate an osteoprogenitor population. The studies presented here employ an approach involving depletion of haematopoietic cells followed by cell sorting based on the marker Stem cell antigen-1 (Sca-1) to isolate an immature mesenchymal stem cell-like population (Sca-1^{+}), and a more mature mesenchymal progenitor cell type (Sca-1^{-}CD51^{+}). Both populations were highly enriched for the formation of CFUs and are able to undergo differentiation down osteogenic, adipogenic, and chondrogenic lineages (P. Simmons, unpublished data). The greater number of Sca-1^{-}CD51^{+} progenitor cells released from the bones of $Y2^{-/-}$ mice indicated an increase in the progression of immature Sca-1^{+} cells towards a more mature Sca-1^{-}CD51^{+} progenitor cell type. This result suggests that the greater mineralisation and adipocyte differentiation observed in stromal cell cultures from $Y2^{-/-}$ mice was due to the presence of a greater proportion of progenitor cells at the initial plating. It would therefore be expected that plating and culturing Sca-1^{+} and Sca-1^{-}CD51^{+} cells from wild type and $Y2^{-}$

$^{/-}$ mice at equal density would abolish the ability of cultures from $Y2^{-/-}$ mice to undergo greater mineralisation and adipogenesis. That experiment was not performed for this thesis but will be required to confirm that the greater mineralisation and adipogenesis of $Y2^{-/-}$ cultures is solely due to a greater proportion of progenitors present at the initial plating.

While this study only investigated the proportion of progenitor cells in germline $Y2^{-/-}$ mice, it would also be of considerable interest to determine whether a similar increase in progenitor number also occurs following conditional deletion of hypothalamic receptors. This would require conditional deletion of hypothalamic Y2 receptors in mice of a maximum of 8-weeks old, in order to allow suitable time for deletion of the Y receptor gene and activation of the anabolic response prior to collection of mice at 14 to 15 weeks of age. To date, the youngest mice used for stereotaxic deletion of hypothalamic Y2 receptors have been 10 to 12 weeks old; however, it is most likely that successful deletion could be achieved in slightly younger mice.

8.4.2 Expression of Y1 receptor in stromal cells

These studies also identified expression of the Y1 receptor in marrow stromal cell cultures from wild type mice, which was virtually abolished in cultures from $Y2^{-/-}$ mice. These findings using RT-PCR are supported by further evidence of Y1 receptor expression within distal femur of wild type, but not $Y1^{-/-}$ mice using *in situ* hybridisation, in which Y1 receptor expression was identified in osteoblasts associated with the endosteal surface of the cortices, and on trabecular bone within the distal metaphyses (P. Lundberg, N. Lee, unpublished data). Y1 receptor expression was also detected by *in situ* hybridisation within bones from $Y2^{-/-}$ mice although quantification of Y1 receptor expression in these bones has not yet been performed.

The direct modulation of bone cell activity by other neuronal factors has been demonstrated by several *in vitro* studies. The sensory peptides calcitonin gene-related peptide (CGRP) and its receptor, substance P (SP) and neurokinin-1 receptors are located on bone cells and have been demonstrated to modify bone cell function (Cornish et al., 1999; Goto et al.,

1998; Mori et al., 1999; Mullins et al., 1993; Togari et al., 1997). Sympathetic neuropeptides in addition to NPY have also been identified within bone tissue. Vasoactive intestinal peptide (VIP), for example, regulates the activity of both osteoblasts and osteoclasts and the presence of VIP receptors has been demonstrated on both these cell types (Lundberg et al., 1999; Lundberg et al., 2000; Lundberg et al., 2001; Togari et al., 1997). These studies demonstrating the presence of neural factors and their receptors within bone cells and the ability of several of these factors to modulate bone cell activity therefore support a potential physiological role for these and other neural factors in the direct regulation of bone function through ligand-receptor interactions.

The down-regulation of Y1 receptors in the $Y2^{-/-}$ model suggests that the increase in osteoblast activity in the absence of Y2 could occur due to reduced signalling through the Y1 receptor subtype on stromal cells within the marrow, suggesting a common pathway through which $Y1^{-/-}$ and $Y2^{-/-}$ models signal to increase osteoblast activity. The ability of reduced Y1 receptor signalling on stromal cells to modulate bone cell activity will require further investigation. This will be approached by future studies in our laboratory by deletion of Y1 receptors *in vitro* via administration of cre-recombinase to stromal cell cultures from Y1 receptor floxed ($Y1^{lox/lox}$) mice, and by using genetic models to ablate Y1 receptor expression from cells of the osteoblastic lineage.

The proposal that the $Y1^{-/-}$ and $Y2^{-/-}$ models share a common signalling pathway is supported by the similar increase in mineral apposition rate and trabecular bone volume in $Y1^{-/-}$ and $Y2^{-/-}$ mice, and the lack of synergistic increase in either mineral apposition rate or trabecular bone volume in $Y1^{-/-}Y2^{-/-}$ double knockout mice. However, despite these similarities between the $Y1^{-/-}$ and $Y2^{-/-}$ models, there are some apparent differences which may indicate they also use alternate pathways to regulate bone remodelling. For instance, parameters of bone resorption are increased in $Y1^{-/-}$ but not $Y2^{-/-}$ mice. It remains possible, however, that the two models use similar pathways to control bone formation but separate mechanisms to control resorption. Interestingly, preliminary data suggests that the Y1-mediated anabolic pathway is not mediated via a central mechanism, with conditional deletion of hypothalamic Y1 receptor signalling failing to elevate bone formation or

trabecular bone volume (unpublished data), supporting a model in which peripheral Y1 receptor signalling, possibly by Y1 receptors located on stromal cells themselves, are responsible for changes in bone cell activity. It is also possible that elevated levels of NPY in the absence of Y2 receptor signalling (King et al., 2000; Sainsbury et al., 2003; Sainsbury et al., 2002b), could inhibit the expression of the Y1 receptor. A recent study used pseudorabies virus-based transneuronal tracing to map trans-synaptically connected neurons from rat bone, providing direct evidence that nerve fibres within bone tissue are under the control of synaptic transmission from the hypothalamus (Denes et al., 2005). Therefore it is possible that alterations in NPY expression within the hypothalamus could directly affect signalling at Y receptors on bone cells. Alternatively, as NPY is produced by cells such as megakaryocytes within bone marrow (Ericsson et al., 1987), it is possible that locally produced NPY affects bone cell activity by direct receptor interactions. Interestingly, NPY mRNA expression was not detected in cultures of bone marrow stromal cells from either wild type or $Y2^{-/-}$ mice by RT-PCR (data not shown), supporting a model in which expression of NPY is altered extramedullary in the absence of Y2. It is of note however that expression of NPY within bone tissue appears to be strain-specific, with evidence for the expression of NPY in bone marrow of C57BL/6 but not in SvJ/129 mice (Z. Zukowska, personal communication). The mice used in this study are on a mixed C57BL6-129/SvJ background, suggesting that levels of NPY in bone marrow stromal cells from these mice may be expressed at particularly low levels.

Importantly, the above model in which the Y1 and Y2 receptors signal through a common pathway to regulate osteoblast activity also fits in with our preliminary assessment of the bone phenotype of $NPY^{-/-}$ mice, which also have greater indices of bone formation and bone volume. These findings indicate that similar increases in bone volume can be achieved in the absence of either the ligand or the receptor. Over-expression of hypothalamic NPY, however, results in decreased bone mass (Baldock et al., 2005; Ducy et al., 2000), contradicting our proposed model in which elevated NPY down-regulates Y1 receptors to increase osteoblast activity. Central administration of adeno-associated viral vector expressing NPY results in massive elevations in hypothalamic NPY, causing a doubling in body weight and adiposity within just 3 weeks, thereby also markedly increasing levels of

serum leptin (Baldock et al., 2005). Therefore it is possible that extremely elevated NPY results in secondary effects which also affect bone cell activity, and which are not observed in the lean $Y2^{-/-}$ model in which hypothalamic NPY levels are increased by only around 20% over wild type (Sainsbury et al., 2002a). Differences between the $Y1^{-/-}$ and $Y2^{-/-}$ models were also observed in the $Y1^{-/-}$/ob and $Y2^{-/-}$/ob crosses and in gonadectomised $Y1^{-/-}$ and $Y2^{-/-}$ mice, in which the greater mineral apposition rate of the $Y1^{-/-}$ model was reduced in both the leptin-deficient, and the sex hormone-deficient state, suggesting that the anabolic activity of the Y1-mediated pathway requires the presence of sex hormones or sympathetic signalling. Thus the degree to which these two models share a common signalling pathway is uncertain at this stage, with some evidence for shared mechanisms, and some evidence for distinct mechanisms using approaches to modify leptin and sex hormone signalling.

8.5 SUMMARY AND POTENTIAL CLINICAL APPLICATION

The findings from these studies suggest that the regulation of osteoblast activity in the absence of either Y1 or Y2 receptors may be mediated by a common pathway, with deletion of Y2 receptors down-regulating expression of Y1 receptors within the bone microenvironment to alter the proportion of mesenchymal progenitors. However, it is also likely that the responses activated in the absence of these two receptors may respond to external influences such as changes levels of leptin and sex hormones differentially, with evidence that the anabolic activity of the $Y1^{-/-}$ model was dependent on leptin signalling and the presence of testicular sex hormones. The downstream effects of the leptin antiosteogenic pathway are mediated by sympathetic signalling, suggesting that sympathetic signalling may also influence the Y1-mediated anabolic pathway. This fits with the known role of the Y1 receptor in the modulation of vasoconstriction in response to altered sympathetic tone. However, the applicability of these findings to the treatment of osteoporosis in humans is not clear. The protective effects of β-blockers on bone in humans remains a matter for debate, and is likely to require placebo-controlled randomised clinical

trials to determine whether alteration of adrenergic signalling may provide suitable benefits for osteoporosis treatment.

The NPY system is involved in numerous physiological processes. A large number of studies have implicated the participation of NPY in the pathophysiology of various diseases including hypertension, intestinal disorders, diabetes, congestive heart failure, feeding disorders, seizures, anxiety, and depression (Balasubramaniam, 2002; Brown et al., 2000; Caberlotto et al., 1998; Colmers and El Bahh, 2003; Vezzani et al., 1999; Zukowska-Grojec et al., 1996). The roles of the specific peptides and Y receptor subtypes involved in these processes have revealed several attractive therapeutic targets for treatment of a variety of human diseases, leading to the development of selective small molecule ligands and assessment of their potential therapeutic applications. For instance, peripheral administration of the Y2 receptor agonist PYY(3-36) acts as a satiety signal and inhibits food intake in rodents and in humans (le Roux and Bloom, 2005), and is currently under further development as an anti-obesity therapy (Brain and Cox, 2006). Antagonists to the Y5 receptor subtypes have also been assessed for their potential as anti-obesity targets, but were unable to reduce feeding (Block et al., 2002). A role for Y1 receptor agonists in the treatment of neurodegeneration, anxiety, and stress disorders has also been proposed (Zukowska and Feuerstein, 2006). The studies presented in this thesis highlight a critical role for the NPY and the Y receptor system also in the regulation of bone formation, and therefore a possible therapeutic benefit of Y1 or Y2 receptor antagonism for the treatment of osteoporosis. However, they also draw attention to potential issues which may arise with the development of treatments for other disorders. For instance, the administration of a Y2 receptor agonist may be effective for the treatment of obesity, but may also result in deteriorative effects on bone density. Thus, further analysis will be required to assess the full potential and limitations of these candidate therapies.

The only anabolic agent currently approved for the treatment of osteoporosis is recombinant PTH 1-34 (teriparatide). This has been shown to have beneficial effects for the treatment of osteoporosis, increasing bone formation and BMD at the lumbar spine and hip, and decreasing the risk of vertebral and non-vertebral fractures (Bradbeer et al., 1992;

Finkelstein et al., 1998; Neer et al., 2001). So far however, PTH treatment is considered primarily for the management of individuals at particularly high risk of fracture and is not recommended for long-term treatment, in part due to the association of PTH administration and the development of osteosarcomas in rodent studies (Hodsman et al., 2005). However, findings from several studies indicate that the efficacy of PTH treatment may be limited in situations of severe osteopenia, most likely resulting from inadequate trabecular template available for the formation of new bone (Qi et al., 1995; Zhou et al., 2003). Of note, while co-administration of PTH 1-34 with an antiresorptive bisphosphonates would be expected to produce synergistic effects on bone volume, evidence from several reports suggests the concurrent use of antiresorptive treatment may reduce the anabolic effects of PTH, suggesting a requirement for osteoclastic activity for the anabolic actions of PTH to follow (Black et al., 2003; Finkelstein et al., 2003). Therefore while the successes of PTH treatment so far have highlighted the benefits of anabolic treatments for osteoporosis, it is clear that the development of novel anabolic treatments is required.

Interestingly, one study has shown an association of a leucine7-to-proline7 (Leu7/Pro7) polymorphism of the NPY gene with protection against postmenopausal loss of femoral neck BMD in women not taking HRT (Heikkinen et al., 2004), suggesting that the NPY system may also be involved in the regulation of bone remodelling within humans. Thus, while the role of the NPY system in the regulation of bone cell activity in humans is still at an early stage of investigation, further studies are required to determine whether alterations in Y receptor signalling which are able to produce such a potent anabolic response in rodents, may also be beneficial for osteoporosis treatment in humans.

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Appendix

PUBLICATIONS ARISING FROM THESIS

Allison SJ, Lundberg P, Brouard N, Lee NJ, Rost S, Enriquez RF, Baldock PA, Sainsbury A, Simmons P, Gardiner EM, Herzog H. Greater bone formation of Y2 knockout mice is associated with down-regulation of Y1 receptors and increased osteoprogenitor number (manuscript in preparation).

Allison SJ, Baldock PA, Sainsbury A, Lin E, Enriquez, RF, Lee NJ, During M, Little DG, Eisman JA, Gardiner EM, Herzog H. Peripheral Actions of Neuropeptide Y1 Receptors Regulate Skeletal and Adipose Homeostasis (manuscript in preparation).

Allison SJ, Baldock PA, Sainsbury A, Enriquez R, Lee NJ, Lin E, Klugman M, During M, Eisman JA, Li M, Pan LC, Herzog H, Gardiner EM. (2006) Conditional deletion of hypothalamic Y2 receptors reverts gonadectomy induced bone loss in adult mice. J Biol Chem. 281:23436-23444.

Baldock PA, Sainsbury-Salis A, Allison S, Lin EJ, Couzens M, Enriquez R, During M, Herzog H, Gardiner EM (2005) Hypothalamic control of bone formation: Distinct actions of leptin and Y2 receptor pathways. J Bone Miner Res. 20(10):1851-1857.

REVIEWS AND CHAPTERS

Allison SJ, Baldock PA, Herzog H. (2006) The control of bone remodelling by neuropeptide Y receptors. Peptides (invited review - submitted).

Baldock P, Allison S, Gardiner E.M. The central control of bone remodelling. In: *Dynamics of Bone and Cartilage Metabolism*, 2nd edition (in press).

Allison S, Herzog H. (2006) NPY and bone. In: G. Feuerstein & Z. Zukowska (eds): *Peripheral Action of NPY*. Humana Press, New Jersey.

ABSTRACTS ARISING FROM THESIS

Oral Presentations

Allison SJ, Baldock PA, Sainsbury-Salis A, Lin EJ, Couzens M, Enriquez R, Boey D, Lin S, During M, Gardiner EM, Herzog H. Beneficial hypothalamic Y2-receptor action in a model of postmenopausal osteoporosis. 8th International NPY Meeting, 22-26th April 2006, St Petersburg, Florida, USA. *Invited Presentation.*

Allison SJ, Baldock PA, Sainsbury-Salis A, Enriquez R, During M, Eisman JA, Herzog H, Gardiner EM. Deletion of hypothalamic neuropeptide Y2-receptors in adult mice protects against continued ovariectomy-induced bone loss. Frontiers of Skeletal Biology 11th Workshop on Cell Biology of Bone and Cartilage in Health and Disease, 18-21st March 2006, Davos, Switzerland.

Allison SJ, Baldock PA, Sainsbury-Salis A, Enriquez R, During M, Eisman JA, Gardiner EM, Herzog H. Adult deletion of hypothalamic Y2 receptors improves bone mass following gonadectomy in mice. Australia and New Zealand Bone & Mineral Society Annual Scientific Meeting, 7-9th September 2005, Perth, Australia.

Allison SJ, Baldock PA, Sainsbury-Salis A, Enriquez R, Couzens M, Herzog H, Gardiner EM. Specific interaction of leptin and Y-receptor pathways in the control of bone formation and marrow fat. 2nd Australian Health and Medical Research Congress Meeting, 21-26th November 2004, Sydney, Australia. *Invited Presentation.*

Allison SJ, Baldock PA, Sainsbury-Salis A, Enriquez R, Couzens M, Herzog H, Gardiner EM. Distinct Y-receptor effects on leptin antiosteogenic and adipogenic pathways. American Society of Bone and Mineral Research 26th Annual Meeting, 1-5th October 2004, Seattle, Washington, USA.

Allison SJ, Baldock PA, Sainsbury-Salis A, Enriquez R, Couzens M, Herzog H, Gardiner EM. Y-receptor and leptin interactions in the central regulation of bone formation and

marrow fat. Australia and New Zealand Bone & Mineral Society Annual Scientific Meeting, 18-20th August 2004, Hunter Valley, NSW, Australia.

Allison SJ, Baldock PA, Sainsbury-Salis A, Lin EJ, Couzens M, Enriquez R, Boey D, Lin S, During M, Herzog H, Gardiner EM. Hypothalamic neuropeptide Y (NPY) Y2 receptors protect cancellous bone from leptin induced bone loss. Australian Society for Medical Research, 13th NSW Scientific Meeting, 7th June 2004, Sydney, Australia.

Poster Presentations

Allison SJ, Baldock PA, Sainsbury-Salis A, Enriquez R, Couzens M, Herzog H, Gardiner EM. Specific interaction of leptin and Y-receptor pathways in the control of bone formation and marrow fat. 2nd Australian Health and Medical Research Congress Meeting, 21-26th November 2004, Sydney, Australia.

AWARDS ARISING FROM THIS THESIS

Young Investigator Award, American Society of Bone and Mineral Research, 26th Annual Meeting, Seattle, USA, 2004.

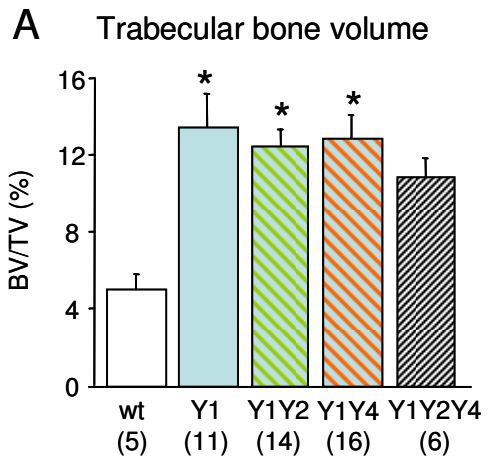
Young Investigator Award, Australia and New Zealand Bone and Mineral Society Annual Scientific Meeting, Hunter Valley, NSW, Australia, 2004.

ANZBMS and Christine & T. Jack Martin Travel Award, 2005.

Page 31, 1.7. Correction: A number of factors, such as insulin, amylin, and leptin circulate at increased concentrations in obese subjects.

Page 32, 1.7. Addition: Insulin, and IGF-1 receptors have been identified on osteoblast-like cells and administration of insulin stimulates a proliferative response.

Page 98. Corrected Figure 3.3A:



Page 101, 3.3.3. Addition: The different sample numbers used for MAR and bone volume measurements in figures 3.3 and 3.4, and 3.5 and 3.6, were due to difficulties encountered with labeling. Samples were excluded from MAR analysis if one or both the fluorescent labels were absent or too weak to analyse, due to ineffective i.p injections. Similarly, samples were excluded from osteoclast surface measurements if problems in staining occurred, evidenced by lack of red cytoplasmic staining. Acid phosphatase staining could be repeated on adjacent sections, however, due to the limited number of sections available per sample, measurements of osteoclast surface for every sample was not always possible.

Page 121, 3.2.3. Addition: Unfortunately, the demeclocycline labels used in this study faded faster than expected, and had nearly disappeared by the time single and double labeled surface were to be measured. As a consequence, calculations of MS and BFR could not be accurately assessed.

Addition to chapters 4 and 5: Number of mice used:

Chapter 4.

Figure 4.3 (wt),($Y1^{-/-}$),(ob/ob),($Y1^{-/-}$/ob); **A-C** (8),(10),(9),(4). **D-F** (4),(4),(7),(6).

Figure 4.4 (wt),($Y2^{-/-}$),(ob/ob),($Y2^{-/-}$/ob); **A-C** (8),(8),(9),(7). **D-F** (4),(5),(7),(5).

Figure 4.5 (wt),($Y4^{-/-}$),(ob/ob),($Y4^{-/-}$/ob); **A-C** (8),(10),(9),(8). **D-F** (4),(5),(7),(4).

Figure 4.6 (wt),(Y1$^{-/-}$),(ob/ob),(Y1$^{-/-}$/ob); **A-C** (4),(11),(10),(5). **D-F** (4),(10),(8),(6).

Figure 4.7 (wt),(Y1$^{-/-}$),(ob/ob),(Y1$^{-/-}$/ob); **A** (5),(9),(5),(5). **B** (5),(9),(10),(4). **C** (4),(9),(7),(6). **D** (4),(8),(6),(5).

Figure 4.8 (wt),(Y2$^{-/-}$),(ob/ob),(Y2$^{-/-}$/ob); **A-C** (4),(7),(10),(7). **D-F** (4),(5),(8),(7).

Figure 4.9 (wt),(Y2$^{-/-}$),(ob/ob),(Y2$^{-/-}$/ob); **A** (5),(7),(10),(7). **B** (5),(7),(5),(6). **C** (4),(5),(7),(7). **D** (4),(12),(6),(7).

Figure 4.10 (wt),(Y4$^{-/-}$),(ob/ob),(Y4$^{-/-}$/ob); **A-C** (4),(8),(10),(8). **D-F** (4),(6),(8),(4).

Figure 4.11 (wt),(Y4$^{-/-}$),(ob/ob),(Y4$^{-/-}$/ob); **A** (5),(9),(10),(8). **B** (5),(-),(5),(4). **C** (4),(6),(7),(4). **D** (4),(-),(6),(4).

Chapter 5.

Figure 5.2 (wt sham),(wt GX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ GX); **A** (15),(16),(8),(8). **B** (15),(18),(7),(8).

Figure 5.3 (wt sham),(wt OVX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ OVX); **A-F** (15),(16),(8),(8).

Figure 5.4 (wt sham),(wt ORX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ ORX); **A-F** (15),(18),(7),(8).

Table 5-1 (wt sham),(wt OVX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ OVX); (12),(10),(8),(8).

Table 5-2 (wt sham),(wt ORX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ ORX); (13),(13),(7),(8).

Figure 5.5 (wt sham),(wt GX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ GX); **B-D** (15),(16),(8),(8). **E-G** (13),(18),(7),(8).

Figure 5.6 (wt sham),(wt OVX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ OVX); **B** (15),(13),(7),(8). **C** (9),(8),(7),(5). **D** (13),(11),(7),(6). **E** (9),(8),(7),(5).

Figure 5.7 (wt sham),(wt ORX),(Y1$^{-/-}$ sham),(Y1$^{-/-}$ ORX); **B** (11),(18),(7),(8). **C** (9),(10),(5),(7). **D** (11),(12),(5),(7). **E** (9),(10),(5),(7).

Figure 5.8 (wt sham),(wt GX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ GX); **A** (15),(16),(5),(9). **B** (15),(18),(10),(10).

Figure 5.9 (wt sham),(wt OVX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ OVX); **A-F** (15),(16),(5),(9).

Figure 5.10 (wt sham),(wt ORX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ ORX); **A-F** (15),(18),(10),(10).

Table 5-3 (wt sham),(wt OVX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ OVX); (12),(10),(5),(9).

Table 5-4 (wt sham),(wt ORX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ ORX); (13),(13),(9),(10).

Figure 5.11 (wt sham),(wt GX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ GX); **B-D** (15),(16),(12),(8). **E-G** (13),(18),(10),(10).

Figure 5.12 (wt sham),(wt OVX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ OVX); **B** (15),(13),(7),(8). **C** (9),(8),(10),(5). **D** (13),(11),(11),(6). **E** (9),(8),(10),(5).

Figure 5.13 (wt sham),(wt ORX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ ORX); **A** (11),(18),(9),(8). **B** (9),(10),(6),(6). **C** (11),(12),(7),(7). **D** (9),(10),(6),(6).

Figure 5.14 (wt sham),(wt GX),(Y2$^{-/-}$ sham),(Y2$^{-/-}$ GX); **B** (12),(10),(12),(8). **C** (12),(9),(12),(8). **D** (7),(8),(10),(8). **E** (11),(7),(8),(8).**F** (10),(6),(7),(7).**G** (4),(5),(5),(7).

Figure 5.15, 5.16 (Sham Cre),(GX empty),(GX Cre); **A** (7),(7),(7). **B** (10),(10),(13).

Figure 5.17 (Sham Cre),(OVX empty),(OVX Cre); (7),(7),(7).

Figure 5.18 (Sham Cre),(ORX empty),(ORX Cre); (10),(10),(13).

Table 5-5 (Sham Cre),(OVX empty),(OVX Cre); (7),(7),(7).

Table 5-6 (Sham Cre),(ORX empty),(ORX Cre); (10),(10),(13).

Figure 5.19 (Sham Cre),(GX empty),(GX Cre); **B-D** (7),(7),(6). **E-G** (10),(10),(12).

Figure 5.20 (Sham Cre),(OVX empty),(OVX Cre); **A** (7),(6),(5). **B** (4),(4),(4). **C** (5),(7),(7). **D** (4),(4),(4).

Figure 5.21 (Sham Cre),(ORX empty),(ORX Cre); **A** (10),(10),(12). **B** (6),(6),(5). **C** (6),(7),(6). **D** (6),(6),(5).

Figure 5.22 (Sham Cre),(GX empty),(GX Cre); **B** (6),(6),(6). **C** (5),(4),(6). **D** (5),(5),(6). **E** (9),(10),(11). **F** (9),(9),(10). **G** (4),(7),(5).

Figure 5.23 (wt sham),(wt GX),(GX empty),(GX Cre); **A** (15),(16),(7),(6). **B** (15),(13),(6),(5). **C** (13),(11),(7),(7). **D** (13),(18),(10),(12). **E** (11),(18),(10),(12). **F** (11),(12),(7),(6).

Figures 7.5 – 7.7. Addition: Experiments were repeated three times.

Page 254, 8.1. Addition: Adipose mass is a major determinant of insulin, amylin, leptin, and adiponectin, which are all factors able to directly modulate bone cell activity as was discussed in the thesis introduction.

Page 322. The correct reference for Maurin et al., 2002 is; Maurin, A.C., Chavassieux, P.M., Vericel, E., Meunier, P.J. (2002). Role of polyunsaturated fatty acids in the inhibitory effect of human adipocytes on osteoblastic proliferation. Bone *31*, 260-6.